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Geochemistry and environmental threats of soils surrounding an abandoned mercury mine

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Abstract

The closure of mercury mining areas is generally associated with a release of Hg and other metals into the environment due to the abandonment of mining wastes. Because of their potential toxic properties, the mobilization of particulate and soluble metal species is of major concern. In the present study, the environmental risks posed by soils surrounding an abandoned mercury mining area in Valle del Azogue (Almeria, Spain) are assessed through the determination of physical-chemical parameters, the quantification of metal concentrations, and the application of aquatic and terrestrial ecotoxicity bioassays. Chemical analysis of soil samples revealed concentrations of Hg, As, Ba, Pb, Sb and Zn above international intervention values. Results from terrestrial tests showed detrimental effects in all studied organisms (*Eisenia foetida*, *Folsomia candida* and different plant species) and revealed the avoidance response of earthworms as the most sensitive endpoint. Surprisingly, the most toxic samples were not the ones with higher metal contents but the ones presenting higher electrical conductivity. Aquatic ecotoxicity tests with *Vibrio fischeri*, *Raphidocelis subcapitata*, *Daphnia magna* and *Danio rerio* were in accordance with terrestrial tests, confirming the need to couple environmental chemistry with ecotoxicological tools for the proper assessment of metal-contaminated sites. In view of the results, a remediative intervention of the studied area is recommended.

Key words: mercury mine, soil contamination, ecotoxicity, water extracts

1. Introduction

One of the main deleterious effects of already closed mines is usually associated with the abandonment of large volumes of wastes (Dudka and Adriano 1997). Tailings formed during the processing of the mineral ore are frequently stored in steep stock piles where they are prone to erosion (Henriques and Fernandes 1991), thus becoming a potential source of pollution to the surrounding environment. Such residues are frequently dispersed by atmospheric emissions, mechanical dispersion or water-leaching from waste deposits (Johnson et al. 1994; Adriano 2001) and are likely to contaminate soils, ground waters, surface waters and stream sediments of the surrounding area. In this context, one of the worst scenarios can occur if the dispersed residues reach agricultural or urban soils and expose humans to heavy metals either directly by suspended dust in air, or indirectly, by transfer into the food chain (Torres and Johnson 2001).

In SE Spain, the Valle del Azogue mine was the main mercury mine in the Betic Range during the 19th century. It was active approximately between 1873 and 1890 and produced about 1000 tons of Hg by means of underground works and small open pits located near two smelter sites. The only existing references to this deposit are by Cortazar (1875) and Becker (1888), who reported the presence of Hg mineralization associated with exhalative deposits. Cinnabar (HgS) was the main ore although high contents of Sb, As, Au, Ag, Pb, Zn and Ba were also reported in the mineralized veins (Navarro et al. 2006). Calcines and secondary Hg and Fe minerals (mainly metacinnabar and Fe oxides) produced during the roasting of the mineral ore were dumped near the metallurgical facilities, where they have become a potential source of particulate and soluble Hg species (Rytuba 2005) that might be transported

as Hg^0 vapor (Navarro et al. 2000; Gustin et al. 2002), ionic soluble phases or colloid particles (Shaw et al. 2001; Lowry et al. 2004).

The environmental risks of metal-contaminated sites were traditionally assessed by means of chemical analysis of metal concentrations and the subsequent comparison with values from quality guidelines. More recently, it was concluded that chemical extractions of metals from multi-contaminated soils did not provide enough information about their bioavailable fractions (Alexander 2000; Ehlers and Luthy 2003; Semple et al. 2004; Harmsen 2007) and were not able to reflect the toxicity of all substances in soil, their synergic and antagonistic effects and their interactions with the soil matrix and organisms (Gruiz 2005). In this context, the application of batteries of terrestrial ecotoxicity tests gained special relevance as complementary, inexpensive, simple and quick tools able to report realistic and non-overestimated effects of contaminated sites to soil organisms (Leitgib et al. 2007; Alvarenga et al. 2008; Maisto et al. 2011; Alvarenga et al. 2012; Agnieszka et al. 2014; Bes et al. 2014; Bori and Riva 2015, Bori et al. 2015). At the same time, aquatic bioassays traditionally applied for the toxicity determination of aquatic pollutants (Lopez-Roldan et al. 2012), industrial effluents (Riva et al. 1993; Riva and Valles 1994; Riva et al. 2007) or extracts of sediments (Pereira-Miranda et al. 2011) were incorporated to assess the impacts of soil composition and runoffs on receiving waters (Loureiro et al. 2005a; Rocha et al. 2011).

The first environmental concerns about the Valle del Azogue mine and its residues were reported by Martínez et al. (1998), Viladevall et al. (1999) and Navarro et al. (2000), who documented the release of Hg vapor into the atmosphere through volatilization as well as the transport of metallic Hg^0 contained in the underground mineralization, soils and mine wastes (calcines, low ore stockpiles and slags). The natural release of Hg into the atmosphere facilitated a near-surface deposition of Hg^0 in soils and sediments, which was added to the Hg^0 accumulated from the furnaces. The threats posed by this area due to the high contents of heavy metals and their potential mobilization were confirmed by subsequent geochemical studies (Navarro et al. 2006; Navarro et al. 2009a). Despite those potential threats, to date the risk assessment of the area has only been performed by chemical and mineralogical analysis.

With this in mind, the major purpose of this work was to assess the risk that the area surrounding the Valle del Azogue mine poses to the environment due to the presence of mercury and other metals. To attain this goal, this study aimed the following: (1) to characterize the area by means of physicochemical and mineralogical determinations; (2) to quantify metal concentrations in soils and in their water extracts; (3) to study the toxicity of the samples through the application of aquatic and terrestrial ecotoxicity tests and (4) to establish relationships between physicochemical parameters, metal contents and toxicity to organisms.

2. Materials and Methods

2.1 Study area and sampling sites

Samples of soils and mine wastes were collected in the Valle del Azogue mine (SE Spain). The sampling area, comprising the North of Sierra Almagrera, is located 90km NE of the city of Almería, in a semi-arid and intensively cultivated region (Fig. 1). Due to mining and metallurgical activities, plants have disappeared from the area or have been severely affected by high contents of mercury and other metals (Viladevall et al. 1999). The main ore is composed of stibnite, cinnabar, arsenic minerals (realgar and orpiment), sphalerite, siderite, chalcopyrite, pyrite, quartz, calcite and barite (Navarro et al. 2006). Together with the Iberian Pyrite Belt and the Cartagena mining district, this abandoned mining area is one of the oldest metallurgical and mining areas in the Iberian Peninsula (Navarro et al. 2006).

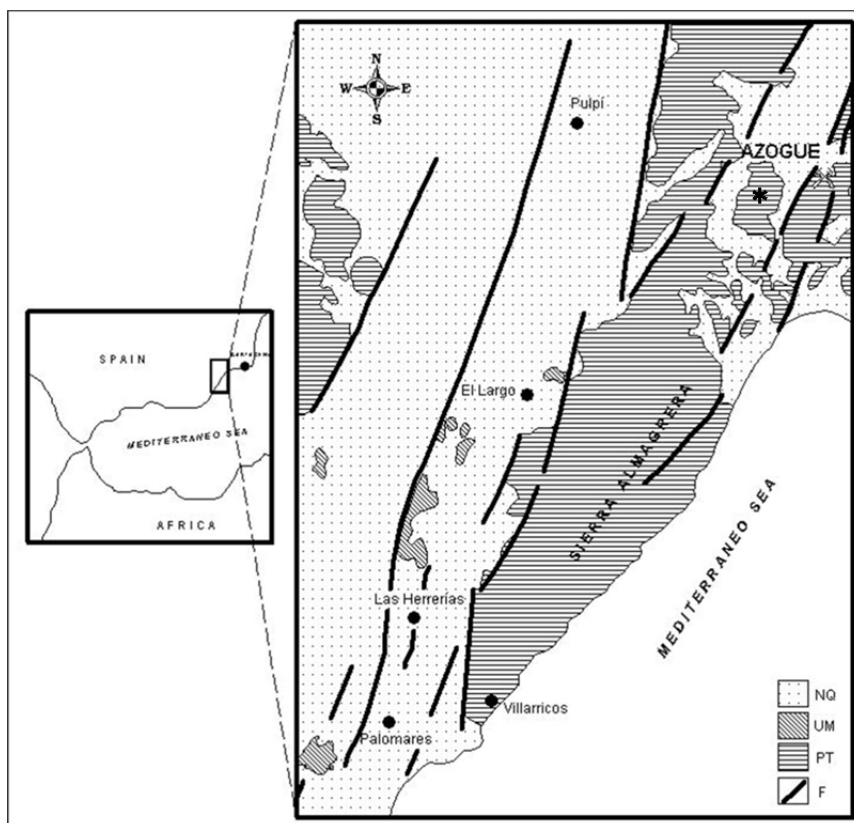


Figure 1. Location map and synthetic geology of the study area. NQ: Quaternary and Tertiary sediments; UM: Volcanic tertiary rocks ; PT: Metamorphic basement; F: Main fractures; *: Study area. Adapted from Navarro et al. (2009).

Samples were collected from seven sites spread throughout the mining district in order to have a representative characterization of the area (Fig. 2). Samples A1, A2 and A6 comprised soils mixed with mining wastes originated by ore extraction and located close to the main open pits. Samples A4, A5 and A7 were soils mixed with calcines derived from metallurgical ore processing and located near the main furnace location. Sample 3 consisted in an anthropogenic soil.

2.2 Soils and mine wastes sampling and analysis

Soil samples (0-0.25 m depth) were crushed, sieved, homogenized and subjected to physical-chemical characterization. The following parameters were evaluated: pH (KCl, 1 mol L⁻¹), electrical conductivity (EC)(1:5 soil:water suspension), soil organic matter (SOM)(by loss on ignition at 550°C for 2 hours), pore size distribution (by sieve analysis through aperture ranges comprised between 4000 µm and <350 µm), porosity (by water displacement in a test tube), bulk density (by test tube assay), field capacity (by empirical methods), sulphur content (by TD-ICP) and calcium content (by TD-ICP). Soil sub-samples for ecotoxicity testing were sieved through a 4 mm mesh and kept refrigerated (4°C) until use.

Samples for chemical analysis were sent to Actlabs (Ontario, Canada) for metal quantification. Au, Ag, As, Ba, Br, Ca, Ce, Co, Cr, Cs, Eu, Fe, Hf, Hg, Ir, La, Lu, Na, Ni, Nd, Rb, Sb, Sc, Se, Sm, Sn, Sr, Ta, Th, Tb, U, W, Y and Yb were quantitatively analyzed by instrumental neutron activation analysis (INAA) and Mo, Cu, Pb, Zn, Ag, Ni, Mn, Sr, Cd, Bi, V, Ca, P, Mg, Tl, Al, K, Y and Be were analyzed by inductively coupled plasma emission spectroscopy (ICP-OES). The quality of the measurements was ensured through reference materials. Hg phases were determined by solid-phase-Hg-thermo-desorption (SPTD) based on the specific thermal desorption or decomposition of Hg compounds from solids at different temperatures (Biester and Scholz 1997; Navarro et al. 2006). Mercury thermo-desorption curves were determined by means of an in-house apparatus, consisting of an electronically controlled heating unit and an Hg detection unit. Measurements were carried out at a heating rate of 0.5°C s⁻¹ and a nitrogen-gas flow of 300 mL min⁻¹. The lowest level

of detection under the given conditions is in the range of 40-50 ng if all Hg is released within a single peak (Biester and Scholz 1997). Results are depicted as Hg thermo desorption curves (Hg-TDC) that show the release of Hg^0 versus temperature. Mine wastes samples were studied using transmitted and reflected light microscopy, X-ray diffraction (XRD) and scanning electron microscopy (SEM) with an attached Energy Dispersive X-Ray Spectroscopy system (EDS) at the Electronic Microscopy Laboratory of the Universitat Autònoma de Barcelona.

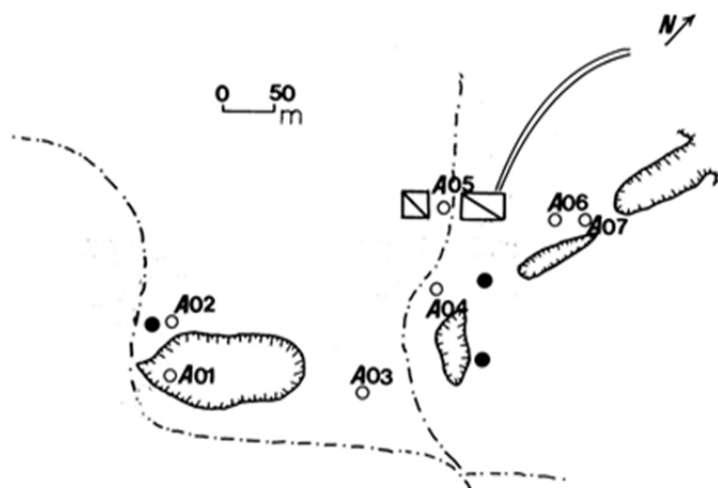


Figure 2. Sampling sites. Open circles: Sampling points; Closed circles: Mine shafts; Striped figures: Open pits. Adapted from Navarro et al. (2009).

2.3 Water extracts collection and analysis

Water extracts were obtained according to the British Standard EN 12457-2 (2002). Sampled soils were incorporated into 2-L glass vessels at a ratio of 1 kg/10 L, corresponding to 0.1 kg of soil per liter of deionized water. Vessels were placed at a rotating apparatus and mixed during 24 hours at a temperature of $20 \pm 2^\circ\text{C}$. After a settling period of 15 minutes, samples were centrifuged (2000g, 10 minutes) and filtered through a $1\ \mu\text{m}$ pore size membrane filter. Supernatants were frozen until use. Values of pH, electrical conductivity and total organic carbon (TOC) were determined with a pH-meter, a conductivity meter and a Total Organic Carbon Analyzer TOC-V_{CSH} (SHIMADZU, Japan) respectively. A subsample of each water extract was sent to Actlabs (Ontario, Canada) for metal quantification through inductively coupled plasma mass spectrometry (ICP/MS).

2.4 Terrestrial ecotoxicity tests

Direct toxicity bioassays were performed using whole soils. When dilution was needed, test soils were mixed with an artificial soil (69% quartz sand, 20% caolinite clay, 10% finely ground sphagnum peat, 1% calcium carbonate and pH adjusted to 6.0 ± 0.5) (ISO 17512 2011) that acted as control soil. In order to obtain different percentages of effect that allowed the calculation of effective and lethal median values (EC50 and LC50), test concentrations ranged from 0 to 100% of sampled soils mixed with artificial soil. All soil bioassays were carried out at 40-60% of the water holding capacity. When possible, EC50 or LC50 values were expressed as the percentage of sampled soil mixed with artificial soil (w/w) reducing by 50% the endpoint measured.

Earthworms from the species *Eisenia foetida* and collembolans from the species *Folsomia candida* were obtained from synchronized cultures maintained at the Centre for Research and Innovation in Toxicology of the Technical University of Catalonia (UPC) in Terrassa (Spain). Earthworms were cultured in 30-liters breeding boxes and a 1:1 mixture of horse manure and peat. Only clitellate adults between 300 and 600 mg of weight were selected for the performance of the tests.

Earthworms were acclimated in control soil during 24 to 48 hours prior to beginning the tests. Collembolans were cultured in vessels filled with a substrate of plaster of Paris and charcoal (8:1, w/w) at 20±2°C. Individuals were fed twice a week with granulated dry yeast added in small amounts to avoid spoilage by fungi. Organisms between 10 and 20 days old were used for toxicity testing.

2.4.1 *E. foetida* acute toxicity test

Acute toxicity tests with earthworms were adapted from the OECD 207 (1984) guideline. Ten organisms were placed in plastic containers (140x140x80 mm) containing 500 g (dry weight (dw)) of test soil. Test containers were kept under constant light (400-800 lux) at a temperature of 20±2°C. Survival was determined after 7 and 14 days of exposure. Each test ran with 6 concentrations (1-10-18-32-57-100%) plus a control and three replicates per treatment. Results were expressed as percentage of mortality. When two consecutive concentrations resulted in 0 and 100% mortality, these two values were considered sufficient to indicate the range within which the LC50 fell (OECD 207 1984).

2.4.2 Avoidance tests with *E. foetida* and *F. candida*

Avoidance tests with *E. foetida* and *F. candida* were adapted from ISO 17512 (2008) and ISO 17512 (2011) standards respectively. Rectangular plastic containers (220x140x50 mm) were used in tests with earthworms while cylindrical vessels (diameter 8 cm; depth 8 cm) were selected for tests with collembolans. Test containers were divided into two equal sections by a vertically introduced plastic card. Each section (control and test) of the test containers was filled with 250 g dw (test with earthworms) or 30 g (wet weight)(test with collembolans) of the corresponding soil and the divider was removed. Ten adult earthworms or twenty adult collembolans were carefully placed on the line separating both soils. Test containers were covered with a transparent plastic lid and incubated for 48 hours in an environmental chamber at 20±2 °C and under a 16:8h light:dark photoperiod. Tests with earthworms ran with 5 concentrations (1-3.1-10-31-100%) plus a control and three replicates per treatment whereas 4 concentrations (17-31-56-100%) plus a control and 5 replicates per treatment were prepared for collembolans. Due to the high toxicity of sample A6, both assays required lower test concentrations (1-1.5-2-2.5-3.1% for earthworms and 10-17-31-56% for collembolans). The plastic card was reinserted at the end of the test period and the number of individuals at each section was counted. In tests with collembolans, the soil from each section was carefully emptied into two different vessels and flooded with water. After gentle stirring, the animals floating on the water surface were counted. Results were expressed as percentage of individuals in the control section at the end of the test.

2.4.3 Seedling emergence and growth tests

Plants bioassays were adapted from the OECD 208 (2006) guideline. The species *Brassica rapa*, *Trifolium pratense* and *Lolium perenne* were selected as test organisms. Plastic containers with 100 grams (wet weight) of test soil (without dilution) were prepared. Twenty seeds of each plant were sown in each test soil and in the control artificial soil (four replicates per treatment i.e. 5 seeds per test container). Tests were performed in an environmental chamber at 24±2°C and under a 16:8 hours light:dark photoperiod. The moisture content and the number of sprouts were checked daily. Fourteen days after 50% of emergence was detected in the controls, plants were harvested and weighed. Results were expressed as percentage of seed emergence and fresh biomass compared to the controls.

2.5 Aquatic toxicity tests

Indirect toxicity bioassays were performed with water extracts from test soils. When required, dilutions were prepared mixing water extracts with the correspondent test medium. Toxicity results were expressed as the percentage of

water extract in the test medium (V/V) reducing by 50% the endpoint measured (EC50 or LC50). Organisms from the species *D. magna* and *R. subcapitata* were cultured in the Centre for Research and Innovation in Toxicology of the Technical University of Catalonia (UPC) in Terrassa (Spain).

2.5.1 Bacteria luminescence inhibition test

Acute toxicity to the bioluminescent bacteria *V. fischeri* was assessed in accordance with the ISO 11348 (2007) standard. Test organisms were reconstituted and exposed to 4 concentrations of water extracts (5.63-11.25-22.5-45%). The luminescence emitted was measured after 15 minutes with a Microtox® 500 system (Microbics®). Three replicates per treatment were used. Results were expressed as percentage inhibition of light emission.

2.5.2 Algal growth inhibition test

Effects on the growth of microalgae were assessed following the OECD 201 (2011) guideline. Cultures of *R. subcapitata* were kept under constant illumination (4000-5000 lux) at a temperature of 20±2°C. Only populations in the exponential phase were used in tests. Assays were carried out in tubes containing 9 mL of test solution and 1 mL of algal inoculums of known concentration that were placed in a controlled room at 20±2 °C and under constant illumination (4000-5000 lux) and agitation. After 72 hours of incubation, the absorbance of each replicate was measured at 665 nm with a CECIL CE9200 spectrophotometer. Tests ran with three replicates per treatment and 7 concentrations (0.1-0.32-1-3.2-10-32-90%) plus a control that consisted in algae culture medium. Lower test concentrations (0.001-0.0032-0.01-0.032-0.1%) were applied to sample A4 due to its high toxicity. In order to avoid interferences in the spectrometric measure of the leachates at the end of the test, one extra tube was prepared with 9 mL of leachate, 1 mL of culture medium and no algae. Results were expressed as percentage of algal growth inhibition.

2.5.3 Daphnia magna acute immobilization test

The acute toxicity test with *D. magna* was carried out according to the OECD 202 (2004) guideline. Bulk cultures of 15 daphnids were kept in 2.5 liters of ASTM hard synthetic water (ASTM 1988). Culture medium was changed three times per week and an organic extract and a concentrate of *Chlorella vulgaris* were added as food. Neonates were removed daily. Cultures were maintained at 20±2°C in a 16:8h light:dark cycle. Only neonates with less than 24 hours old were used for toxicity testing. Assays were performed in glass tubes containing 10 mL of test medium and 5 daphnids. Test vessels were kept in an incubator at 21±2°C and in the dark. Immobilization was visually recorded after 24 and 48 hours of exposure. Daphnids were exposed to 7 dilutions of water-extracts (1-2.2-4.8-10-22-48-100%) plus a control in four replicates per treatment. Lower test concentrations were required for samples A4 (0.01-0.022-0.048-0.1-0.22-0.48-1%) and A6 (0.82-1-1.3-1.7-2.2-2.9%). Mortality at the end of the test was expressed as a percentage.

2.5.4 Fish, acute toxicity test

The acute toxicity to fish was tested in accordance with the OECD 203 (1992) guideline for semi-static test conditions. Organisms from the species *Danio rerio* were supplied by P&S Piscicultura Superior sl (Barcelona, Spain). Individuals with the same age and between 2 and 4 cm long were kept at 21-24°C under a 16:8 light/dark photoperiod. Seven individuals were placed in 5-L aquariums containing 3 L of test medium (1 g fish L⁻¹). Dissolved oxygen was kept above 60% of air saturation and a 16:8-h light/dark photoperiod and a temperature of 20-24°C were set. Test organisms were not fed during the assay and mortality was recorded after 24, 48, 72 and 96 hours of exposure. Four concentrations (10-22-48-100%) were tested with most samples except for A2, A4 and A6, which required additional lower

test concentrations (1-2.2-4.8-10-22-48-100%). Mortality at the end of the test was expressed as percentage.

2.6 Statistical analysis

Data were checked for their homogeneity of variances and normality. Differences between means were tested with one-way ANOVA. Whenever significant differences were found ($p < 0.05$), the Tukey post hoc test was applied. Non-normal data were transformed and when the assumption of normality was not reached, non-parametric Kruskal-Wallis tests alongside with Mann-Whitney post hoc tests were performed.

Median effective and lethal concentration values (EC50 and LC50 respectively) and their 95% confidence intervals were calculated by Probit regression. A normal or logistic distribution was assumed depending on results from normality tests.

Relationships between soil physical-chemical parameters, metal concentrations and ecotoxicological factors were studied. Simple correlations between variables were calculated with the Pearson's test. Additionally, soil data were processed by means of principal component analysis (PCA). The PCA is concerned with interrelations between variables and operates by extracting eigenvalues and eigenvectors from a covariance or correlation matrix. The PCA facilitates the reduction, transformation and organization of the original data creating a new set of uncontrolled variables which are the linear combinations of the original ones.

Statistical analysis was performed using SPSS software (SPSS 15.0 for Windows; SPSS Inc., Chicago, IL, USA) and Minitab Statistical Software (Minitab 15.0; Minitab Inc., State College, PA, USA).

3. Results and Discussion

3.1 Physicochemical parameters and geochemistry of soils and mine wastes

Soil physicochemical characteristics markedly varied between sites (Table 1). Samples A2 and A4 were very strongly acidic ($\text{pH} < 3.9$) while the remaining samples presented pH values closer to neutrality (6.83 to 7.55). Electrical conductivity ranged from moderate (0.59 mS cm^{-1} in A5) to rather high values (8.25 mS cm^{-1} in A6). Organic matter contents remained below 10% (5.20 to 9.82%), which correspond to contents usually found in mineral soils. Soil pore sizes (in terms of equivalent diameter) ranged from 0.8 mm in soil A5 to 3.4 mm in A1, revealing that the studied soils were largely comprised of a sandy material fraction. The coarser sample A1 was associated to mining wastes and possibly, to overburden ore deposit. Average values of porosity, bulk density and field capacity of the sampled area were 0.40, 1450 kg m^{-3} and 8% respectively. Sulphur content ranged from 0.28% (A7) to 2.81% (A5) while that of calcium ranged from 0.12% (A7) to 7.33% (A5).

Table 1. Physical-chemical characteristics (mean \pm sd; $n=2$ when possible) of sampled soils. Values within the same row followed by the same letter are not significantly different ($p > 0.05$). EC: electrical conductivity (mS cm^{-1}); SOM: soil organic matter (%); d_e : equivalent diameter (mm); BD: bulk density (kg m^{-3}); FC: field capacity (%); S: sulphur content (%); Ca: calcium content (%).

	A1	A2	A3	A4	A5	A6	A7
pH	$6.84 \pm 0.06c$	$2.90 \pm 0.01a$	$7.35 \pm 0.03cd$	$3.83 \pm 0.09b$	$7.55 \pm 0.14d$	$6.83 \pm 0.05c$	$7.33 \pm 0.18cd$
EC	$1.14 \pm 0.04b$	$2.47 \pm 0.03cd$	$2.28 \pm 0.07c$	$2.64 \pm 0.04d$	$0.59 \pm 0.04a$	$8.25 \pm 0.08f$	$4.75 \pm 0.04e$
SOM	$6.40 \pm 0.09bc$	$9.82 \pm 0.12f$	$7.35 \pm 0.20d$	$6.81 \pm 0.09cd$	$5.42 \pm 0.25a$	$5.88 \pm 0.16ab$	$5.20 \pm 0.06a$
d_e	3.4	1.8	1.0	0.8	0.5	1.9	1.3
Porosity	-	0.34	0.45	-	-	-	0.41
BD	1330	1430	1310	1410	1470	1400	1490
FC	8	8	10	10	7	8	8
S	1.8	2.05	0.47	2.02	2.81	1.49	0.28
Ca	1.34	0.27	2.49	3.09	7.33	2.28	0.12

Total contents of most studied metals were extremely high in the sampled soils, calcines and mining wastes (Figure 3) and were similar to values reported by Navarro et al. (2000) and Navarro et al. (2006) for the same area. As reached 1550 mg kg^{-1} in A4 and exceeded by two orders of magnitude the intervention values for soil remediation of Dutch regulations (55 mg kg^{-1})(VROM 2000) whereas Ba in A1

(110000 mg kg⁻¹) surpassed by three orders of magnitude its remediation values (625 mg kg⁻¹). Hg and Sb concentrations markedly varied between sites although their intervention values (10 mg kg⁻¹ and 15 mg kg⁻¹ respectively) (VROM 2000) were exceeded by several orders of magnitude in most sites. Pb and Zn were the only metals whose intervention values according to Dutch regulations (530 mg kg⁻¹ and 720 mg kg⁻¹ respectively) were not surpassed in all sites.

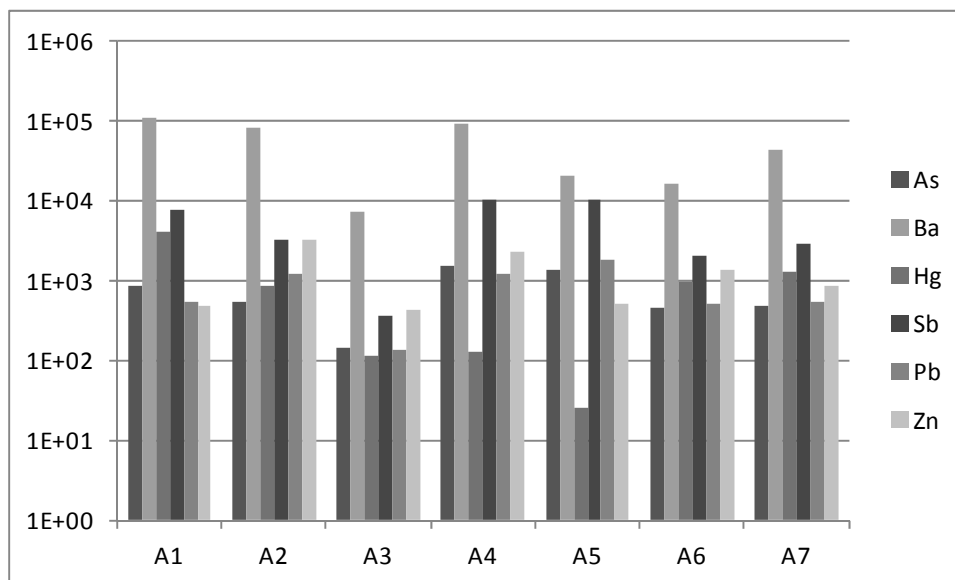
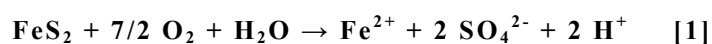


Figure 3. Total metal concentrations (mg kg⁻¹ dry weight) in test soils.

The studied soil samples presented physical-chemical characteristics typical from tailings usually found surrounding mining sites: neutral to acidic pH, high EC, low fertility and high total concentrations of heavy metals (Conesa et al. 2006; Navarro et al. 2008; Carmona et al. 2009). The low pH of soils A2 and A4 was explained by pyrite oxidation and could explain their high Zn contents, which showed a negative significant correlation with soil pH ($r = -0.96$, $p < 0.01$). Thus, the possible main hydrogeochemical reactions associated to sulphide oxidation are pyrite and sphalerite oxidation:



Such low pH represented one of the main threats of the area since it can lead to the solubilization of metals and consequently to the spread of contamination towards the water compartment (Navarro Flores and Martínez Sola 2010). Besides, the samples with lower pH (A2 and A4) showed high S contents, possibly associated with arsenian pyrite. However, samples with the highest pH value (A3 and A5) showed high Ca contents, which could be associated with significant amounts of calcite in the soil. Additionally, statistically significant positive correlations were found between Sb concentrations and As ($r = 0.97$, $p < 0.01$), Ba ($r = 0.93$, $p < 0.05$) and Hg ($r = 0.97$, $p < 0.01$) contents, which might be indicative of a common origin. The particular structure of the soils comprising the study area was another major cause of concern as the high percentage of sand and the absence of a proper soil structure due to the high presence of mine tailings and wastes can further increase the leaching of heavy metals (Conesa et al. 2006).

Hg-thermodesorption curves (Hg-TDC) belonging to mining wastes, soils and calcine samples showed predominant release of Hg in two temperature ranges: 200-250°C and 300-330 °C. The first temperature range was assigned to a release of Hg from the soil matrix components based on the Hg-TDCs of standard materials (Biester and Scholz 1997). Thus, we assume that most Hg present in the calcine material is bound to mineral components mainly by iron oxides, which were formed when the cinnabar-bearing ore was being roasted. Earlier studies already suggested that Hg⁰ formed during thermal breakdown of cinnabar is re-condensed during

cooling of the material and adsorbed to iron oxide surfaces (Biester et al. 2000). In addition to matrix-bound Hg, some calcine samples contained traces of cinnabar. This could be explained by an incomplete breakdown of cinnabar ore during the roasting process. The second temperature range was assigned to Hg release from cinnabar, which was the predominant Hg mineral in contaminated soils and mining wastes (host rock and low grade stockpiles). Cinnabar and Hg sulphates were also detected in several samples (Navarro et al. 2006). No free metallic Hg, which is typically released at temperatures below 100°C, was found in any of the samples studied.

Mercury phase characterization by X-ray showed the presence of cinnabar (HgS), corderoite (Hg₃S₂Cl₂), laffittite (AgHgAsS₃), metacinnabar ((Hg)S), shakhovite (Hg₄SbO₅(OH)₃), schuetteite (Hg₃(SO₄)O₂) and tiemannite (HgSe) (Table 2). The proportionally Hg predominant phase was cinnabar, which was concordant with the SPTD analyses. The detailed SEM and EDS systems study of mine wastes samples showed the presence of primary and secondary cinnabar associated with barite, pyrite and botryoidal pyrite. Also, SEM observations showed several small particles containing both Hg and Cl that may be associated to calomel (Hg₂Cl₂). Moreover, some particles containing both Hg and Br were observed and may be associated to kuzminite (Hg₂(Br,Cl)₂)(Navarro et al. 2009b; Navarro et al. 2012). Additionally, main minerals in the gangue were quartz, barite and silicates.

Table 2. Minerals identified by DRX in the Valle del Azogue soil and mine wastes. Modified from Navarro et al. (2012). *: high-medium abundant minerals. a: detected by DRX in calcines; b: detected by DRX in mining wastes.

	Minerals		Secondary minerals	
Calcines	Quartz ^{a*}	SiO ₂	Hematite*	Fe ₂ O ₃
	Barite ^{a*}	Ba(SO ₄)	Hg ⁰	Hg
	Illite ^{a*}	KAl ₂ Si ₃ AlO ₁₀ (OH) ₂ · 3H ₂ O		
	Calcite*	Ca(CO ₃)		
	Cinnabar	HgS		
	Orthoclase	K(Al, Fe)Si ₂ O ₈		
	Mineralization, wastes and soil	Quartz ^{b*}	SiO ₂	Hg ⁰
Barite ^{b*}		Ba(SO ₄)	Goethite	FeOOH
Cinnabar-		HgS	Jarosite	KFe ₃ (SO ₄) ₂ (OH) ₆
Metacinnabar*				
Dolomite ^{b*}		CaMg(CO ₃) ₂	Hematite	Fe ₂ O ₃
Calcite ^{b*}		Ca(CO ₃)	Inyoite ^b	CaB ₃ O ₃ (OH) ₅ · 4H ₂ O
Huntite ^{b*}		Mg ₃ Ca(CO ₃) ₄	Ferrihydrite	Fe(OH) ₃
Stibnite*		Sb ₂ S ₃	Kaolinite	KAl ₂ Si ₃ AlO ₁₀ (OH) ₂ · 3H ₂ O
Realgar*		As ₂ S ₃	Gypsum	Ca(SO ₄) · 2H ₂ O
Oripiment		As ₂ S ₃	Schuetteite	Hg ₃ (SO ₄)O ₂
Calcopyrite		CuFeS ₂	Tiemannite	HgSe
Arsenian pyrite*		Fe(S _{1-x} As _x) ₂	Corderoite	Hg ₃ S ₂ Cl ₂
Esfalerite		ZnS	Shakhovite	Hg ₄ SbO ₅ (OH) ₃
Orthoclase		K(Al, Fe)Si ₂ O ₈	Calomel	Hg ₂ Cl ₂
Au		Au	Kuzminite	Hg ₂ (Br, Cl) ₂
Illite ^{b*}	Al ₄ (Si ₄ O ₁₀)(OH) ₈			

3.2 Analysis of water extracts

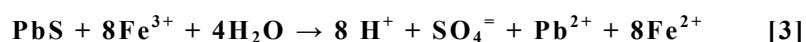
Data from water extracts are presented in Table 3. Similarly to soils, pH differed significantly from the strong acidity of samples A2 and A4 to the neutrality of the rest. All sampling sites were clearly differentiated by the salinity of their extracts, with the highest values determined in samples A6 (5.29 mS cm⁻¹) and A7 (3.53 mS cm⁻¹). Organic matter content in soils was not mirrored in water extracts, where the lowest value of total organic carbon was determined in sample A5 (1.36 mg L⁻¹) and the highest in the sample A3 (6.47 mg L⁻¹). Metal concentrations in water extracts markedly varied depending on the metals and samples. With the exception of Zn, metal concentrations in all extracts represented less than 1% of their soil contents, thus revealing the low concentration of water-soluble metal species in soils. In the case of Zn, the average recovery rate in water extracts was 4%. Even so, high metal concentrations were detected in water extracts due to their extremely high contents

in test soils. Between samples, arsenic, mercury and zinc concentrations were significantly higher in samples A1 (571 $\mu\text{g L}^{-1}$), A6 (1220 $\mu\text{g L}^{-1}$) and A4 (>50000 $\mu\text{g L}^{-1}$) respectively. Ba and Sb contents were similar among samples, with concentrations that ranged between 79.2 $\mu\text{g L}^{-1}$ (A1) and 306 $\mu\text{g L}^{-1}$ (A6) in the case of Ba and between 24.1 $\mu\text{g L}^{-1}$ (A2) and 154 $\mu\text{g L}^{-1}$ (A1) for Sb. Lead concentrations markedly varied from 0.22 $\mu\text{g L}^{-1}$ in soil A1 to 297 $\mu\text{g L}^{-1}$ in soil A2. The acidity of A2 and A4 extracts was associated to their markedly higher contents of Pb and Zn, whose concentrations were negatively correlated ($r = -0.977$, $p < 0.01$ for Pb and $r = -0.572$, $p = 0.18$ for Zn) with pH of aquatic samples. At the same time, soluble Hg was found responsible ($r = 0.88$, $p < 0.01$) of the higher electrical conductivity of extracts A6 and A7 whereas the contents of As and Sb correlated as seen with soil samples ($r = 0.83$, $p < 0.05$).

Table 3. Physical-chemical characteristics (mean \pm sd; n=2) and total concentrations of metals (in $\mu\text{g L}^{-1}$) in water samples extracted from test soils. Values within the same row followed by the same letter are not significantly different ($p > 0.05$). EC: electrical conductivity (mS cm^{-1}); TOC: total organic carbon (mg L^{-1}).

	A1	A2	A3	A4	A5	A6	A7
pH	6.90 \pm 0.08c	3.08 \pm 0.02a	7.14 \pm 0.12cd	4.18 \pm 0.06b	7.46 \pm 0.05d	7.58 \pm 0.04d	8.16 \pm 0.14e
EC	1.57 \pm 0.01a	2.70 \pm 0.02d	2.07 \pm 0.01b	2.51 \pm 0.03c	2.01 \pm 0.01b	5.29 \pm 0.03f	3.53 \pm 0.02e
TOC	1.48 \pm 0.02a	2.28 \pm 0.04c	6.47 \pm 0.06f	1.95 \pm 0.02b	1.36 \pm 0.08a	4.16 \pm 0.02e	2.54 \pm 0.12d
As	571	69.4	25.9	4.83	5.53	4.61	4.84
Ba	79.2	111	140	87.4	280	306	304
Hg	2.1	2.8	0.3	42.9	19.1	1220	28.7
Sb	154	24.1	24.4	32.3	78.7	62.5	68.4
Pb	0.22	297	1.68	199	1	1.17	0.6
Zn	27.4	>5000	871	>50000	1040	345	181

Pb concentrations in the extracts (samples A2 and A4) were associated with high amounts of lead in soils (Table 1), which was possibly originated by galena weathering:



The higher concentrations of Zn in water samples (samples A2 and A4) were also associated with high contents of Zn in soils. Zn may be mobilized by sphalerite weathering (reaction 2). Higher As concentrations in water extracts (samples A1 and A2) was associated with moderate As contents in soils (Table 1), while high Sb concentrations (samples A1 and A5 to A7) could be associated with elevated contents of Sb in soil (Table 1). Thus, the mobilization of Sb in leaching experiments could be originated by stibnite weathering.

3.3 Ecotoxicological evaluation

Terrestrial and aquatic ecotoxicological bioassays presented different sensitivities depending on the test endpoints and organisms. Within terrestrial assays, *E. foetida* mortality test showed the lesser sensitivity, was unable to estimate median lethal concentration values for soils A1 and A5 and provided the highest EC50 (LC50) values (i.e less toxicity detected)(Table 4). Despite its low sensitivity, the mortality of *E. foetida* was significantly and positively correlated with sublethal effects (avoidance response) observed in earthworms ($r=0.833$; $p < 0.05$) and collembolans ($r=0.838$; $p < 0.05$). In contrast, the behavioral test with *E. foetida* presented an extreme sensitivity, was able to estimate median effective concentration values for all soils and provided EC50 values as low as 0.33% (sample A6). According to Hund-Rinke and Wiechering (2001), all samples should be considered to have a limited habitat function because the percentage of earthworms in control sections was higher than 80% at the end of the tests. Results from avoidance tests with earthworms were in agreement with previous studies that highlighted the higher sensitivity of sublethal endpoints in general (Hund-Rinke et al. 2002; Davies et al. 2003) and of avoidance tests with earthworms in metal-contaminated soils in particular (Alvarenga et al. 2012). EC50 values estimated for avoidance tests with earthworms were significantly and positively correlated ($r = 0.984$, $p < 0.01$) with those from collembolans, thus indicating the potential and suitability of behavioral

responses as endpoints in terrestrial ecotoxicology. However, sensitivity decreased (Table 4) and only four soils (A2, A3, A6 and A7) showed a limited habitat function when soil arthropods were used as test organisms. Thus, our study points out the higher sensitivity of avoidance tests with earthworms, which is in agreement with results from Hentati et al. (2013) and Natal da Luz et al. (2012) after exposing earthworms and collembolans to soils contaminated with petroleum compounds and the pesticide diazinon respectively.

Table 4. EC50 values (95% confidence intervals) of terrestrial ecotoxicity tests with soil invertebrates. Results expressed as percentage weight of soil sample mixed with artificial soil (w/w). '-': non-applicable.

	<i>E. foetida</i> Acute Toxicity	<i>E. foetida</i> Avoidance Behavior	<i>F. candida</i> Avoidance Behavior
A1	-	19.61 (14.13-27.20)	-
A2	74.54 (-)	5.29 (3.63-7.70)	17.93 (12.02-26.75)
A3	61.92 (49.17-83.91)	4.04 (2.91-5.63)	38.33 (26.48-55.48)
A4	74.54 (-)	20.92 (15.33-28.55)	75.65 (44.54-128.48)
A5	-	79.60 (44.41-142.66)	-
A6	16.00 (12.51-22.34)	0.33 (0.24-0.45)	4.21 (3.40-5.21)
A7	24.48 (18.43-32.13)	4.95 (3.54-6.92)	42.43 (27.42-65.66)

Seed germination and growth rates are presented in Figure 4. Emergence and growth of the three studied species was totally inhibited in soils A2, A4, A6 and A7, which was expected due to the absence of a plant cover in the sampling area. It is important to emphasize that toxic effects of soils contaminated by mining tailings should not be exclusively associated to the presence of metals, but also to the fact that these anthropogenic soils are the product of a relatively rapid accumulation of mine wastes and consequently have not been formed through the complex and long process of rock erosion and materials accumulation that supplies the parameters needed for the proper development of flora (Dudka and Adriano 1997). Only *Lolium perenne* was able to germinate and grew in sample A3 (average of 15 and 12% as percentage of the controls respectively)(data not shown). Results from tests with plants confirmed those from ecotoxicity tests with invertebrates, revealing soils A1 and A5 as the least toxic. Both A1 and A5 soils presented similar percentages of emergence when compared with the controls, with values that ranged from 26 to 70% in soil A1 and from 32 to 85% in soil A5 depending on the test species. Among species, the highest inhibition was found in *T. pratense* while no statistical differences were found between the germination of *B. rapa* and *L. perenne*. These results are in accordance with those from Ramírez et al. (2008), who estimated lower EC50 values (i.e higher sensitivity) for *T. pratense* than for *B. rapa* and *L. perenne* when exposed to different sewage sludges. Regarding plant growth, it was significantly higher in soil A5 (48 to 61%) than in A1 (32 to 36%). No significant statistical differences in growth rate were appreciated between test species within the same soil.

In this study, total metal concentrations suggested soils A4, A1 and A2 as the most toxic. However, results from bioassays identified soils A6, A3, and A7 as the most detrimental for terrestrial organisms. The low toxicity of samples A4, A1 and A2 was explained by differences in the bioavailability of the studied metals between sites. The sample A1, for example, was composed of soil mixed with mining wastes and presented high mercury contents. However, results from the mineralogical analysis pointed out that Hg was mainly found in the form of cinnabar, thus becoming inaccessible to soil organisms. On the other hand, the high toxicity of soils A6 and A7 was attributed to their high salinity, which was positively and significantly correlated with the toxicity to soil invertebrates ($r = 0.98$, $p < 0.01$ for earthworms mortality; $r = 0.89$, $p < 0.01$ for earthworms avoidance; $r = 0.89$, $p < 0.05$ for collembolans avoidance). According to Alvarenga et al. (2012), the high salinity of metal-contaminated soils could be indicative of a high bioavailability of metals. Since EC was positively correlated with Hg concentrations and the

mineralogical analysis detected the presence of mercury sulfates, we attributed the high electrical conductivity and consequently high toxicity of samples A6 and A7 to their high concentration of mercury in the form of salts. No clear explanation was found for the high toxicity shown by sample A3.

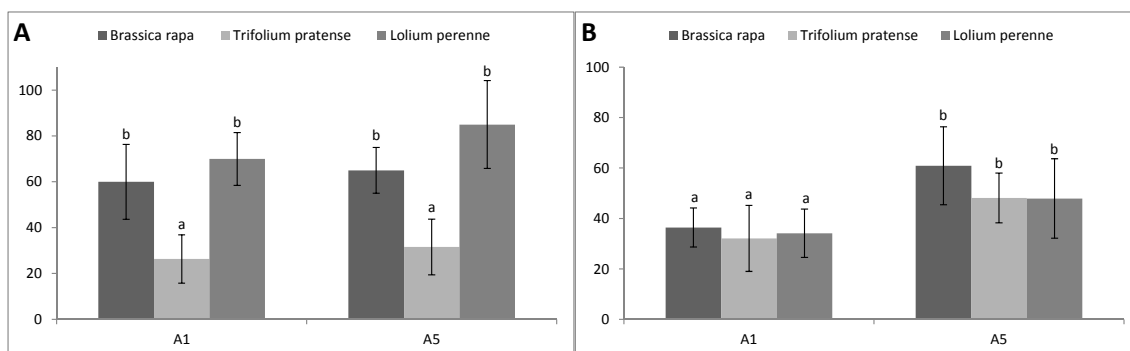


Figure 4. *Brassica rapa*, *Trifolium pratense* and *Lolium perenne* seeding emergence (A) and fresh biomass (B) as percentage of the controls. Means and standard deviations from four replicates. Values presenting the same letter are not statistically different ($p > 0.05$).

Toxicity of water extracts to aquatic organisms is shown in Table 5. The observed toxic effects in the bacterial bioluminescence inhibition assay were not sufficient to estimate EC50 values for samples A1, A3, A5 and A7 whereas extracts A2 and A4 were very toxic and A6 presented moderate toxicity. The lesser sensitivity of *V. fischeri* luminescence towards leachates from mine soils was previously documented (Alvarenga et al. 2008; Maisto et al. 2011) and might be related with the pH correction of the leachate suggested by standard methods (Alvarenga et al. 2013). In contrast, the growth inhibition of the microalgae *R. subcapitata* showed an extreme sensitivity that correlated significantly with Zn content in water extracts ($r = 0.996$, $p < 0.01$). Consequently, tests with algae estimated the lowest EC50 values for all samples and became the most metal-sensitive among the aquatic ecotoxicity tests applied, as previously reported by Maisto et al. (2011) and de Paiva Magalhães et al. (2014). *D. magna* was moderately affected by water extracts, showing significant correlations with their pH ($r = -0.913$, $p < 0.01$) and Pb contents ($r = 0.94$, $p < 0.01$). Samples A3 and A5 caused no mortality to the crustaceans while extremely high toxicity was observed for samples A2, A4 and A6, thus becoming more sensitive than *V. fischeri* to this type of contamination (Alvarenga et al. 2013) but not as metal-sensitive as algal growth rate (Maisto et al. 2011). As expected, bioassays with fish were the least sensitive among all tested species due to their particular resistance to most metals (de Paiva Magalhães et al. 2014). Even so, fish lethality was significantly correlated with pH ($r = -0.855$, $p < 0.05$) and Pb content ($r = 0.904$, $p < 0.01$) and occurred in the samples that proved more toxic to the other aquatic species tested (A2, A4 and A6).

Table 5. EC50 values (95% confidence limits) of aquatic ecotoxicity tests. Results expressed as percentage volume of water extract mixed with test medium (v/v). ‘-’: non-applicable.

	Bacteria Luminescence Inhibition	Algal Growth Inhibition	<i>Daphnia magna</i> Immobilization	<i>Danio rerio</i> Acute Toxicity
A1	>45	29.1 (20.6-44.3)	69.6 (50.1-120.7)	>100
A2	0.71	5.7 (4.7-6.8)	0.39 (0.29-0.52)	14.8 (-)
A3	>45	36.6 (25.3-58.2)	>100	>100
A4	1.2	0.015 (0.009-0.025)	0.47 (0.38-0.59)	52.7 (-)
A5	>45	9.2 (5.2-16.1)	>100	>100
A6	20.3	1.2 (1-1.4)	1 (0.9-1.1)	69.3 (-)
A7	>45	26.6 (20.7-35.6)	24 (17-34)	>100

Results from ecotoxicological bioassays with water extracts confirmed the high toxicity of sample A6 and detected remarkable deleterious effects by samples A2 and A4, which were the ones that presented higher metal contents. In addition, leachates from samples A2 and A4 showed the greater mobilization of Pb and Zn, which may indicate that aquatic toxicity was directly related with these metals. The toxicity of sample A6 was therefore attributed to its high salinity, while that of samples A2 and A4 resulted from its high acidity and metal concentrations.

3.4 Multivariate analysis

Principal component analysis for the terrestrial compartment was carried out on 10 variables: As, Ba, Hg, Pb, Sb, Zn, pH, Earthworms survival (ES), Earthworms avoidance (EA) and Collembola avoidance (CA). Variables were reduced to 4 principal components that explained 96.8% of the total variance (Table 6). Principal component 1 (PC1) was responsible for 47.4% of the total variance and was best represented by As and inversely related with Sb, Zn, pH, ES and EA. PC1 is explanatory of the toxicity of samples A6, A3 and A7, reflecting the role of pH and As content in soil toxicity and the detrimental effects observed through the survivability and avoidance of earthworms. Component 2 was responsible for 25.9% of the total variance and showed a direct correlation between Ba, Hg and Zn, which is frequent in soils contaminated by mining activities and related with ES and CA. Component 3 explained 18.7% of the total variance and was positively represented by Hg and inversely by Pb, indicating the influence of lithogenic geochemistry over soil composition. Component 4 explained 4.6% of the total variance and was represented by As and inversely by CA, indicating the possible influence of As in the behavior of collembolans.

Table 6.- Principal components loadings of soils.

Variables	PC1	PC2	PC3	PC4
As	0.4215	0.2244	0.0267	0.4459
Ba	0.0697	0.3228	-0.0752	-0.0077
Hg	-0.0874	0.5042	0.6851	0.0078
Sb	-0.5006	-0.1134	-0.0034	-0.3401
Pb	0.1603	0.0601	-0.5955	0.0391
Zn	-0.4002	0.4398	-0.2821	-0.257
pH	-0.3788	0.1143	-0.0322	0.3457
ES	-0.2364	0.3505	-0.2441	0.4297
EA	-0.3561	-0.174	-0.0055	0.4764
CA	0.2171	0.4638	-0.1713	-0.2945

Principal component analysis of leachates was applied using the same 6 geochemical variables of the soil multivariate analysis plus pH and electrical conductivity. The following ecotoxicological variables were also considered: *V. fischeri* luminiscence inhibition (VFLI), Algal growth inhibition (AGI), *D. magna* immobilization (DMI) and *D. rerio* acute toxicity (DRAT). The loadings of the first four principal components are shown in Table 7 and explain 93.4% of the total variance. PC1 was responsible for 48% of the total variance and was directly related with Ba, Hg, and DRAT and inversely with DMI, indicating a geochemical factor associated with the ecotoxicity of Hg. Component 2 explained 27.4% of the total variance and was associated with Ba, Pb and Zn and inversely with AGI and DMI, suggesting the ecotoxicity of these dissolved metals. Component 3 explained 11.9% of the total variance and was represented by Hg, Zn, VFLI and inversely by Ba. Component 4 explained 6% of the total variance and was associated with As (possibly), Hg, Pb and EC indicating the effect of metal concentration in electrical conductivity (EC). Since component 4 is inversely associated with DRAT, it may explain the ecotoxicity related with *D. rerio*.

Table 7. Principal components loadings of water extracts.

Variables	PC1	PC2	PC3	PC4
As	0.1584	0.1385	-0.0421	0.2613
Ba	0.3089	-0.4734	-0.4621	0.1034
Hg	0.6116	-0.1533	0.3274	0.5841
Sb	-0.0714	-0.0054	0.0149	0.1826
Pb	-0.0745	0.3910	-0.1628	0.2960
Zn	0.0286	-0.3847	0.6000	-0.2446
VFLI	0.1183	0.3159	0.3114	-0.0248
AGI	-0.2993	-0.4079	-0.1852	0.2804
DMI	-0.4572	-0.3531	0.2472	0.1607
DRAT	0.3453	-0.1935	-0.2609	-0.4260
pH	0.1066	-0.0489	0.1617	-0.0634
EC	-0.2255	-0.0396	-0.0644	0.3294

4. Conclusions

The environmental risks posed by the studied area were successfully evaluated. The Valle del Azogue mining area presented physical-chemical parameters typical from abandoned mining areas. High concentrations of mercury (mainly bound to the matrix or released from cinnabar) were detected throughout the area. Besides mercury, several other metals were quantified in amounts exceeding international intervention values. The risk of metals leaching towards the surrounding aquatic compartment due to the particular characteristics of the studied soils was identified.

The application of a battery of bioassays with organisms from different species proved to be a very valuable tool for the assessment of metal-contaminated sites. Most soil samples exerted severe toxic effects to terrestrial organisms, including the death of soil invertebrates and the total inhibition of plant growth. The avoidance test with earthworms was the most sensitive terrestrial bioassay, identifying almost all test soils as toxic after only 48 hours of exposure. To a lesser extent, aquatic bioassays confirmed the high toxicity detected by terrestrial tests and the growth inhibition of microalgae was identified as the most sensitive test.

This study successfully helped in the interpretation of the complexity associated to metal-contaminated soils. Relationships between physical-chemical parameters of soils and water extracts, heavy metals concentrations, and toxicity were established. Interestingly, the most contaminated soils were not identified as the most toxic by terrestrial tests, thus emphasizing the importance of ecotoxicological tests as complementary tools for the reliable risk assessment of contaminated sites. Furthermore, the electrical conductivity of terrestrial and aquatic samples was established as one of the main source of toxicity. In view of the results, an intervention on the studied area is encouraged due to the threat presented by the contaminated soils and the risk of spreading the contamination to agricultural areas located close to the studied site and/or towards the groundwater systems.

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Compliance with ethical standards

The authors declare that they have no conflict of interest.

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