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RESEARCH ARTICLE

# Scenario-targeted toxicity assessment through multiple endpoint bioassays in a soil posing unacceptable environmental risk according to regulatory screening values

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**Abstract** Lanestosa is a chronically polluted site (derelict mine) where the soil (Lanestosa (LA) soil) exceeds screening values (SVs) of regulatory policies in force (Basque Country; Europe) for Zn, Pb and Cd. A scenario-targeted toxicity assessment was carried out on the basis of a multi-endpoint bioassay approach. Acute and chronic toxicity bioassays were conducted with selected test species (*Vibrio fischeri*, *Dictyostelium discoideum*, *Lactuca sativa*, *Raphanus sativus* and *Eisenia fetida*) in combination with chemical analysis of soils and elutriates and with bioaccumulation studies in earthworms. Besides, the toxicity profile was compared with that of the mine runoff (RO) soil and of a fresh artificially polluted soil ($LA_{APS}$) resembling LA soil pollutant profile. Extractability studies in LA soil revealed that Pb, Zn and Cd were highly available for exchange and/or release into the environment. Indeed, Pb and Zn were accumulated in earthworms and LA soil resulted to be toxic. Soil respiration, *V. fischeri*, vegetative and developmental cycles of *D. discoideum* and survival and juvenile production of *E. fetida* were severely affected. These results confirmed that LA soil had unacceptable environmental risk and demanded intervention. In contrast, although Pb and Zn concentrations in RO soil revealed also unacceptable risk, both metal extractability and toxicity were much lower than in LA soil. Thus, within the polluted site, the need for intervention varied between areas that posed dissimilar risk. Besides, since $LA_{APS}$, with a high exchangeable metal fraction, was the most toxic, ageing under in situ natural conditions seemingly contributed to attenuate LA soil risk. As a whole, combining multi-endpoint bioassays with scenario-targeted analysis (including leaching and ageing) provides reliable risk assessment in soils posing unacceptable environmental risk according to SVs, which is useful to optimise the required intervention measures.

**Keywords** Metal · Chronic pollution · Toxicity tests · Availability · Bioaccumulation · Leaching · Ageing

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## Introduction

An increasing number of countries have incorporated soil screening values (SVs) for a preliminary risk assessment distinguishing soils that could or actually pose risks for the uses intended (Carlon 2007). In the Law for Prevention and Correction of Soil Pollution from the Basque Autonomous Community, SVs are termed Indicative Values for Assessment (IVAs; Eusko Jaurlaritza/Gobierno Vasco 2007). Three IVAs (A, B and C) were defined based on literature data on toxicity

testing for single pollutants and theoretical extrapolation models (Ihobe 1998a; Ihobe 1998b; Urzelai et al. 2000). When the total concentration of individual contaminants in soil is above IVA-C, the risk is considered unacceptable and intervention is required (Ihobe 1998b).

Yet, the notion that toxicity can be readily extrapolated from pollutant concentrations in a complex matrix is hardly defensible (Vasseur et al. 2008; Reijnders 2009). Pollutant mobility, availability and uptake and metabolisation by living organisms are crucial issues neglected by SVs (Achazi 2002). As a general rule, risk assessment cannot be solely based on the analytical determination of total contents of specific contaminants because information on unexpected chemicals or bioavailability is not provided and the risk can be underestimated (Achazi 2002; Maisto et al. 2011). Moreover, SVs can also result in risk overestimations, as demonstrated upon the application of different batteries of multiple endpoint bioassays with different test species. Thus, although a flare pit soil required remediation according to chemically based soil criteria, neither the soil nor its leachates were significantly toxic after applying a battery of multiple endpoint bioassays with earthworms, plants and microorganisms (Cook et al. 2002). Likewise, an industrial soil heavily contaminated with hydrocarbons at concentrations largely above French SVs was not toxic as regards earthworm survival, collembolan reproduction and plant growth, only earthworm reproduction being slightly affected (Vasseur et al. 2008). Overall, it seems that the risk of historically contaminated soils can be overestimated when the total concentration of contaminants in soil is used for regulatory purposes (Diez et al. 2009). In contrast, there exist evidences that combining chemical and toxicological approaches leads to more reliable environmental risk estimates, in which soil physicochemical properties, total and available soil contaminants, bioaccumulation and multiple endpoint toxicity with species of different trophic levels are considered as a whole (Achazi 2002; Bierkens et al. 1998; Critto et al. 2007; Diez et al. 2009; Hund-Rinke et al. 2002a; b; Oleszczuk et al. 2014; Renoux and Sunahara 2002; Rodríguez-Ruiz et al. 2014).

Presently, a derelict zinc mine site (Lanestosa, Basque Country) was selected as a case study of historically contaminated soil with unacceptable environmental risk according to SVs in force. Total metal concentrations in Lanestosa (LA) soil (67,874 mg Pb/kg soil dry wt, 21,929 mg Zn/kg soil dry wt and 31 mg Cd/kg soil dry wt; Barrutia et al. 2010; Epelde et al. 2010) exceeded IVA-C values, and therefore the soil posed an unacceptable risk for the environment and required intervention (Ihobe 1998b). An scenario-targeted toxicity assessment was carried out based on multiple endpoint bioassays (according to Rodríguez-Ruiz et al. 2014) in order to help in deciding to which extent intervention was needed and in designing optimal intervention measures. Multiple endpoint bioassays included the assessment of bioavailability and lethal and sublethal toxic responses in a battery of test organisms (*Vibrio fischeri*, *Dictyostelium discoideum*, *Lactuca sativa*, *Raphanus sativus* and *Eisenia fetida*), together with chemical analyses of pollutants in total soil and extracted fractions. In addition, the study was extended to the runoff area in the vicinity of the mine runoff (RO) soil, in an attempt to obtain a scenario-specific spatial view of the environmental risk of the mine site (Fernández and Tarazona 2008). Finally, in order to assist the interpretation of the toxic effects and to determine the relevance of ageing in this chronically polluted site, the toxicity of an artificially polluted soil ($LA_{APS}$) resembling the pollutant levels found in LA soil was also investigated.

Though restricted to a particular case study, the present investigation was conceived as a pilot exercise, so that an equivalent approach might be applied in those cases in which applicable national/regional soil legislation is based on SVs.

## Material and methods

### Soil collection

#### *Control soils*

OECD artificial soil (70 % quartz sand, 20 % kaolinite clay and 10 % sphagnum moss; pH 6.0±0.5; OECD 1984) and Italian rural soil (IRS; texture=loam, OC=1.4 %, CEC=14 meq/100 g soil dry wt, pH=7.9; Rapp Prova Analisi Terreno N°76/07/T, 03/07/2007, Cadir Lab SRL, UNIPM, Alessandria) were used as control soils to produce dilutions of the reference and test soils in different experimental sets as described by OECD (1984, 2004) and by INIA et al. (2007).

#### *Reference and test soils*

Soils were collected from two localities in the Basque Country (Spain): (a) a rural pristine site at Delika (42° 57′ N, 2° 59′ W) that was used as reference site (Delika (DE) soil) and (b) a highly degraded site in the vicinity of a derelict mine in Lanestosa (43° 13′ N, 3° 25′ W) (LA soil) with chronic Zn, Pb and Cd pollution (about 100,000 mg Zn/kg dry weight (dw) soil, 24,000 mg Pb/kg dw soil, 33 mg Cd/kg dw soil; Epelde et al. 2010). Additionally, soil was also collected from an apparently less impacted site in the runoff area of Lanestosa mine (RO soil). Soil samples were collected from the top layer (0–15 cm) and sieved (6.3-mm mesh) immediately in the field to remove larger particles and organisms.

#### *Artificially polluted soil*

A portion of DE soil was spiked with a metal solution containing Zn, Cd and Pb in order to obtain an artificially polluted soil with the concentration of these metals similar to those

found in LA soil ($LA_{APS}$). $LA_{APS}$ was left to equilibrate for 14 days prior to experimentation (Pietrantonio et al. 2003).

## Soil characterisation

Upon arrival at the laboratory, DE, LA and RO soils were again sieved through a 2-mm mesh, characterised and kept at 4 °C until use. Briefly, texture was determined by mechanical analysis of air-dried soil (Brown 2003). Humidity and organic content as loss on ignition (LOI) were measured by gravimetric method according to ISO 11465 and NEN 5754, respectively (ISO 1993; NEN 1994). Soil pH (ISO 10390; ISO 2005) and electrical conductivity (EC) were determined in a 1:5 soil to $H_2O$ slurry by potentiometric titration. Soil respiration ($CO_2$ evolution) was measured by a NaOH trap (Alef and Nannipieri 1995; Rodríguez-Ruiz et al. 2014).

## Chemical analysis

### *Metals in soils*

**Total metals** Total concentration of metals was measured in soils, as well as in their experimental dilutions used in the adapted OECD 207 assay. Air-dried soil samples were digested with aqua regia and analysed by inductively coupled plasma atomic emission spectroscopy (ICP/AES; Perkin Elmer Optima 2100 DV spectrophotometer, Shelton, CT, USA) for As, Zn, Pb, Cd, Fe, Cr, Cu, Al, Mo, Sb, Co, Ba, Mn and Ni. Quality control was maintained by including certified reference material (7004, loam with elevated analyte levels, Czech Metrology Institute, Praha, Czech Republic) and by repetitive measurements of standard curves (USEPA 1996a; USEPA 2007), as detailed in Rodríguez-Ruiz et al. (2014).

**Water-soluble fraction of metals** Soil elutriates were obtained following the German standard method DIN 38414-S4 (DIN 1984); EC, pH and the concentrations of Zn, Pb and Cd in the water-soluble fraction were determined as described above.

**Exchangeable fraction of metals** Unbuffered 0.1 M $CaCl_2$ and 1 M $NH_4NO_3$ were used to extract the exchangeable fraction of soil contaminants by a single step (Gleyzes et al. 2002), following an adaptation of Tessier et al. 1979 and DIN 19730, respectively, as detailed in Rodríguez-Ruiz et al. (2014). The concentrations of Cd, Pb and Zn in the exchangeable fraction were determined by ICP/AES as described above.

**Sequential extraction of metals** In order to study metal speciation in DE, LA and RO soils, the modified *Bureau Commune de Reference* (BCR) sequential extraction protocol was applied (Rauret et al. 1999).

### *Metals in earthworms*

Freeze-dried earthworms ("5 per treatment; see 'Toxicity testing'") were digested individually (Rodríguez-Ruiz et al. 2014) and Zn, Pb and Cd measured by ICP/AES as described above.

### *Total petroleum hydrocarbons in soil*

Total petroleum hydrocarbons were extracted from air-dried soil (DE, LA and RO soils) by sonication (USEPA 3550B; USEPA 1996b). The florisil-cleaned up extract was measured for the sum of the fraction C10-C40 by FID-GC (Agilent Technologies SYS-GC-6890, Santa Clara, CA, USA; NEN 5733; NEN 1997).

## Toxicity testing

The solid phase and the DIN 38414 (water-soluble) fraction of DE, LA and RO soils and $LA_{APS}$ were used for toxicity testing with biomarker-based tests. Wherever possible, EC50 values were calculated (see "Data treatment and statistical analyses").

### *Test organisms*

*D. discoideum* and *E. fetida* were obtained from our laboratory stocks. *D. discoideum* (AX2 strain) was grown in axenic medium (14.3 g/L peptone, 7.15 g/L yeast extract, 18 g/L maltose, 0.419 g/L $Na_2HPO_4$, 0.486 g/L $KH_2PO_4$, pH 6.6) with 10 μg/mL tetracycline and developed in orbital incubator (Sanyo Gallenkamp Plc., Loughborough, UK) at 21 °C/180 rpm. Cultures were maintained at logarithmic phase of growth, keeping the cell density at about $2–4\times10^6$ cell/mL. Healthy adult clitellated *E. fetida*, of similar size (0.3–0.6 g fresh weight), were selected from a stock population reared under laboratory conditions (kept in tanks at 21 °C and 12:12 light/dark cycle with horse manure as food source ad libitum). Pesticide-free (Greene et al. 1989) lettuce and radish seeds were obtained from a local dealer.

### *Microtox®*

Toxicity to *V. fischeri* was determined for the water-soluble fraction (DIN 38414) and for the solid phase of DE, LA and RO soils and $LA_{APS}$, as well as in experimental dilutions of LA soil used in the adapted OECD 207 assay. Microtox® was conducted according to the manufacturer's standard procedures (Azur Environmental 1995a; Azur Environmental 1995b). IC50s and associated values for the 95 % confidence limits were normalised for moisture content and values were shown as milligramme per liter (Environment Canada 2002).

*D. discoideum*

***D. discoideum* inhibition of fruiting body formation (IFBF) solid phase assay** DE, LA and RO soils were mixed with IRS to obtain a series of soil dilutions (0, 10, 25, 50, 75 and 100 %). *D. discoideum* cells ($2.7\times10^7$) were exposed to test soils after being washed following Fey's protocol (details in Rodríguez-Ruiz et al. 2013). The number of fruiting bodies was counted on the micrographs and used to calculate the percentage IFBF (Balbo and Bozzaro 2008).

***D. discoideum* developmental cycle (DDDC) solid phase assay** DE, LA and RO soils were uniformly mixed with distilled water to saturation (100 % water holding capacity—WHC) and air-dried. Then, *D. discoideum* cells resuspended in 80 % of soil WHC PAS were spread over the air-dried DE, LA and RO soils, covered and kept in a humid dark chamber at 21 °C. After 24 h, at least 12 micrographs (190 $mm^2$) were obtained per test soil (6 optical fields×2 Petri dishes) using a stereo microscope at ×7.1 magnification. Aggregates (AG), slugs (migrating forms; MF), culminants (CC) and fruiting bodies (FB) were counted. The percentage of multicellular units (MU%) (MU=AG+MF+CC+FB) and the fruiting body size factor (FBSF=$r^3$) were calculated. For each micrograph, the aggregation arrest index (AAI=AG/MU), the migration arrest index (MAI=MF/(MF+CC+FB+1)) and the culmination arrest index (CAI=CC/(CC+FB+1)) were calculated in order to evaluate the progress of the developmental cycle of *D. discoideum* through critical checkpoints (Rodríguez-Ruiz et al. 2013).

***D. discoideum* elutriate toxicity** Cells in their logarithmic growth phase ($2–4\times10^6$ cell/mL) were diluted to 0.75 $10^6$ cell/mL in exposure medium (DE, LA and RO soil elutriates) together with 25 % AX2 medium with 10 μg/mL tetracycline. Cell viability, lysosomal membrane stability, endocytosis rate and cell replication rate were measured (Dondero et al. 2006; Sforzini et al. 2008).

*Lettuce seed germination and root elongation test*

Solid phase and elutriate effect on seed germination and root elongation was measured, as detailed in Rodríguez-Ruiz et al. (2014). Lettuce (*L. sativa*) seeds ($n$=15) were spread on open-air-dried LA and RO soils mixed with OECD artificial soil to obtain the same dilutions than in the above described OECD 207 assay (0, 0.3, 0.6, 1.25, 2.5, 25, 50 and 100 %) ($n$=4). Besides, 15 lettuce seeds were spread on open-air-dried $LA_{APS}$ mixed with DE soil at 0, 0.3, 0.6, 1.25, 2.5, 25 and 100 % dilutions ($n$=4). Finally, lettuce (*L. sativa*) ($n$=15) or radish (*R. sativus*) seeds ($n$=10) were spread on the elutriates of DE, LA and RO soils and $LA_{APS}$ ($n$=4). Procedure blank (distilled water processed like the elutriates) was used as control. After 72 h, the number of germinated seeds was counted and the root length measured. Seed germination (%) was estimated relative to the initial number of seeds in each treatment. Root elongation was evaluated as the root length to the nearest millimetre and expressed as average millimetre per seed. The percentages of relative seed germination (RSG) and relative root growth (RRG) were determined to calculate the Germination Index (GI), expressed as GI=[RSG×RRG]/100 (Hoekstra et al. 2002).

*Earthworm assays*

**Earthworm acute toxicity test (OECD 207)** LA soil was homogenously mixed with OECD artificial soil to obtain eight dilutions of soil (0, 0.3, 0.6, 1.25, 2.5, 25, 50 and 100 %) recommended by the Spanish Methodological guidelines to calculate L(E)C50 values (INIA et al. 2007; OECD 1984). Undiluted DE soil served as a reference soil to compare to the effects of non-diluted LA and OECD soils. A total of 400 g of soil (dry wt) was added to each 1-L glass container, moistened to 60 % WHC and let equilibrate for 24 h before initiating the assay ($n$=4). On days 7 and 14, external pathologies, behavioural changes (phototropism, atypical mobility events), loss of weight and mortality were recorded. Humidity, organic matter (OM), EC, pH and metals in soils and total metal tissue concentrations in earthworms were measured, according to the procedures described above.

**Earthworm reproduction test (ISO 11268-2)** This test was performed in line to ISO 11268-2 guidelines (ISO 2012), an updated revision of the OECD test guideline 222 recommended by the Spanish Methodological guidelines (INIA et al. 2007). LA soil was homogenously mixed with OECD artificial soil (0, 0.3, 0.6, 1.25, 2.5, 10, 30 and 60 %), and EC50 values for mortality and juvenile number were calculated using probit analysis. DE soil was used as reference soil. Soil (500 g of soil dry wt) was added to each container, moistened to 60 % WHC and let equilibrate for 24 h before initiating the assay ($n$=4). On day 28, adult individuals were removed and changes in weight and mortality were recorded (OECD 2004). On day 56, juveniles were counted. During the assay, the temperature was maintained at 22 C±2 C under natural summer photoperiod (May–July).

**Data treatment and statistical analyses**

Homogeneity of variances (Levene's test) as well as normality of data (Kolmogorov-Smirnov's test) were tested before statistical analysis. Normal datasets were subject to either Student's $t$ test or one-way ANOVA followed by Duncan's test or to two-way ANOVA. Non-parametric Mann-Whitney $U$, Kruskal-Wallis followed by Mann-Whitney $U$ or Scheirer-Ray-Hare's tests were applied to non-normal data sets (e.g., heterogeneous variance). In all cases, statistically significant

differences were established at $p<0.05$. Statistical tests were performed using SigmaStat v2.03. The median effective (EC50) and lethal (LC50) concentrations were calculated according to the probit method (SPSS Statistic v17.0). No observed effect concentration (NOEC) and lowest observed effect concentration (LOEC) were determined by comparison with the control experimental set through one-way ANOVA followed by Dunnett's test as recommended by OECD (OECD 2004), using SigmaStat v2.03 (Systat Software, San Jose, CA, USA).

## Results

### Characterisation of the reference soil (Delika)

DE soil had high OM (10.8 %), high pH (8.0) and moderate levels of Cd, Co and Ni (Table 1). Zn, Pb, Co, Ba and Mn were associated with the reducible fraction or bound to oxides and hydroxides (Fig. 1). Ni, Cr and Cu were more or less equally distributed between the fraction associated with reducible phases and that of metals bound to sulphides and organic matter (Fig. 1). Cd was below detection limits in all fractions analysed. Zn, Pb and Cd were below detection limits in DIN 38414, DIN 19730 and $CaCl_2$ extracts.

DE soil resulted to be non-toxic according to both solid phase and basic Microtox® tests performed on DIN 38414, DIN 19730 and $CaCl_2$ extracts (Tables 2 and 3).

### Properties and toxicity profile of LA and RO soils and $LA_{APS}$

LA soil was sandy with significantly lower respiration, OM, pH and EC than DE soil (Table 1). LA soil presented significantly higher concentrations of Zn, Pb, Cd and Cu than DE soil (Table 1). Particularly, Zn and Pb concentrations in LA soil were 170 and 3000 times higher than those recorded in DE soil, respectively. Likewise, Cd concentration was 10 times higher, and Cu concentration was twice that in DE soil. In LA soil, where the total soil concentrations of Zn, Pb and Cd were above or close to their IVA-C values, only the 34.9 % of the recovered Zn (1591.3 ±367.5 mg Zn/kg soil dry wt), the 10.2 % of the Pb (263.9± 35.8 mg Pb/kg soil dry wt) and the 55 % of the Cd (7.2±1.5 mg

**Table 1** Physicochemical properties of Delika (DE), Lanestosa (LA), runoff (RO) and artificial Lanestosa ($LA_{APS}$) soils: texture, humidity (%), organic matter (OM) measured as loss on ignition (LOI, %), pH, electrical conductivity (EC; μS/cm), soil respiration (mg $CO_2$/g dw/day) and total concentration (mg/kg soil dry wt) of metals and total petroleum hydrocarbon (TPH)

| | | Source soil | | Artificial soil | |
|---|---|---|---|---|---|
| | | DE | LA | RO | $LA_{APS}$ |
| Soil properties | Texture | Sandy loam | Sand | Silt loam | Sandy loam |
| | Humidity | 8.42±0.07 | 12.64±0.77* | 17.67±0.44* | 21.21±1.72* |
| | OM | 10.80±0.10 | 4.99±0.15* | 6.86±0.66* | 11.62±0.29 |
| | pH | 8.0±0.1 | 6.7±0.2* | 6.8±0.0* | 6.7±0.1* |
| | EC | 152±38.7 | 29.36±18.2* | 63.2±1.93* | 6,800±0.0* |
| | Respiration | 0.058±0.018 | 0.023±0.007* | 0.057±0.018 | – |
| Contaminants and trace elements (mg/kg dry wt soil) | As | 3.9±0.2 | 1.2±0.5* | 6.6±0.5* | – |
| | Zn | 70.2±0.2 | 11,841±388* | 962±63.2 | 12,874±362* |
| | Pb | <2.5 | 3,353±311* | 588±2.0 | 3,844±105* |
| | Cd | 1.8±0.1 | 16.7±0.4* | 1.7±0.0 | 21.1±0.3* |
| | Fe | 26,235±643 | 12,220±240* | 8,447±628* | – |
| | Cr | 38.0±0.2 | 8.3±1.8* | 13.5±4.8* | – |
| | Cu | 7.5±1.9 | 18.4±4.7* | 6.2±0.2 | – |
| | Al | 26,445±1 | 10,033±5,668* | 14,108±4 | – |
| | Mo | <2.5 | <2.5 | <2.5 | – |
| | Sb | <2.5 | <2.5 | 3.2±0.2* | – |
| | Co | 6.8±0.2 | <2.5 | 2.9±0.1 | – |
| | Ba | 103±5.8 | 27.0±0.7* | 35.7±21.6* | – |
| | Mn | 384±5.8 | 40.6±1.3* | 41.7±1.7* | – |
| | Ni | 18.2±0.7 | 2.4±0.1* | <2.5 | – |
| | TPH | <25 | <25 | <25 | – |

Values are shown as average±standard deviation. En dash means not measured

*$p<0.05$, significant differences in comparison with DE soil, according to one-way ANOVA followed by Dunnett's test

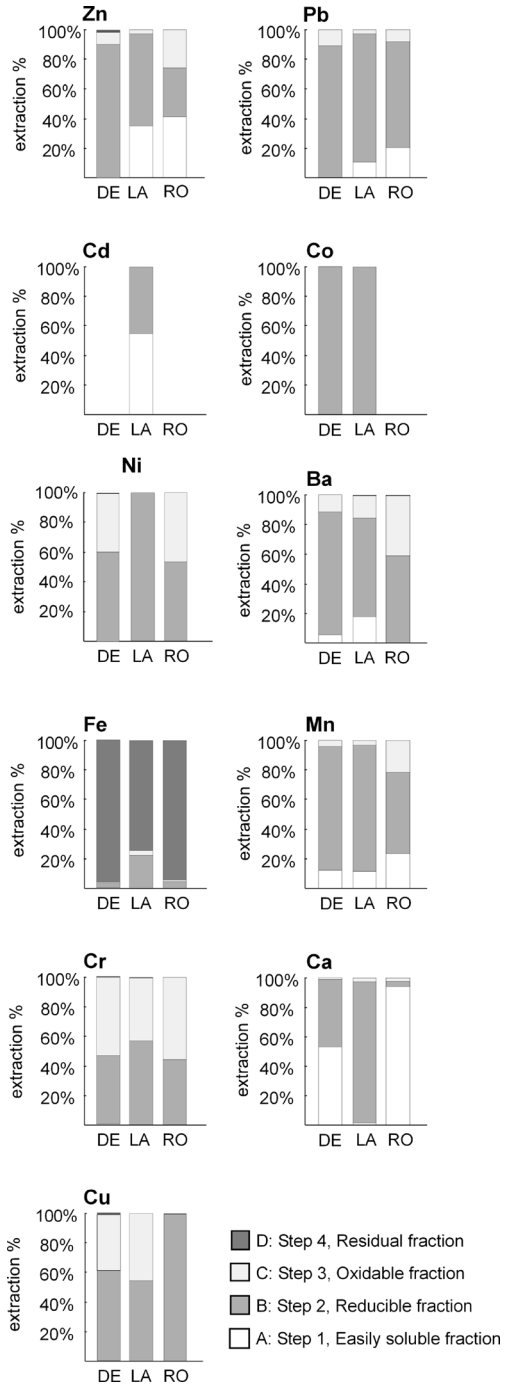


**Fig. 1** Zn, Pb, Cd, Co, Ni, Ba, Fe, Mn, Cr, Ca and Cu distributions in Delika (DE), Lanestosa (LA) and runoff (RO) soils, shown as the extractable element concentrations related to the total recovered elements (%) performed after the BCR protocol: ▨ step 1 is extracted with acetic acid, ▭ step 2 with hydroxylamine hydrochloride, ▨ step 3 is extracted with in $H_2O_2$ and ammonium acetate and ■ step 4 is the residual fraction

Cd/kg soil dry wt) was present in the easily soluble fraction, with the Zn concentration above its IVA-C value, and Pb and Cd concentrations above IVA-B values (Fig. 1). The 62.5 % of the Zn (2847.2±1193.5 mg Zn/kg soil dry wt), 87.0 % Pb (2247.7± 261.6 mg Pb/kg soil dry wt) and 45.0 % Cd (5.9±3.0 mg Cd/kg soil dry wt) was in the reducible fraction, with the Zn and Pb concentrations remaining above their IVA-C values and Cd concentration above its IVA-B value. In contrast, only 2.6–2.8 % of the recovered Zn and Pb were present in the oxidable fraction but both (120.1±22.3 mg Zn/kg soil dry wt and 72.0± 9.1 mg Pb/kg soil dry wt) were above their respective IVA-B values (Zn, 106 mg Zn/kg soil dry wt). No difference was observed in the sequential extraction pattern of Co and Mn with respect to the reference soil. Similar concentrations of Zn, Pb and Cd were extracted by DIN 19730 and $CaCl_2$, and they were higher than those extracted with DIN 38414 (Table 3).

RO soil was silt loamy with significantly lower OM, pH and EC than DE soil (Table 1). RO soil presented significantly higher concentrations of Zn (14 times), As and Sb than DE soil (Table 1). For Zn, Pb, Mn and Ca, over 20 % of the recovered metal was present in the easily soluble fraction (Fig. 1). In addition, the reducible fraction of Pb, Ni, Ba, Mn, Cr and Cu was over 40 % of the recovered metal. No difference was observed in the sequential extraction pattern for Cr, Mn and Ca with respect to the reference soil (DE soil), and Cd was not recovered in any fraction. A similar concentration of Zn was extracted by DIN 19730 and $CaCl_2$, and it was higher than that extracted with DIN 38414 (Table 3). Pb and Cd were very low or under detection limit in DIN 38414 extract.

$LA_{APS}$ had a significantly higher EC and lower pH than DE soil although resulted to have the same pH than LA soil but much higher EC (Table 1). $LA_{APS}$ presented the same concentrations of Zn, Pb and Cd than LA soil, which were significantly higher than in DE soil (Table 1). Zn, Pb and Cd were recovered with DIN 38414, DIN 19730 and $CaCl_2$ methods. The water-soluble fraction of Zn and Cd was much higher than in LA soil, whereas that of Pb was only slightly higher. Likewise, the exchangeable fraction of Zn was 2–4 times higher in $LA_{APS}$ than in LA soil, and the exchangeable fraction of Cd was twice that in LA soil, whereas those of Pb were not dissimilar. DIN 19730 extracted more Zn and Pb than $CaCl_2$ (Table 3).

## Toxicity screening

### *Solid phase toxicity*

LA soil and $LA_{APS}$ resulted toxic after solid phase Microtox® (IC50 <1000 mg/L; Environment Canada 2002), while RO soil was found to be non-toxic (Table 2). The estimated *V. fischeri* EC50 was 18.8 % LA soil (2748 mg Zn/kg soil dry wt, 1140 mg Pb/kg soil dry wt, Cd under detection limit (u.d.l.)) and NOEC and LOEC estimates were 2.5 and 25 % LA soil, respectively. Overall, the concentrations of Zn and Pb in the 18.8 % LA soil were much above their respective IVA-C values (Ihobe 1998b; Table 4). The estimated *V. fischeri* EC50 resulted to be 36.5 % RO soil (351 mg Zn/kg soil dry wt, 215 mg Pb/kg soil dry wt, 0.6 mg Cd/kg soil dry wt). These concentrations of Zn and Pb were over their respective IVA-B values, whereas that of Cd was just below its IVA-B value (Table 4, Ihobe 1998b).

Regarding DDDC assay results, a significant reduction in MU%, FBSF, MAI and CAI was found in LA soil in comparison with DE soil (Table 2). Overall, less aggregates, slugs and culminants and smaller fruiting bodies were recorded in LA soil than in DE soil, which rendered the total number of multicellular units reduced beyond 50 %. Besides, a significant decrease in MU% and AAI and a significant increase in FBSF and CAI were found in RO soil when compared with DE soil (Table 2). Overall, significantly fewer aggregates and more culminants were recorded in RO soil than in DE soil, which rendered a significantly smaller total number of multicellular units (Table 2).

*Elutriate toxicity*

The results of the basic Microtox® test on LA soil DIN 38414 elutriate (low EC values and neutral pH; Table 3) and on RO soil DIN 38414 elutriate (high EC values and acid pH; Table 3) did not reveal any significant toxicity. In contrast, LA soil DIN 38414 extracts produced significantly shorter roots and lower GI (%) in lettuce and RO soil DIN 38414 extract produced significant effects on lettuce root elongation and GI (%), and in radish GI (%). $LA_{APS}$ DIN 38414 elutriate (high EC and low pH) revealed significant effects on lettuce and radish root elongation and GI (%) (Table 3).

## Critical toxic values

*D. discoideum toxicity*

IRS, used as control soil for dilutions of LA soil, significantly inhibited fruiting body formation after 24 h in comparison with the reference soil (DE, Fig. 2a). In order to avoid the influence of confounding factors other than LA soil toxicity on the effects of LA soil dilutions, the toxicity of this soil was estimated relative to DE soil for each experimental dilution of IRS. Thus, a dose response curve was clearly obtained for IFBF, depending on LA dilutions (Fig. 2b). EC50 was 48.2 % LA soil (23.5–74.7 %) and the estimated NOEC and LOEC values were 25 and 50 % LA soil, respectively.

The effect of RO soil was estimated also relative to DE soil for each experimental dilution in IRS. A clear dose response curve was obtained for the corrected IFBF values (Fig. 2c). EC50 was 36.5 % LG soil. Estimated NOEC and LOEC values were 0 and 10 %, respectively.

*Lettuce toxicity*

Exposure of seeds to 25 % LA soil and $LA_{APS}$ resulted in statistically significant reduction in root elongation and GI (%) (Figs. 3c, e and 5b, c), while 100 % $LA_{APS}$ significantly affected germination (Figs. 3, 4 and 5). Besides, exposure of seeds to 50 % RO soil resulted in significant decrease in root elongation and GI (%), while 100 % RO soil resulted in a significant decrease in seed germination (Fig. 4a, c, e). When 100 % OECD (control), DE (reference) and LA (test) soils were compared, the three soils were significantly different among them as regards root elongation and GI (%) (Fig. 3d, f). The estimated EC50 values (as % LA soil) were >100 for germination, 66.5 for root elongation and 65.6 for GI (%) (NOEC=100 % LA soil for germination; NOEC=2.5 % LA soil and LOEC=25 % LA soil, for root elongation and GI (%)). When 100 % OECD (control), DE (reference) and RO (test)

**Table 2** Median inhibiting concentration (30-min IC50) in *V. fischeri* after the Microtox solid phase test and effects on *D. discoideum* after the DDDC assay for Delika (DE), Lanestosa (LA), runoff (RO) and artificial Lanestosa ($LA_{APS}$) soils

| Organism | Endpoint (units) | DE | LA | RO | $LA_{APS}$ |
|---|---|---|---|---|---|
| *V. fischeri* | IC50 at 30 min (mg/L) | 4080 | 696[a] | 35,087 | 523[a] |
| | (95 % C.R.) | (3316–5021) | (568–852) | (29,349–41,953) | (402–681) |
| *D. discoideum* | Aggregates (no.) | 89.08±24.82 | 33.25±17.65* | 65.33±13.79* | – |
| | Slugs (no.) | 3.25±2.05 | 0.08±0.29* | 3.50±0.80 | – |
| | Culminants (no.) | 6.08±2.57 | 1.83±1.64* | 10.00±4.13 | – |
| | Fruiting bodies (no.) | 7.92±5.92 | 6.33±2.06 | 5.42±2.15 | – |
| | Total MU (no.) | 106.3±28.97 | 41.5±19.47* | 84.25±14.45* | – |
| | MU (%) | 100.0±27.24 | 39.03±18.31* | 79.23±13.59* | – |
| | FBSF | 0.03±0.01 | 0.01±0.00* | 0.07±0.01* | – |
| | AAI | 0.84±0.06 | 0.78±0.08 | 0.77±0.06* | – |
| | MAI | 0.19±0.12 | 0.01±0.04* | 0.18±0.04 | – |
| | CAI | 0.44±0.14 | 0.18±0.14* | 0.59±0.13* | – |

Values are shown as average±standard deviation

*C.R.* confidence range

*$p<0.05$, significant differences in comparison with DE soil, according to one-way ANOVA followed by Dunnett's test

[a] Toxic to *V. fischeri* following the Canada Environment interim guideline (Canada Environment 2002)

**Table 3** Concentrations of Zn, Pb and Cd in DIN 19730, $CaCl_2$ and DIN 38414 elutriates of Delika (DE), Lanestosa (LA), runoff (RO) and artificial Lanestosa ($LA_{APS}$) soils; and the pH, electrical conductivity (EC, μS/cm) and toxicity, after standard (Microtox; plant seed germination and root elongation tests) and novel (*D. discoideum* biomarker-based tests) bioassays, of the water-soluble fraction (DIN 38414 elutriate) of DE, LA, RO soils and $LA_{APS}$

| Matrix | | Endpoint | Source soil | | Artificial soil | |
|---|---|---|---|---|---|---|
| | | | DE | LA | RO | $LA_{APS}$ |
| DIN 19730 | Metals | Zn (mg/kg) | <0.1 | 1086 | 7.3 | 4710 |
| | | Pb (mg/kg) | <0.1 | 38.8 | 0.4 | 59.1 |
| | | Cd (mg/kg) | <0.1 | 4.4 | 0.1 | 14.0 |
| $CaCl_2$ | Metals | Zn (mg/kg) | <0.5 | 996 | <0.5 | 2398 |
| | | Pb (mg/kg) | <0.5 | 16.8 | <0.5 | 14.1 |
| | | Cd (mg/kg) | <0.5 | 7.2 | <0.5 | 14.2 |
| DIN 38414 | Metals | Zn (mg/kg) | <0.1 | 27.4 | 2.76 | 592 |
| | | Pb (mg/kg) | <0.1 | 1.48 | <0.1 | 2.61 |
| | | Cd (mg/kg) | <0.1 | <0.1 | <0.1 | 4.09 |
| | Physicochemical properties | pH | 7.5 | 7.3 | 2.8 | 6.8 |
| | | EC (μS/cm) | 180 | 35 | 822 | 6500 |
| | *V. fischeri* | IC50 (15 min) mg/L | 564,000 | 266,700 | 77,660 | |
| | | 95 % C.R. | (545,800–582,800) | (213,900–332,600) | (73,390–82,180) | |
| | *L. sativa* | Germination (% initial) | 97.50±4.96 | 95.83±7.07 | 95.00±4.71 | 95.00±6.38 |
| | | Root elongation (mm/seed) | 14.73±3.15 | 10.54±1.07* | 10.69±1.68* | 2.48±0.32* |
| | | GI (% DE) | 99.61±22.68 | 70.16±10.26* | 70.10±10.54* | 16.26±1.78* |
| | *R. sativus* | Germination (% initial) | 100.0±0.00 | 95.00±5.77 | 95.00±5.77 | 97.50±5.00 |
| | | Root elongation (mm/seed) | 25.95±1.24 | 26.95±3.52 | 21.18±4.10 | 7.50±0.63* |
| | | GI (% DE) | 100.0±4.78 | 99.94±8.49 | 75.82±17.39* | 33.67±2.47* |
| | *D. discoideum* | Mortality (% initial) | 16.10±3.63 | 11.29±2.79* | 11.66±2.96* | – |
| | | LMS (% DE) | 100.0±37.13 | 84.74±30.68 | 83.90±29.45 | – |
| | | Endocytosis (% DE) | 100.0±20.55 | 94.99±24.07 | 90.77±19.92 | – |
| | | Replication (% DE) | 100.0±6.24 | 89.22±6.80 | 77.04±10.84* | – |

Germination Index (GI), lysosomal membrane stability (LMS), endocytosis and replication estimates are relative (%) to those recorded in DE soil. Values are shown as average±standard deviation

*IC50* median inhibiting concentration (30 min) after Microtox solid phase test, *C.R.* confidence range

*$p<0.05$, significant differences in comparison with DE soil, according to one-way ANOVA followed by Dunnett's test

**Table 4** Classification of Lanestosa (LA), runoff (RO) and artificial Lanestosa ($LA_{APS}$) soils according to soil screening values (SVs; IVA-A, IVA-B or IVA-C values) in the Basque legislation (Ihobe 1998b) and toxicity profiles based on critical toxicity values (EC50 or NOEC only when EC50 could not be calculated) determined after multiple endpoint toxicity testing of the soils and their DIN 38414 elutriates with *V. fischeri*, *D. discoideum*, *L. sativa* and *E. fetida*

| | | | | LA | RO | $LA_{APS}$ |
|---|---|---|---|---|---|---|
| Classification of soil according to regulatory SVs (IVA values) | | | As (mg/kg) | IVA-A | IVA-A | – |
| | | | Zn (mg/kg) | IVA-C | IVA-C | IVA-C |
| | | | Pb (mg/kg) | IVA-C | IVA-C | IVA-C |
| | | | Cd (mg/kg) | IVA-B | IVA-B | IVA-C |
| | | | Cu (mg/kg) | IVA-A | IVA-A | – |
| | | | Mo (mg/kg) | IVA-A | IVA-A | – |
| | | | Co (mg/kg) | IVA-A | IVA-A | – |
| | | | Ni (mg/kg) | IVA-A | IVA-A | – |
| | | | TPH (mg/kg) | IVA-A | IVA-A | – |
| Toxicity profiles in soil (multiple endpoint toxicity testing) | Solid phase | *V. fischeri* | Microtox IC50 | 696[a] | 35,087 | 523 [a] |
| | | | Microtox IC50 | 18.8 % | 36.5 % | – |
| | | *D. discoideum* | EC50 | 48.2 | 34.0 % | – |
| | | *L. sativa* | Germination | 100 % Ω | 50 % Ω | 46.4 % |
| | | | Root elongation | 66.5 % | 50 % Ω | 17.9 % |
| | | | GI | 65.6 % | 87.1 % | 17.4 % |
| | | *E. fetida* | Reproduction (RT) | 17.4 % | – | – |
| | | | Mortality (RT) | 26.8 % | – | – |
| | DIN 38414 elutriate | *V. fischeri* | Microtox IC50 | 266,700 | 77,660 | – |

Ω refers to NOEC value. En dash means not available

[a] Toxic to *V. fischeri* following the Canada Environment interim guideline (Canada Environmental 2002)

soils were compared, the three soils were significantly different among them as regards root elongation and GI (%) (Fig. 4d, f). EC50 values for germination and root elongation were >100 % RO soil. EC50 value for GI (%) was 87.11 % RO soil (NOEC=50 % RO soil and LOEC=100 % RO soil, for the three analysed parameters). Calculated EC50 for $LA_{APS}$ resulted to be 46.4 % $LA_{APS}$ for germination, 17.9 % $LA_{APS}$ for root elongation and 17.4 % $LA_{APS}$ for GI (%) (NOEC=25 % $LA_{APS}$ and LOEC=100 % $LA_{APS}$, for germination; NOEC=0 % $LA_{APS}$ and LOEC=0.3 % $LA_{APS}$, for root elongation and GI (%)).

*Earthworm acute toxicity*

Marked differences in physicochemical characteristics were found among the different experimental soils (Online Resource 1). OM varied from 5.0 % in OECD soil to 8.2 % in LA soil. pH decreased at increasing concentrations of LA soil, being DE soil and OECD soil (0 % LA) pH similar.

*E. fetida* loss of weight at the end of the test was evident in all experimental dilutions as well as in the reference soil (>20 %; Online Resource 2). No obvious physical or pathological symptoms or distinct changes in behaviour were observed in the test organisms. Mortality in control animals, as well as in those exposed up to 2.5 % LA soil, did not exceed 10 % at the end of the test (Fig. 6a; Online Resource 2). However, after 7-day exposure, they accumulated Zn and Pb from soils (Fig. 6a; Online Resource 3). Cd was not accumulated. LC50 was 25.1 % LA soil (NOEC=2.5 % LA soil; LOEC=25 % LA soil), whereas the lowest concentration causing 99 % mortality was 50 % LA soil. Mortality recorded in OECD (control) soil was significantly smaller than DE (reference) and 100 % LA (test) soil (Fig. 6b).

*V. fischeri toxicity*

After applying Microtox® solid phase test to the dilutions of LA soil, there were significant effects at 25, 50 and 100 % LA soil, according to the interim guidelines recommended by Environment Canada (2002) (Online Resource 2). Thus, estimates of NOEC and LOEC were 2.5 and 25 % LA soil, respectively. The estimated EC50 was 18.8 % LA soil.

*Earthworm reproduction test*

No difference was found in humidity along the experimental time for each experimental group (ca. 5–7 % LA soil and its dilutions, 7–9 % DE soil), although humidity was especially

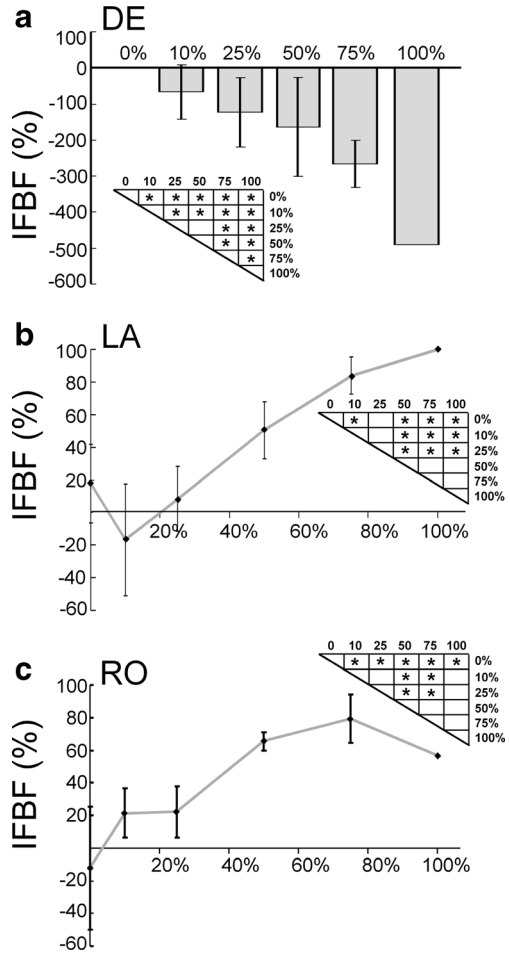


**Fig. 2** Inhibition of fruiting body formation (IFBF; %) in *D. discoideum* exposed to DE soil mixed with IRS (**a**) and IFBF (%) of *D. discoideum* estimated relative to DE soil when exposed to Lanestosa (LA; **b**) and runoff (RO; **c**) soils mixed with IRS. Values are shown as average± standard deviation. *$p \leq 0.05$, significantly different according to one-way ANOVA followed by Duncan's test

low in the 0.6 % LA soil (3–4 %, Online Resource 1). Although none of the replicates in the control produced at least 30 juveniles by the end of the test and the coefficient of variation (CV) was higher than 30 %, mortality was rare and these validity conditions were fulfilled by the reference soil (>30 juveniles; CV=16 %). Thus, the test results were considered valid. In addition, neither obvious pathological symptoms nor distinct changes in behaviour were recorded, and live adult earthworms did not decrease in body weight after 28 days (Online Resource 2). Significant adult mortality occurred at 25 % LA soil within 28 days, which was significantly higher than that in OECD and LG soil (Fig. 7a, b). LC50 was 26.8 % LA soil (NOEC=10 % LA soil, LOEC=25 % LA soil). EC50 for juvenile numbers was 17.4 % (NOEC=25 % LA soil, LOEC=10 % LA soil). Juvenile number recorded in both OECD and LA soils was significantly lower than that recorded in DE soil (Fig. 7c, d; Online Resource 2).

## Discussion

It was confirmed that the total concentration in LA soil of Zn, Pb and Cd was just around (16.7 mg Cd/kg soil dry wt) or above (11,842 mg Zn/kg soil dry wt, 3353 mg Pb/kg soil dry wt) their respective IVA-C values (Barrutia et al. 2010; Epelde et al. 2010). Likewise, RO soil presented a high concentration of Zn, As and Sb, and though Zn and Pb concentrations were lower than in LA soil, they were still above IVA-C values. Therefore, both LA soil and, to a lesser extent, RO soil posed an unacceptable risk for the environment. Albeit soils from mining areas with Cd, Zn and Pb concentrations higher than those recorded in LA soil were shown to be toxic to a variety of test organisms (Van Gestel et al. 2001), the present scenario-targeted toxicity assessment using multiple endpoint bioassays revealed that the environmental risk was lower than intended from SVs, as discussed below.

### Availability of metals

In LA soil, the easily soluble fraction of Zn, Pb and Cd extracted with the two single-stepped methods (DIN 19730 and 0.1 M $CaCl_2$) was similar, and comparable with the first step of the sequential extraction, where over 10 % of the recovered metal was present in the easily soluble fraction. The recovered Zn in this fraction was above its IVA-C value, while the recovered Pb and Cd concentrations were above their IVA-B values. Overall, the non-residual fraction (exchangeable + reducible + oxidable) of Zn, Pb and Cd were ~100 %, which indicates that these metals are potentially available for exchange and/or release into the environment (Velimirovic et al. 2010). In particular, LA soil shows signs of low-medium risk for Pb, high risk for Zn and very high risk for Cd, according to the Risk Assessment Code (RAC; Velimirovic et al. 2010).

RO soil sequential extraction pattern was found to be different from LA soil pattern. Cd was not recovered in any fraction. Over 20 % Zn and Pb was present in the easily soluble fraction, whereas >30 % Zn and 70 % Pb was in the reducible fraction. Besides, the recovered Zn oxidable fraction was 10 times higher in RO soil than in LA soil. These different retention capacities of the two soils seem to be related to the differences in soil OM content because the pH was similar in both soils (Meers et al. 2007; Pueyo et al. 2003).

$LA_{APS}$ resulted to have the highest metal concentrations in the water-soluble and exchangeable fractions. This suggests that the retention capacity was much higher for aged soil (LA) than for freshly made soil ($LA_{APS}$). Accordingly, chemical speciation, bioavailability, bioaccumulation, toxicity and mixture effects are known to be significantly affected by ageing (Alexander 2000; Peijnenburg and Vijver 2009; Smolders et al. 2009).

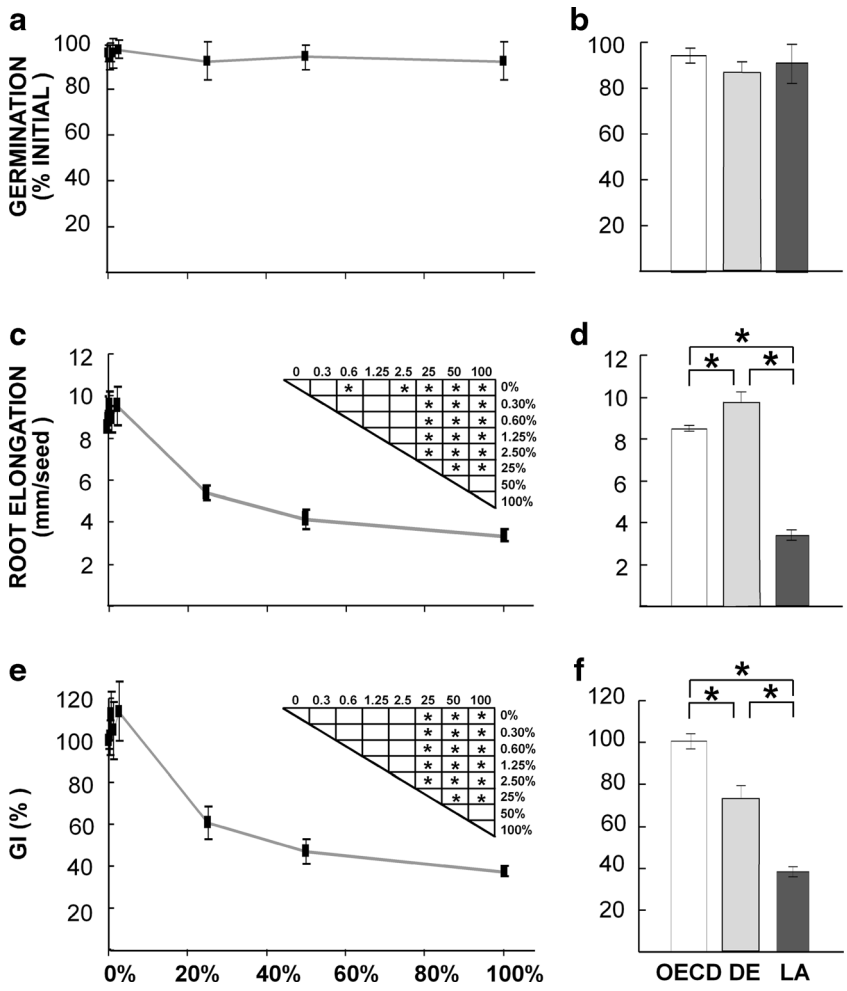


**Fig. 3** Lettuce seed germination (% of initial seed number; **a**, **b**), root elongation (mm/seed; **c**, **d**) and Germination Index (GI, %; **e**, **f**) after exposure to Lanestosa (LA) soil mixed with OECD artificial soil in a dilution series (**a**, **c**, **e**) and comparison of the effects on the lettuce seeds germination (**b**), root elongation (**d**) and GI (%, **f**) exposed to control soil (OECD), reference soil (DE) and test soil (LA). Values are shown as average± standard deviation. *$p \leq 0.05$, significantly different according to one-way ANOVA followed by Duncan's test

### Bioaccumulation in earthworms

*E. fetida* exposed to 2.5–25 % LA soil (350–3500 mg Zn/kg soil dry wt) accumulated Zn in the short term (3 days) but further on (7–14 days) this metal was fully regulated, reaching steady state tissue concentrations similar to previously reported (~170 μg Zn/g tissue dry wt; Asensio et al. 2013). Nevertheless, bioconcentration factor (BCF) was below "1" (BCF=0.48) on exposure to 25 % LA, which reflects that bioaccumulation was low. Similar low BCF values were also found in *E. fetida* and *Eisenia veneta* exposed to soil with high levels of Zn pollution whereas BCF over 1 were described on exposure to low Zn concentrations in soil (Hankard et al. 2004; Hønsi et al. 2003). Presently, the 14-day BCF was approximately "2" on exposure to 0.3–0.6 % Zn (~90 mg Zn/kg soil dry wt). Overall, Zn is known to be regulated in lumbricids (Beeby 1991; Lock and Janssen 2001a; Peijnenburg et al. 1999). Nevertheless, Zn regulation and metabolisation may provoke extra energetic demand and may result in oxidative stress (Asensio et al. 2013; Maity et al. 2008). Indeed, Zn toxicity has been reported in *E. fetida* (Asensio et al. 2013; Conder and Lanno 2000; Rodríguez-Ruiz 2010; Spurgeon and Hopkin 1996a).

The Pb tissue concentration was very variable but increased on exposure to 25 % LA soil (~1550 mg Pb/kg soil dry wt) after 3 and 7 days exposure in absence of food supply. Some regulation of Pb uptake may occur at low exposure levels (Davies et al. 2003), but this is especially relevant when OM% >10 % (Asensio 2009; Scaps et al. 1997). In LA soil, OM% is 4.99 and therefore regulation would be less effective, which could explain the high variability found in tissue Pb concentrations at concentrations lower than 25 % LA soil. The Pb 7-day BCF on exposure to 25 % LA soil is high (BCF=2.8), whereas the 14-day BCF on exposure to 2.5 % LA soil is much lower (BCF= 0.63). Previous studies in *E. fetida* have reported values of BCF lower than 1 (Asensio 2009; Spurgeon and Hopkin 1996b). This metal is known to provoke toxic effects in this species (14-day LC50=2827–7000 mg Pb/kg soil dry wt; Davies et al. 2003; Spurgeon et al. 1994; body weight loss 14-day EC50=6670±162 mg Pb/kg soil dry wt; Nahmani et al. 2007). These critical concentrations are in the range of those measured in 50 and 100 % LA soil, which could explain,

at least partially, the mortality recorded in this experiment (see below).

A linear increase in Cd earthworm tissue concentration was described in *E. fetida* exposed to Avenmouth soil (222 μg Cd/g tissue dry wt; bioaccumulation factor (BAF)=4.09) and to Shipham soil (493 μg Cd/g tissue dry wt; BAF=1.42) (Nahmani et al. 2009). However, we were unable to determine whether Cd was accumulated in earthworms maybe due to the mortality recorded in the high concentration exposure groups. Although *E. fetida* survives and bioaccumulates Cd after single exposure to higher concentrations of the metal than those presently recorded (10–1000 mg Cd/kg soil dry wt; BCF=0.79–5.69; Asensio 2009), it is conceivable that toxicity is enhanced by the presence of other pollutants (Asensio 2009; Conder and Lanno 2000; Lukkari et al. 2005; Rodríguez-Ruiz 2010).

## Toxicity

### *Microbes*

Alterations in soil metabolism constitute a general indication of perturbations in soils (Epelde et al. 2009). Soil respiration represents the sum of all soil metabolic processes producing carbon dioxide (Margesin et al. 2000; Singh and Gupta 1977). The presence of high levels of metals in soil provokes toxic effects on soil microorganisms and impedes their biological activity (Giller et al. 1998). Accordingly, soil respiration in LA soil was significantly lower than in the reference soil (DE soil), but respiration was not affected in RO soil.

LA soil and $LA_{APS}$ were found to be toxic to *V. fischeri* (IC50 <1000 mg/L). However, neither RO soil nor LA and RO soil elutriates were toxic (Environment Canada 2002). Similarly, *V. fischeri* exposed to a soil from a former industrial area containing Cr, Cu, Ni and Pb at maximum concentrations of 7.35 mg Cr/kg, 51.65 mg Cu/kg, 4.02 mg Ni/kg and 1052 mg Pb/kg showed high toxicity although no effects were recorded when it was exposed to the water-soluble fraction (4.3 μg Cr/kg, 260 μg Cu/kg, 4.5 μg Ni/kg and 200 μg Pb/kg; Maisto et al. 2011). Nonetheless, *V. fischeri* exposed to a soil collected at an adjacent roadside urban park (3.52 mg Cr/kg, 147.94 mg Cu/kg, 3.27 mg Ni/kg and 181.48 mg Pb/kg) showed slight toxic effects, and it showed moderate toxic effects when exposed to its water-soluble fraction (1.8 μg Cr/kg, 550 μg Cu/kg, 7.7 μg Ni/kg and 70 μg Pb/kg; Maisto et al. 2011). Gray and O'Neill (1997) calculated an IC50 of

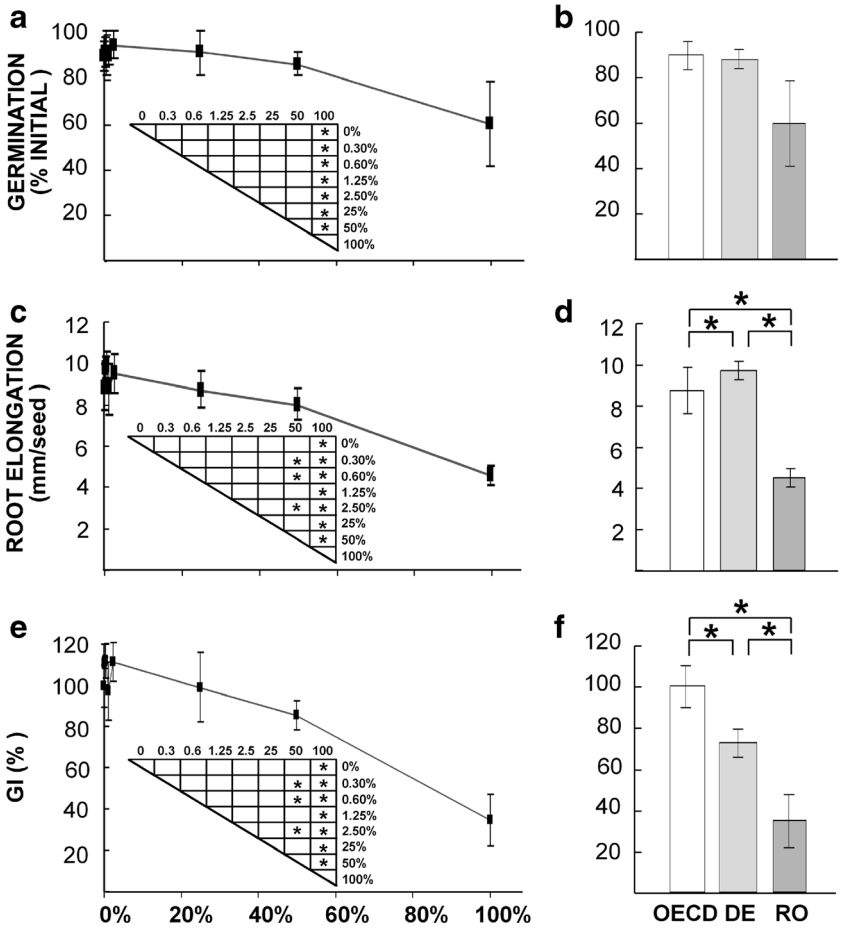


**Fig. 4** Lettuce seed germination (% of initial seed number; **a**, **b**), root elongation (mm/seed; **c**, **d**) and Germination Index (GI, %; **e**, **f**) after exposure to runoff (RO) soil mixed with OECD artificial soil in a dilution series (**a**, **c**, **e**) and comparison of the effects on the lettuce seeds germination (**b**), root elongation (**d**) and GI (%, **f**) exposed to control soil (OECD), reference soil (DE) and test soil (RO). Values are shown as average± standard deviation. *$p \leq 0.05$, significantly different according to one-way ANOVA followed by Duncan's test

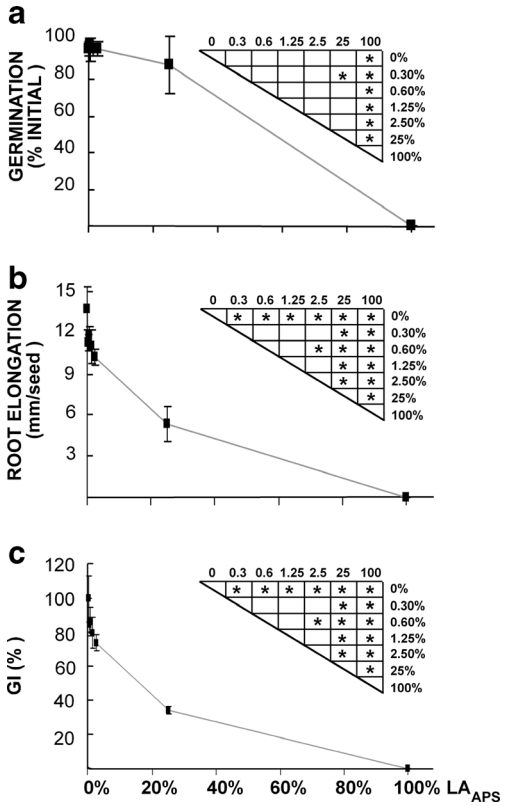


**Fig. 5** Lettuce seed germination (% of initial seed number, **a**), root elongation (mm/seed, **b**) and Germination Index (GI, %, **c**) after exposure to artificial Lanestosa ($LA_{APS}$) soil mixed with DE soil in a dilution series. Values are shown as average±standard deviation. *$p \leq 0.05$, significantly different according to one-way ANOVA followed by Duncan's test

2.20 % after exposing *V. fischeri* to the acid mine drainage from Avoca mines (88.2 mg Zn /L, 1.5 mg Pb/L and 5.6 mg Mn/L). LA soil Zn and Pb concentration were much above Avoca mine drainage concentrations but the Zn-to-Pb and Cd-to-Pb ratios were similar (Zn/Pb~50; Cd/Pb~0.2).

The concentrations of Zn and Pb in the estimated EC50 (18.8 % LA soil, 2748 mg Zn/kg soil dry wt, 1140 mg Pb/kg soil dry wt, Cd u.d.l.) were above their respective IVA-C values (Ihobe 1998b; Table 4) and therefore LA soil possessed an unacceptable risk according to this test. In RO soil, although IC50 did not reach values indicative of solid phase toxicity (Environment Canada 2002), the concentrations of Zn and Pb were over their respective IVA-B values, whereas that of Cd was just below its IVA-B value (Table 4, Ihobe 1998b).

*Protists*

Exposure to LA soil provoked severe toxic effects in *D. discoideum*. These included a significant reduction in the number of multicellular units (lowered MU%), stepping up of the migration and culmination stages and shrinkage of the fruiting bodies. Accordingly, it resulted in a significant IFBF after 24 h. This would indicate a severe impact in the potential to produce spores and it might therefore compromise the population fitness. The profile is similar to that obtained after exposure to a brownfield soil polluted with metals and hydrocarbons except that then enlargement of fruiting bodies was recorded instead (Rodríguez-Ruiz et al. 2014). Overall, it seems that LA soil affected viability of amoeba in soil and aggregation (i.e. cAMP signalling or cell movement; Gross 1994; Meima and Schaap 1999; Ponte et al. 2000), since both the number of multicellular units and the size of fruiting bodies resulted severely reduced after exposure to this soil. Accordingly, cell replication was significantly affected in amoeba treated with the LA soil DIN 38414 elutriate, which suggests that cell viability was compromised by the water-

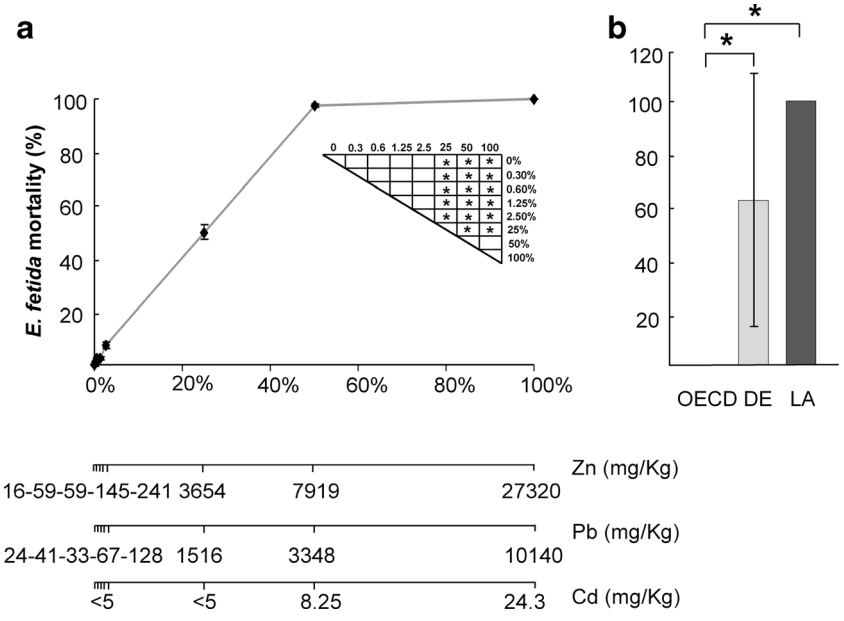


**Fig. 6** Cumulative mortality (%) of *E. fetida* after 14-day exposure to LA soil (test soil) and its dilutions in OECD soil (control soil) with indication of Zn, Pb and Cd concentrations at each experimental dilution (**a**). Cumulative mortality after 14-day exposure to 100 % OECD soil (control soil), 100 % DE soil (reference soil) and 100 % LA soil (test soil) (**b**). Values are shown as average±standard deviation. *$p \leq 0.05$, significant difference according to one-way ANOVA followed by Duncan's test

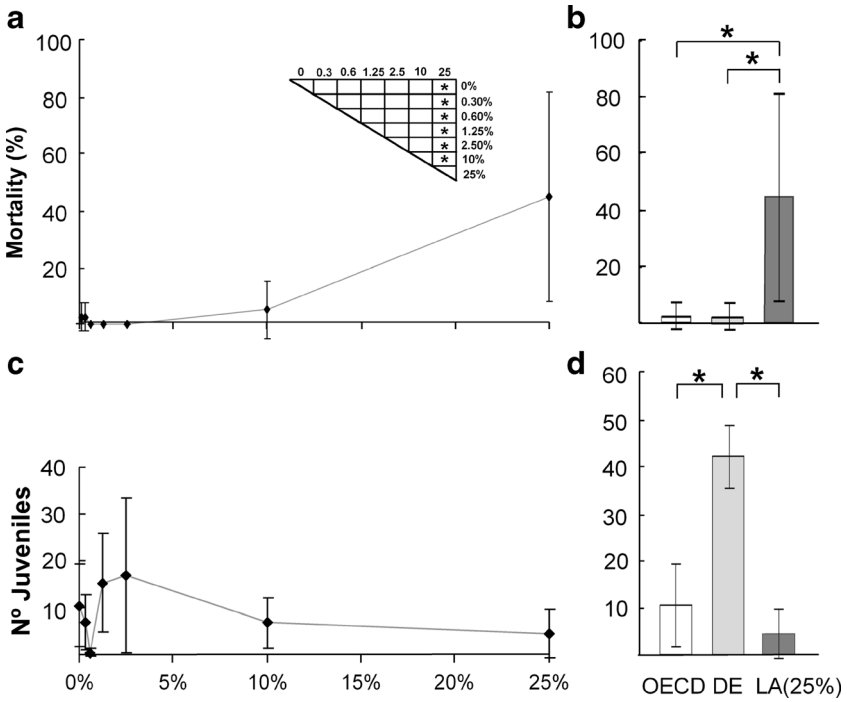


**Fig. 7** Cumulative mortality (%) of *E. fetida* after 28-day exposure to LA soil (test soil) and its dilutions in OECD soil (control soil) (**a**). Cumulative mortality after 28-day exposure to 100 % OECD soil (control soil), 100 % DE soil (reference soil) and 25 % LA soil (test soil) (**b**). *E. fetida* juvenile numbers after 56-day exposure to LA soil and its dilutions in OECD soil (**c**). Juvenile numbers after 56-day exposure to 100 % OECD, 100 % DE and 25 % LA soil (**d**). Values are shown as average±standard deviation. *$p \leq 0.05$, significantly different according to one-way ANOVA followed by Duncan's test

soluble fraction of pollutants and less and smaller colonies were established. In contrast, it seems that successful multicellular units progressed more quickly into migration and culmination phases, which can be either the result of less competence at lower densities of individuals or an adaptation mechanism to compensate the impact on colony formation (Rodríguez-Ruiz et al. 2014). Since fruiting body size was reduced, we cannot exclude that, despite of the apparent stepping up, toxicity might have affected $H^+$-ATPases, membrane receptors and cytoskeleton components needed to progress to migration and culmination, respectively (Alvarez-Curto 2007; Gross 1994; Sroka et al. 2001). *D. discoideum* IFBF EC50 was 48.2 % LA soil. This IFBF EC50 value is higher than the one calculated for LG soil (Rodríguez-Ruiz et al. 2014), which indicates that LA soil is less toxic to *D. discoideum* than LG soil. However, like other soils polluted with heavy metals and organic pollutants, 100 % LA soil provokes 100 % IFBF and must therefore be considered "very toxic" (Balbo and Bozzaro 2008). It is worth noting that the concentration of individual pollutants in 48.2 % LA soil (7634 mg Zn/kg soil dry wt, 3227 mg Pb/kg soil dry wt, 8 mg Cd/kg soil dry wt) was either above IVA-B (Cd) or IVA-C (Zn and Pb) values (Ihobe 1998b; Table 4). As a whole, it is concluded that the ecological fitness of *D. discoideum* (capacity to resist starvation and start a new vegetative cycle in presence of food) is severely reduced in LA soil.

RO soil was marginally toxic to *D. discoideum*, with a slight but significant reduction in the number of multicellular units (lowered MU%), little quickening in aggregation, marked arrest in culmination and prominent enlargement of fruiting bodies. The profile is different from those obtained after exposure to LA soil and to other polluted soils (Rodríguez-Ruiz et al. 2014). It seems that RO soil had a certain effect on viability of amoebae. Indeed, cell replication was severely affected by the RO soil elutriate, even more than by the LA soil elutriate. Besides, arrest in the progress from culminant to fruiting body was the most outstanding effect and might explain the presence of larger fruiting bodies. These large fruiting bodies might contribute to improve the potential to produce spores and thus might attenuate the consequences for population fitness. Nevertheless, as a result of these changes in DDDC, a significant inhibition IFBF was observed after 24 h. Considering the toxicity scale proposed by Balbo and Bozzaro (2008) for IFBF, RO soil would be graded as "toxic" (57 % IFBF in 100 % RO soil). Thus, RO soil would be less toxic than LA soil. Finally, IFBF EC50 was 34 % RO soil, very similar to the EC50 estimate for *V. fischeri* and hence would correspond to soil concentrations of Zn and Pb over their respective IVA-B values (Table 4; Ihobe 1998b).

### *Plants*

Germination of *L. sativa* exposed to LA soil was less sensitive (EC50 >100 % LA soil) than root elongation and GI (%) (EC50=66.5 and 65.6 % LA soil, respectively). Accordingly, seed germination is known to be less sensitive than root elongation (Eom et al. 2007; Plaza et al. 2005). Some metals like Pb affect root elongation but not germination (Chang et al. 1997). Likewise, LA soil DIN 38414 elutriate only affected lettuce root elongation. However, it was not

toxic to radish. Similarly, Escoto Valerio et al. (2007) found no effect on lettuce germination after testing the water-soluble fraction of Zn, Pb and other metals present at high concentrations in two soils affected by the Aznalcóllar pyrite-mine spill. Presently, despite of the moderate toxicity to lettuce and the lack of toxicity to radish, individual pollutants in 66.5 % LA soil (10,532 mg Zn/kg soil dry wt, 4453 mg Pb/kg soil dry wt, 11 mg Cd/kg soil dry wt) were over their respective IVA-C values (Zn and Pb) and IVA-B value (Cd).

RO soil seemed to be only marginally toxic to lettuce, as only GI EC50 could be estimated (87.11 % RO soil). Lettuce root elongation has been previously inhibited by 18 mg Zn/L (Wang 1987). Presently, the concentration of individual pollutants in 87.11 % RO soil (838 mg Zn/kg soil dry wt, 512 mg Pb/kg soil dry wt, 1.5 mg Cd/kg soil dry wt) was above IVA-C (Pb), just below IVA-C (Zn) and over IVA-B (Cd) values (Table 4; Ihobe 1998b). Thus, IVA values would be almost overestimating toxicity to lettuce, even in presence of mixtures that may modify the toxicity of individual pollutants. Interestingly, RO DIN 38414 elutriate resulted to be toxic to lettuce and radish, with around 70 and 75 % effect on GI (%), respectively.

When exposed to $LA_{APS}$, lettuce EC50 was 46.4 % $LA_{APS}$ for germination, 17.9 % $LA_{APS}$ for root elongation and 17.4 % $LA_{APS}$ for GI (%). The concentration of individual pollutants in 17.4 % $LA_{APS}$ (2240 mg Zn/kg soil dry wt, 669 mg Pb/kg soil dry wt, 3.7 mg Cd/kg soil dry wt) was above IVA-C (Zn and Pb) or IVA-B (Cd) values (Table 4; Ihobe 1998b). In addition, in soils with high EC (EC >2 mS/cm), such as $LA_{APS}$, a salinity effect can provoke additional effects on plant germination and root elongation (Hoekstra et al. 2002), which might interact with the effects exerted by metals in $LA_{APS}$. On the other hand, $LA_{APS}$ DIN 38414 elutriate provoked dramatically significant effects in lettuce and radish root elongation and GI (%). Consequently, for similar concentration of metals in the soil, the toxicity for different test species is markedly lower in the aged (LA) than in the freshly made ($LA_{APS}$) soil, in agreement with previous studies (Alexander 2000; Lock and Janssen 2001b; Peijnenburg and Vijver 2009; Smolders et al. 2009).

*Earthworms*

LA soil was highly toxic to *E. fetida* as it impaired survival and juvenile production already at 25 % LA soil and 10 % LA soil, respectively. In previous works, 100 % LA soil provoked severe affection in enzyme activity, metabolic status, cellular integrity and individual condition in *E. fetida* after 3-day exposure ex situ, which finally resulted in massive death beyond 5-day exposure (Asensio et al. 2013). In contrast, Alvarenga et al. (2008) did not observe earthworm mortality after exposing *E. fetida* to the acid metal-contaminated soil from the Aljustrel mining area (2.6 mg Cd/kg total, 0.20 mg Cd/L exchangeable; 1250 mg Pb/kg total, 2.90 mg Pb/L exchangeable; 254 mg Zn/kg total, 1.06 mg Zn/L exchangeable). Likewise, although reproduction was affected, no mortality was recorded in *E. fetida* exposed to soils with high Zn, Pb, Cu and Cd concentrations (up to 32,871 mg Zn/kg, 15,996 mg Pb/kg, 2609.4 mg Cu/kg and 312.2 mg Cd/kg dry wt soil) collected from seven sites in the vicinity of smelting works in Avonmouth (England) (Spurgeon and Hopkin 1995). Presently, toxicity was elicited at much lower concentrations of Zn, Pb and Cd. LC50 was 25–27 % LA soil (3917 mg Zn/kg soil dry wt, 1625 mg Pb/kg soil dry wt and Cd under detection limit) and EC50 (juvenile number) was 17.4 % LA soil (2543 mg Zn/kg, 1055 mg Pb/kg and Cd under detection limit). Nevertheless, the concentration of Zn and Pb was over their respective IVA-C values (Ihobe 1998b; Table 4). Moreover, although Cd concentration in soil was under detection limits in 25 % LA soil, earthworms accumulated the metal with tissue levels of 11.4 μg Cd/g tissue dry wt after 7-day exposure to 25 % LA soil and of 10.8 μg Cd/g tissue dry wt after 14-day exposure to 2.5 % LA soil. It cannot be disregarded that Cd may result toxic even at so low soil concentrations. Indeed, it has been demonstrated that, when compared with single Cd exposure (which is the basis of IVA values), ten times lower soil Cd concentrations are sufficient to elicit toxicity in presence of Zn and diesel (Rodríguez-Ruiz 2010).

## Concluding remarks

Upon the application of a battery of toxicity bioassays with a selected set of test species (*V. fischeri*, *D. discoideum*, *L. sativa*, *R. sativus* and *E. fetida*) in combination with chemical analysis of soils, elutriates and earthworm tissues, the soil at Lanestosa mine site was revealed to be toxic, although the toxicity was spatially heterogeneous. LA soil resulted to be toxic according to the majority of the endpoints investigated (soil respiration, solid phase Microtox®, *D. discoideum* vegetative and developmental cycle, lettuce root elongation and Germination Index, earthworm acute toxicity and reproduction tests). However, lettuce and radish were not severely affected neither by LA soil nor by its water-soluble fraction. Thus, the multi-endpoint bioassay approach confirmed that LA soil possessed an unacceptable risk for the environment and demanded intervention. In contrast, although RO soil posed an unacceptable risk for the environment as regards the soil concentrations of Pb and Zn, metal extractability and toxicity were much lower than in LA soil. Thus, the environmental risk was seemingly attenuated towards the runoff area. Therefore, the need for intervention varied between different areas of the same polluted site. Besides, $LA_{APS}$, with a high exchangeable fraction of metals, led to much higher toxicities to *V. fischeri* and to plants than LA soil. Accordingly, it

was concluded that ageing under the natural conditions of the investigated scenario contributed to attenuate the environmental risk of LA soil, which might be relevant to decide whether intervention or simply natural attenuation is needed.

As a whole, the combination of multiple endpoint bioassays conducted with a suite of test organisms from different taxonomical groups and ecological levels (*V. fischeri*, *D. discoideum*, *L. sativa*, *R. sativus* and *E. fetida*) and scenario-targeted analysis (including the consequences of leaching -e.g., runoff- and ageing) provides a reliable environmental risk assessment in soils posing unacceptable environmental risk according to SVs and can be useful to optimise the design of the required intervention measures. An equivalent approach is suitable to be applied for scenario-targeted soil risk assessment in those cases in which national/regional soil legislation is based on SVs, as previously suggested by Rodriguez-Ruiz et al. (2014).

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## References

Achazi R (2002) Invertebrates in risk assessment development of a test battery and of short term biotests for ecological risk assessment of soil. J Soils Sediments 2:174–178

Alef K, Nannipieri P (1995) Estimation of microbial activities. In: Alef K, Nannipieri P (eds) Methods in applied soil microbiology and biochemistry. Academic, London, pp 193–270

Alexander M (2000) Aging, bioavailability, and overestimation of risk from environmental pollutants. Environ Sci Technol 34:4259–4265

Alvarenga P, Palma P, Gonçalves A, Fernandes R, de Varennes A, Vallini G, Duarte E, Cunha-Queda A (2008) Evaluation of tests to assess the quality of mine-contaminated soils. Environ Geochem Health 30: 95–99

Alvarez-Curto E (2007) Developmental regulation and evolution of cAMP signalling in *Dictyostelium*. PhD Dissertation, Fac Science, Leiden University

Asensio V, Rodríguez-Ruiz A, Garmendia L, Andre J, Kille P, Morgan AJ, Soto M, Marigómez I (2013) Towards an integrative soil health assessment strategy: a three tier (integrative biomarker response) approach with *Eisenia fetida* applied to soils subjected to chronic metal pollution. Sci Total Environ 442:344–365

Asensio V (2009) Health assessment of polluted soils after *Eisenia foetida* ex situ bioassays based on conventional and in vitro cellular biomarkers and microarray technology. PhD Dissertation, Univ Basque Country (UPV/EHU)

Azur Environmental (1995a) Microtox acute toxicity basic test procedures (basic SPT). Carlsbad, CA

Azur Environmental (1995b) Microtox acute toxicity solid-phase test procedures (solid-phase SPT). Carlsbad, CA

Balbo A, Bozzaro S (2008) A novel bioassay for evaluating soil biohazards using *Dictyostelium* as biosensor: validation and application to the Bio-Bio Project. Fresenius Environ Bull 17:1137–1143

Barrutia O, Garbisu C, Hernandez-Allica J, Garcia-Plazaola JI, Becerril JM (2010) Differences in EDTA-assisted metal phytoextraction between metallicolous and non-metallicolous accessions of *Rumex acetosa* L. Environ Pollut 158:1710–1715

Beeby A (1991) Toxic metal uptake and essential metal regulation in terrestrial invertebrates: a review. In: Newman MC, McIntosh AW (eds) Metal ecotoxicology: concepts and applications. Lewis, Chelsea, pp 65–89

Bierkens J, Klein G, Corbisier P, Van Den Heuvel R, Verschaeve L, Weltens R, Schoeters G (1998) Comparative sensitivity of 20 bioassays for soil quality. Chemosphere 37:2935–2947

Brown RB (2003) Soil texture. Univ Florida, IFAS Extension. Available: http://ufdc.ufl.edu/IR00003107/00001 (last access, 11/03/2014)

Carlon C (2007) Derivation methods of soil screening values in Europe. A review and evaluation of national procedures towards harmonisation. European Commission, Joint Research Centre, Ispra, EUR 22805 EN: 306

Chang LW, Meier JR, Smith MK (1997) Application of plant and earthworm bioassays to evaluate remediation of a lead-contaminated soil. Arch Environ Contam Toxicol 32:166–171

Conder JM, Lanno RP (2000) Evaluation of surrogate measures of cadmium, lead and zinc bioavailability to *Eisenia fetida*. Chemosphere 41:1659–1668

Cook SV, Chu A, Goodman RH (2002) Leachability and toxicity of hydrocarbons, metals and salt contamination from flare pit soil. Water Air Soil Pollut 133:297–314

Critto A, Torresan S, Semenzin E, Giove S, Mesman M, Schouten AJ, Rutgers M, Marcomini A (2007) Development of a site-specific ecological risk assessment for contaminated sites: part I. A multicriteria based system for the selection of ecotoxicological tests and ecological observations. Sci Total Environ 379:16–33

Davies NA, Hodson ME, Black S (2003) The influence of time on lead toxicity and bioaccumulation determined by the OECD earthworm toxicity test. Environ Pollut 121:55–61

Diez M, Simon M, Martin F, Dorronsoro C, Garcia I, Van Gestel CAM (2009) Ambient trace element background concentrations in soils and their use in risk assessment. Sci Total Environ 407:4622–4632

DIN (1984) DIN 38414-S4 Deutsche Einheitsverfahren zur Wasser-Abwasser- und Schlammuntersuchung-Schlamm und Sedimente-Bestimmung der Eluierbarkeit mit Wasser. Deutsches Institut für Normung e.V, Berlin

Dondero F, Jonsson H, Rebelo M, Pesce G, Berti E, Pons G, Viarengo A (2006) Cellular responses to environmental contaminants in amoebic cells of the slime mould *Dictyostelium discoideum*. Comp Biochem Physiol 143C:150–157

Environment Canada (2002) Biological test method: reference method for determining the toxicity of sediment using luminescent bacteria in a solid-phase test EPS 1/RM/42.

Eom IC, Rast C, Veber AM, Vasseur P (2007) Ecotoxicity of a polycyclic aromatic hydrocarbon (PAH)-contaminated soil. Ecotoxicol Environ Saf 67:190–205

Epelde L, Becerril JM, Barrutia O, Gonzalez-Oreja JA, Garbisu C (2010) Interactions between plant and rhizosphere microbial communities in a metalliferous soil. Environ Pollut 158:1576–1583

Epelde L, Mijangos I, Becerril JM, Garbisu C (2009) Soil microbial community as bioindicator of the recovery of soil functioning derived from metal phytoextraction with sorghum. Soil Biol Biochem 41:1788–1794

Escoto Valerio M, Fernandez Garcia J, Martin Peinado F (2007) Determination of phytotoxicity of soluble elements in soils, based on a bioassay with lettuce (*Lactuca sativa* L.). Sci Total Environ 378:63–66

Eusko Jaurlaritza/Gobierno Vasco (2007) Plan de suelos contaminados del Pais Vasco (2007–2012). 1–149

Fernández MD, Tarazona JV (2008) Complementary approaches for using ecotoxicity data in soil pollution evaluation: risk based versus direct toxicity assessment. In: Dominguez, J. V. (ed) Soil contamination research trends, pp 1–50

Giller KE, Witter E, McGrath SP (1998) Toxicity of heavy metals to microorganisms and microbial processes in agricultural soils: a review. Soil Biol Biochem 30:1389–1414

Gleyzes C, Tellier S, Astruc M (2002) Fractionation studies of trace elements in contaminated soils and sediments: a review of sequential extraction procedures. TrAC Trends Anal Chem 21:451–467

Gray NF, O'Neill C (1997) Acid mine-drainage toxicity testing. Environ Geochem Health 19:165–171

Greene JC, Bartels CL, Warren-Hicks WJ, Parkhurst BR, Linder GL, Peterson SA, Miller WE (1989) Protocols for short term toxicity screening of hazardous waste sites, USEPA, Corvallis, OR, 1989, EPA 600/3-88/029

Gross JD (1994) Developmental decisions in *Dictyostelium discoideum*. Microbiol Mol Biol Rev 58:330–351

Hankard PK, Svendsen C, Wright J, Wienberg C, Fishwick SK, Spurgeon DJ, Weeks JM (2004) Biological assessment of contaminated land using earthworm biomarkers in support of chemical analysis. Sci Total Environ 330:9–20

Hoekstra NJ, Bosker T, Lantinga EA (2002) Effects of cattle dung from farms with different feeding strategies on germination and initial root growth of cress (*Lepidium sativum* L.). Agric Ecosyst Environ 93:189–196

Hønsi TG, Stubberud HE, Andersen S, Stenersen J (2003) Lysosomal fragility in earthworms (*Eisenia veneta*) exposed to heavy metal contaminated soils from two abandoned pyrite ore mines in southern Norway. Water Air Soil Pollut 142:27–37

Hund-Rinke K, Kördel W, Hennecke D, Achazi R, Warnecke D, Wilke BM, Winkel B, Heiden S (2002a) Bioassays for the ecotoxicological and genotoxicological assessment of contaminated soils (results of a round robin test). Part II. Assessment of the habitat function of soils—tests with soil microflora and fauna. J Soils Sediments 2: 83–90

Hund-Rinke K, Kördel W, Hennecke D, Eisenträger A, Heiden S (2002b) Bioassays for the ecotoxicological and genotoxicological assessment of contaminated soils (results of a round robin test). Part I. Assessment of a possible groundwater contamination: ecotoxicological and genotoxicological tests with aqueous soil extracts. J Soils Sediments 2:43–50

Ihobe SA (1998a) Valores indicativos de evaluación (VIE-A). Nivel de referencia. Eusko Jaurlaritza/Gobierno Vasco Vitoria-Gasteiz, Autonomous Community of the Basque Country (Spain), 45 pp

Ihobe SA (1998b) Valores indicativos de evaluación (VIE-B, VIE-C) para la protección de los ecosistemas. Eusko Jaurlaritza/Gobierno Vasco. Vitoria-Gasteiz, Autonomous Community of the Basque Country (Spain), 104 pp

INIA, IGME, Ministerio de Medio Ambiente (2007) Anexo E: Metodología para la caracterización toxicológica de una muestra de suelo. Version Web Guía metodológica de aplicación del RD 9/2005, de 14 de enero, por el que se establece la relación de actividades potencialmente contaminantes del suelo y los criterios y estándares para la declaración de suelos contaminados (BOE núm. 15, de 18 enero 2005). 114–119

ISO (1993) Soil quality—determination of dry matter and water content on a mass basis—gravimetric method. ISO 11465

ISO (2005) Soil quality—determination of pH. International Standard. 10390, 1–7

ISO (2012) Soil quality—effects of pollutants on earthworms—part 2: determination of effects on reproduction of *Eisenia fetida/Eisenia andrei*. 11268–2

Lock K, Janssen CR (2001a) Zinc and cadmium body burdens in terrestrial oligochaetes: use and significance in environmental risk assessment. Environ Toxicol Chem 20:2067–2072

Lock K, Janssen CR (2001b) Cadmium toxicity for terrestrial invertebrates: taking soil parameters affecting bioavailability into account. Ecotoxicology 10:315–322

Lukkari T, Aatsinki M, Väisänen A, Haimi J (2005) Toxicity of copper and zinc assessed with three different earthworm tests. Appl Soil Ecol 30:133–146

Maisto G, Manzo S, De Nicola F, Carotenuto R, Rocco A, Alfani A (2011) Assessment of the effects of Cr, Cu, Ni and Pb soil contamination by ecotoxicological tests. J Environ Monit 13:3049–3056

Maity S, Roy S, Chaudhury S, Bhattacharya S (2008) Antioxidant responses of the earthworm *Lampito mauritii* exposed to Pb and Zn contaminated soil. Environ Pollut 151:1–7

Margesin R, Walder G, Schinner F (2000) The impact of hydrocarbon remediation (diesel oil and polycyclic aromatic hydrocarbons) on enzyme activities. Acta Biotechnol 20:313–333

Meers E, Du Laing G, Unamuno V, Ruttens A, Vangronsveld J, Tack FMG, Verloo MG (2007) Comparison of cadmium extractability from soils by commonly used single extraction protocols. Geoderma 141:247–259

Meima M, Schaap P (1999) *Dictyostelium* development—socializing through cAMP. Semin Cell Dev Biol 10:567–576

Nahmani J, Hodson ME, Black S (2007) Effects of metals on life cycle parameters of the earthworm *Eisenia fetida* exposed to field-contaminated, metal-polluted soils. Environ Pollut 149:44–58

Nahmani J, Hodson ME, Devin S, Vijver MG (2009) Uptake kinetics of metals by the earthworm *Eisenia fetida* exposed to field-contaminated soils. Environ Pollut 157:2622–2628

NEN (1994) Determination of organic matter content in soil as loss-on-ignition. NEN 5754

NEN (1997) Soil—determination of mineral oil content in soil and sediments with gas chromatography. NEN 5733

OECD (1984) Earthworm, acute toxicity tests. OECD guideline for the testing of chemicals No. 207: 1–9

OECD (2004) Earthworm reproduction test (*Eisenia fetida/andrei*). OECD guideline for the testing of chemicals No. 222: 1–18

Oleszczuk P, Jośko I, Kuśmierz M, Futa B, Wielgosz E, Ligęza S, Pranagal J (2014) Microbiological, biochemical and ecotoxicological evaluation of soils in the area of biochar production in relation to polycyclic aromatic hydrocarbon content. Geoderma 213:502–511

Peijnenburg WJGM, Baerselman R, de Groot AC, Jager T, Posthuma L, Van Veen RPM (1999) Relating environmental availability to bioavailability: soil-type-dependent metal accumulation in the oligochaete *Eisenia andrei*. Ecotoxicol Environ Saf 44:294–310

Peijnenburg WJGM, Vijver MG (2009) Earthworms and their use in eco(toxico)logical modeling. In: Devillers, J. (ed) Ecotoxicology modeling. Emerging topics in ecotoxicology: principles, approaches and perspectives 2, pp 177–204

Pietrantonio M, Calace N, Petronio BM, Pietroletti M (2003) Laboratory-polluted soils: a methodological approach to establish equilibrium conditions for different metal chemical forms in soils. J Environ Monit 5:451–454

Plaza G, Nalecz-Jawecki G, Ulfig K, Brigmon RL (2005) The application of bioassays as indicators of petroleum-contaminated soil remediation. Chemosphere 59:289–296

Ponte E, Rivero F, Fechheimer M, Noegel A, Bozzaro S (2000) Severe developmental defects in *Dictyostelium* null mutants for actin-binding proteins. Mech Dev 91:153–161

Pueyo M, Sastre J, Hernandez E, Vidal M, Lopez-Sanchez JF, Rauret G (2003) Prediction of trace element mobility in contaminated soils by sequential extraction. J Environ Qual 32:2054–2066

Rauret G, Lopez-Sanchez JF, Sahuquillo A, Rubio R, Davidson C, Ure A, Quevauviller P (1999) Improvement of the BCR three step

sequential extraction procedure prior to the certification of new sediment and soil reference materials. J Environ Monit 1:57–61

Reijnders L (2009) Are soil pollution risks established by governments the same as actual risks? Appl Environ Soil Sci 237038. doi: 10.1155/2009/237038

Renoux AY, Sunahara GI (2002) Introduction. In: Renoux GI, Thellen AY, Gaudet, CL and Pilon A (ed) Environmental analysis of contaminated sites, Wiley, pp 3–8

Rodríguez-Ruiz A, Asensio V, Zaldibar B, Soto M, Marigómez I (2014) Toxicity assessment through multiple endpoint bioassays in soils posing environmental risk according to regulatory screening values. Environ Sci Pollut Res. doi:10.1007/s11356-014-2915-7

Rodríguez-Ruiz A, Marigómez I, Boatti L, Viarengo A (2013) *Dictyostelium discoideum* developmental cycle (DDDC) assay: a tool for Hg toxicity assessment and soil health screening. Sci Total Environ 450–451:39–50

Rodríguez-Ruiz A (2010) Risk assessment in real soils from the Basque Country after soil health screening through toxicity profiles based on standard and novel multiple endpoint bioassays. PhD Dissertation, Univ Basque Country

Scaps P, Grelle C, Descamps M (1997) Cadmium and lead accumulation in the earthworm *Eisenia fetida* (Savigny) and its impact on cholinesterase and metabolic pathway enzyme activity. Comp Biochem Physiol 116C:233–238

Sforzini S, Dagnino A, Torrielli S, Dondero F, Fenoglio S, Negri A, Boatti L, Viarengo A (2008) Use of highly sensitive sublethal stress responses in the social amoeba *Dictyostelium discoideum* for an assessment of freshwater quality. Sci Total Environ 395:101–108

Singh J, Gupta S (1977) Plant decomposition and soil respiration in terrestrial ecosystems. Bot Rev 43:449–528

Smolders E, Oorts K, Van Sprang P, Schoeters I, Janssen CR, McGrath SP, McLaughlin MJ (2009) Toxicity of trace metals in soil as affected by soil type and aging after contamination: using calibrated bioavailability models to set ecological soil standards. Environ Toxicol Chem 28:1633–1642

Spurgeon DJ, Hopkin SP (1995) Extrapolation of the laboratory-based OECD earthworm toxicity test to metal-contaminated field sites. Ecotoxicology 4:190–205

Spurgeon DJ, Hopkin SP (1996a) Effects of metal-contaminated soils on the growth, sexual development, and early cocoon production of the earthworm *Eisenia fetida*, with particular reference to zinc. Ecotoxicol Environ Saf 35:86–95

Spurgeon DJ, Hopkin SP (1996b) Risk assessment of the threat of secondary poisoning by metals to predators of earthworms in the vicinity of a primary smelting works. Sci Total Environ 187:167–183

Spurgeon DJ, Hopkin SP, Jones DT (1994) Effects of cadmium, copper, lead and zinc on growth, reproduction and survival of the earthworm *Eisenia fetida* (Savigny): assessing the environmental impact of point-source metal contamination in terrestrial ecosystems. Environ Pollut 84:123–130

Sroka J, Madeja Z, Galanty A, Michalik M, Przestalski S, Rakoczy L, Korohoda W (2001) Trimethyltin inhibits the chemotaxis of *Dictyostelium discoideum* amoebae. Eur J Protistol 37:313–326

Tessier A, Campbell PGC, Bisson M (1979) Sequential extraction procedure for the speciation of particulate trace metals. Anal Chem 51: 844–851

Urzelai A, Vega M, Angulo E (2000) Deriving ecological risk-based soil quality values in the Basque Country. Sci Total Environ 247:279–284

USEPA (1996a) Microwave assisted acid digestion of siliceous and organically based matrices. Method 3052:1–20

USEPA (1996b) Ultrasonic extraction. Method 3550B:1–11

USEPA (2007) Inductively coupled plasma-atomic emission spectrometry. Method 6010C:1–34

Van Gestel CAM, Van der Waarde JJ, Derksen JGM, van der Hoek EE, Veul MFXW, Bouwens S, Rusch B, Kronenburg R, Stokman GNM (2001) The use of acute and chronic bioassays to determine the ecological risk and bioremediation efficiency of oil-polluted soils. Environ Toxicol Chem 20:1438–1449

Vasseur P, Bonnard M, Palais F, Eom IC, Morel J-L (2008) Bioavailability of chemical pollutants in contaminated soils and pitfalls of chemical analyses in hazard assessment. Environ Toxicol 23: 652–656

Velimirovic MB, Prica MD, Dalmacija BD, Roncevic SD, Dalmacija MB, Becelic MD, Trickovic JS (2010) Characterisation, availability, and risk assessment of the metals in sediment after aging. Water Air Soil Pollut 214:219–229

Wang W (1987) Root elongation method for toxicity testing of organic and inorganic pollutants. Environ Toxicol Chem 6:409–414