Risk assessment of an abandoned pyrite mine in Spain based on direct toxicity assays

Concepción García-Gómez[1]
[1]Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria (INIA)

Beatriz Sánchez-Pardo[2]
[2]Facultad de Ciencias. Universidad Autónoma de Madrid

Elvira Esteban[2]
[2]Facultad de Ciencias. Universidad Autónoma de Madrid

[2]Jesús Manuel (Double name) Peñalosa[2]
[2]Facultad de Ciencias. Universidad Autónoma de Madrid

María Dolores (Double name) Fernández[1]
[1]Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria (INIA)

Postal address of each affiliation:
Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria (INIA)
Environment Department
Ctra. A Coruña, km 7.5
28040 Madrid (Spain)

Facultad de Ciencias. Módulo 10.
C/ Francisco Tomas y Valiente, nº 7
Universidad Autónoma de Madrid
28049 Cantoblanco. Madrid

Corresponding author:
María Dolores Fernández
Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria (INIA)
Environment Department
Ctra. A Coruña, km 7.5
28040 Madrid (Spain)
Phone:34913471483
Fax: 34913572293
mdfdez@inia.es

Abstract
This research reports the risk assessment of an abandoned pyrite mine using direct toxicity assays of soil and groundwater samples taken at the site. The toxicity of As and heavy metals from mining soils to soil and aquatic organisms was studied using the Multispecies Soil System (MS-3) in soil columns. Ecotoxicological assessment was performed with soil samples diluted with a control soil at concentrations of 12.5, 25, 50 and 100% test soil/ soil (w/w). In this way, changes in the mobility and bioavailability of soil contaminants due to changes in geochemical soil properties via soil dilution were studied. The toxicity of water samples was tested on algae and Daphnia magna. The assessment of the mining area indicated that the current presence of As and heavy metals at the site may cause injuries to soil and aquatic organisms in the entire research area. Moreover, this investigation demonstrated that changes in geochemical conditions can increase the availability of arsenic and, consequently, the environmental risk of these soils. A good correlation was not found between toxicity parameters and the concentrations of soil contaminants based on total and extracted element concentrations. This finding reinforces the usefulness of direct toxicity assays for evaluating environmental risk.

1. Introduction

Today, there is a growing concern towards the environmental effects of mine tailing sites (Antunes et al., 2011; Frouz et al., 2011; Kapusta et al., 2011; Cerqueira et al., 2012; Ribeiro et al., 2013). Tailings are produced during ore processing and are characterized by elevated levels of heavy elements, which can be released into the environment by erosion and leaching processes. In most cases, mining areas show high levels of hazardous elements in surface water, groundwater and soil, which can pose serious geochemical risks to human health and the environment (Anawar et al., 2006; Hofmann et al., 2008). This threat is aggravated by the long-term persistence of hazardous elements in the environment.

Risk assessment methodologies have been widely applied for the management of contaminated sites. However, their application to mining sites affected by tailing contamination is more limited (Kim et al., 2005; Lee et al., 2006; Moreno-Jimenez et al., 2011). In a first-tier approach, an ecological risk assessment is based on geochemical analysis, where concentrations measured in soil are compared to established thresholds. These accepted levels are based on the worst possible scenarios: toxicity data on the most sensitive species and the application of the most protective safety factors. Under these assumptions, the risk may be overestimated and may result in unnecessary remediation (Alexander, 2000; Ollson et al., 2009). A risk assessment based on geochemical analyses is highly simplified and does not take into account factors such as the bioavailability of a contaminant or the simultaneous presence of different contaminants, which can affect toxicity and exposure estimates (McLaughlin et al., 2000; De Zwart and Posthuma, 2005; O'Halloran, 2006). These facts are of the utmost importance for hazardous elements. The availability of hazardous elements in the soil is highly dependent on soil properties (Cerqueira et al., 2011). Moreover, hazardous elements can be present in different geochemical forms with different geochemical and toxicological properties that can be transformed from one into another due to environmental changes. To have a more complete impression of the degree of pollution and toxicity of a contaminated site, it is recommended to

consider alternative approaches that more accurately reflect specific site conditions. Thus, risk assessment in mining sites should integrate both chemical and toxicological assays (Antunes et al., 2011; Chapman et al., 2012).

Direct toxicity assessment, conducted with natural samples taken at the site, allows the measurement of the toxicity of complex mixtures of contaminants and can enhance the realism and certainty of the risk assessment. Although these techniques have an increasingly important role, they are not generally available in existing guidelines for ecological risk assessment on contaminated sites (Fernandez et al., 2006; Semenzin et al., 2008). Therefore, the ecotoxicological risk assessments based on direct toxicity assays need to be validated through field studies performed with natural samples.

The application of bioassays to contaminated site assessments requires selecting an appropriate set of test species and measurement endpoints to be applied at the investigated site. Microcosms are considered one of the higher-tier options to assess toxic substances and contaminated soils (Schaeffer et al., 2010). More complex than single toxicity assays, microcosms consist of systems in which an assemblage of species is exposed simultaneously. This allows one to consider the interactions among species that may influence toxicity, hence increasing realism.

In this research, a multispecies system in soil microcosms, termed a "Multispecies Soil System" (MS-3), was used for the ecotoxicological evaluation of an abandoned pyrite mine in Bustarviejo (Madrid, Spain). This system has previously been applied to characterize geochemical substances and polluted soils (Fernandez et al., 2005; Garcia Frutos, et al., 2010). In the MS-3 system, the organisms were selected from different trophic levels and included taxonomic groups that cover essential ecological roles for sustainable soil use. The mobility of contaminants and possible risks to the aquatic environment (groundwater and surface water) can also be determined through watering and subsequent leaching processes.

In this paper, a case study that utilized direct toxicity assays to assess site-specific risk in an abandoned pyrite mine is presented. The aims of this work were: i) evaluating the potential of direct toxicity assays using the microcosm system (MS-3) for assessing multi-element

contaminated soils in a mining area, ii) assessing changes in mobility and bioavailability of soil contaminants due to changes in geochemical soil properties via soil dilution and iii) comparing results based on direct toxicity assays and geochemical data.

2. Materials and methods

2.1. Site description

Our research site was the area surrounding the Mónica pyrite mine, near the village of Bustarviejo (Sierra de Guadarrama, Madrid, Spain). Mining activities were carried out here from 1427 until 1980, and a group of galleries and pyritic dumps remain. The site extends across 200,000 m^2 within the La Mina stream valley, between the following UTM coordinates: 30 T - X= 0438606, Y= 4524302; X= 0437797, Y= 4523518, where a shrub land (higher sites) and a woodland (lower sites) are developed. Two freshwater streams (with water depth between 10 and 15 cm) were present at the studied site: La Mina and La Barranca.

2.2. Soil samples

Based on the total element concentrations derived from a previous research (Moreno-Jimenez et al., 2011), the sampling points were designed to represent the entire area. Four soil samples were selected to represent different zones according to mine distance and the level of As and metals in the soils 2 and 3 (S2 and S3) had the highest hazardous elements concentrations and were found nearest the mine (3-312 m); soil 4 (S4) denoted intermediate distance sites with intermediate contaminant levels (459-657 m) and soil 5 (S5) possessed contaminants levels representative of distant sites (771 -1229 m) with soil contaminant levels close to background levels in this zone (De Miguel et al., 2002). The sampling points are shown in Figure 1. Control soil was collected from a field located near Madrid (Spain). This soil was also used to prepare the dilution series of contaminated soils. Control and contaminated soils were each taken from the topmost soil layer (0-20 cm), air-dried and sieved (2 mm mesh). Table 1 details the main physico-chemical characteristics and element concentrations of control and test soils.

Figure 1 here

Table 1 here

2.3. Water samples

Water samples were taken from streams in the surroundings of the mine and sampling locations are detailed in Fig. 1. Samples A1 and A2 were taken from a stream that goes through the mine and leaves without being diluted by adjacent streams; A1 is within and A2 is outside of the mine. Sample A3 was taken upstream of the mine to obtain background levels for waters in the site. Samples A4 and A5 were taken in the La Mina and La Barranca streams, which cross the mine's surroundings. Sample A9 was taken downstream from the confluence of adjacent streams at the most distant site. Surface waters (100 mL) were sampled in plastic flasks. Samples for ecotoxicological assessment were stored at 4ºC and analyzed as soon as possible. Samples for chemical analyses were supplemented with HNO_3 at a ratio of 1 mL of HNO_3 per 40 mL of water. Non-filtered samples were stored at 4ºC for a maximum of 20 days before further analysis.

2.4. Ecotoxicity assay

Contaminated soils were tested at four dilutions (100, 50, 25 and 12.5% test soil, w/w). Dilutions of polluted soil with control soil were prepared on a dry-weight basis and were obtained by mechanically mixing the soils in a B50 Solid V-mixer (Lleal, S.A.). Soils were assessed on the Multispecies Soil System MS-3 (Fernández et al., 2005). Soils were placed in 15 cm height x 15 cm diameter methacrylate columns (2.0 kg soil dry wt. per column) and three replicates of each concentration were examined. Adult Eisenia fetida (Oligochaeta: Lumbricidae) from our laboratory cultures, at between 300 to 600 mg of wet weight, were washed with distilled water, kept for 24 hours on moist filter paper to depurate the gut contents and weighed. Then, earthworms (10 individuals per column) were added on day 0 to each soil microcosm and used to represent soil invertebrates. Seven seeds of three plant species (i.e., wheat, Triticum aestivum, radish, Raphanus sativus and vetch, Vicia sativa) were sown into the

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soil in each microcosm. Seeds were obtained from the Spanish National Centre for Seeds and Vegetal Varieties (Madrid). Columns were incubated in a climate controlled room at a temperature of $20 \pm 2^{\circ}C$ and illuminated with fluorescent bulbs (18 W) with a photoperiod of 16 h daylight and 8 h darkness; the light intensity was 3000-4000 lux. Enough water was added to the soil to reach its water-holding capacity. Columns were watered 5 days a week with 50 ml of dechlorinated water, simulating 1000 mm rainfall/year, allowing the soils to drain to field capacity.

After 21 days, the MS-3 columns were opened and the earthworms were counted for survival assessment, washed with distilled water, kept for 24 hours on moist filter paper and weighed. Seedling emergence and above-ground biomass production, measured as wet mass of shoots, were recorded. Soil samples from the superficial layer were collected for microbial activity assays. Toxic effects on microorganisms were determined using a soil respiration test induced by glucose and soil enzyme activity, specifically dehydrogenase (DHA) and acidic phosphatase. Microbial respiration was determined following the principles of standardized methods (OECD, 2000). Samples were amended with 4 mg glucose/g soil (dry weight) and carbon dioxide release was measured using a BacTrac 4000 SY-Lab (Microbiological Analysers). Dehydrogenase and acidic phosphatase activities were measured according to Carbonell et al. (2000) and Freeman et al. (1995) respectively. Treated and control soils were run in triplicate, and duplicates of each sample were taken for analysis.

Leachates were collected for 21 days, in association with watering events. Successive leachate fractions of each microcosm were mixed and kept refrigerated at $4^{\circ}C$. At the end of the assay, leachates were stored at $-20^{\circ}C$ for further chemical and biological analysis when immediate analysis was not possible. Toxicity of the leachates to aquatic organisms was determined for algae (OECD, 2006; Ramos et al., 1996) and daphnids (OECD, 2004). Daphnia is very sensitive to low pH, but pH adjustment of the samples was not performed to avoid changes in As and metal bioavailability.

2.5. Chemical analyses

After drying, sieving and homogenizing the soils, dichromate-oxidizable organic matter (OM) and pH for a 1:2.5 (soil:water) suspension were measured following the protocols of the Spanish Ministry of Agriculture (MAPA, 1994). Pseudo-total concentrations of elements were assayed after $HNO_3:H_2O_2$ digestion in an autoclave (Wenzel et al., 2001). Extracts were filtered (num. 42 filter paper, Whatman) and diluted with milli-Q water. The extractable trace element content of the soils was obtained by shaking 2 g of soil with 20 mL of 0.1 M $(NH_4)_2SO_4$ for 4 h, then the suspension was filtered and the filtrate analyzed (Vázquez et al., 2008). Extraction was performed in triplicate at the beginning and the end of the toxicity test.

Elements concentration in samples of leachates and soils (total and extractable) were analyzed by atomic absorption spectrometry (Perkin Elmer Analyst 800 for Cd, Cu and Zn) or atomic fluorescence (P S Analytical 10.055 Millennium Excalibur System). Three analytical replicates were measured per sample.

2.6. Statistical analysis

The data were analyzed statistically using STATGRAPHICS software (Version 5.0). Statistically significant differences for chemical and toxicological data were established by analysis of variance (ANOVA) with Fisher's least significant difference procedure (LSD, $P<0.05$). Log-probit methods were used to calculate L(E)C50. Linear regression analysis was performed for chemical soil analysis and toxicity data.

3. Results and discussion

Soil quality assessment of the mine's surroundings was performed with a multispecies assay using the MS-3 system. Toxicity to aquatic organisms was determined with the soil leachates to assess the risk of contaminated soils to ground and surface waters. The results were analyzed using two approaches. In the first, the toxicity of undiluted samples was measured as a percentage of effect compared with control soil. In the second, a dose-response curve was measured similarly to the one obtained when assaying pure chemical substances.

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3.1. Physico-chemical soil characteristics

The physico-chemical soil characteristics, total and available concentrations of As and hazardous metals in the soil samples are shown in Table 1. The soil pH varied from 3.8 to 5.9, indicating the acidic nature of the contaminated soil, which is usual in mining sites. Lower pH was found at the sites nearest the mine (S2 and S3) with respect to S4 (intermediate distance) and S5 (most distant). Organic matter content was very low, with the highest levels found at S4. Soil texture was very similar for all soil samples, with sand comprising the largest fraction. Samples S2, S3 and S4 showed a sandy-loam texture, while S5 presented a loamy-sand texture. Soils were contaminated with a mixture of As and hazardous metals (e.g. Cd, Zn and Cu). Arsenic and Zn were the most concentrated elements, and Cd levels were approximately 2 orders of magnitude lower than the rest of the elements.

The total concentrations of As and metals in the soils decreased with increasing distance from the mine, in the order S3, S2, S4 and S5. The median values for the hazardous elements were compared with the limits developed by the Autonomous Community of Madrid (2006) for the protection of environmental health in natural areas. The levels of As and metals were well above those of soil quality criteria. Concentrations of As were higher than contamination standards even in the farthest sites. Cadmium and Cu standards were exceeded in all soil samples with the exception of S5. Conversely, the standard for Zn is more permissive and was only exceeded in the most contaminated soils (S2 and S3). Therefore, soils 2, 3 and 4 require risk assessment as well as S5 due to their As levels. Moreover, the previous site-specific assessment based on geochemical analysis alone (Moreno-Jimenez et al., 2011) showed that As and metals may cause a range of impacts over the entire research area, affecting all potential receptors.

The available fraction of contaminants was determined using a low concentration of ammonium sulfate, which is a relevant and frequently applied technique for As and metals (Adriano, 2001) analysis. Extractions with neutral salt solutions based on desorption or ionic exchange processes result in low concentrations of elements. However, different studies show that this fraction is a good predictor for element uptake by plants and earthworms, which absorb toxicants

predominantly via a soil solution/dermal route, especially in the case of metals [McLaughlin et al., 2000; Berthelot et al., 2008).

The available concentration of As followed a different pattern than Cd, Zn and Cu (Table 1). Available As concentrations decreased with distance from the mine, similar to the total concentrations (S3 > S2 > S4 > S5). However, the available concentrations of Cd, Zn, and Cu were higher in S2 than in S3 despite the total concentrations of these metals being 2, 6 and 4-fold higher, respectively, than in soil 3. A high availability of hazardous elements is expected in these soils due to pH values below or close to 5.5 and low organic matter content. Soil 2 showed the highest availability for metals with available concentrations of 10% for Cd and Zn, and 4% for Cu. In contrast, S3's metal availability was the lowest, with values of 1, 0.1 and 0.5% of total Cd, Zn and Cu, respectively, despite having the lowest pH measured. This fact indicates that metals in soil 3 were in a poorly soluble form. S3 was taken from the base of a tailings pile; receiving aqueous runoff and particles released from this waste by the weathering of mineralized rock. Consequently, an important fraction of the hazardous elements would be held within a crystal lattice from the original mineral, which is not easily separated or removed. Additionally, more soluble fractions can be quickly washed away in runoff. With regard to arsenic, the amount extracted by $(NH_4)_2SO_4$ was lower than 0.1% of the total concentration in all soils, concurring with values found by other authors (Adriano, 2001). This might be due to the anionic character of As, which favors adsorption at low pH.

Arsenic and metal levels were also measured in the streams on the site (Table 2). Samples A1 and A2 reported the highest concentrations of As and metals and the lowest pHs due to their proximity to the mine, where they have not yet undergone dilution by other streams. The concentrations of As, Cu and Zn in all of the sampling points exceeded Spain's maximum allowed values for surface waters (Ministerio de Medio Ambiente, 2000). Values for Cd have not been legislated yet.

Table 2 here

3.2. Effects of soil dilution on pH and on As and metal availability

Mixing contaminated soils with control soil increased pH inverse to the percent of contaminated soil in the different series (Table 3). The pH increased from 3.8 to 5.9 in the undiluted soils to 5.7 to 7.5 in the diluted soils. Moreover, a slight rise of pH was observed at the end of the microcosm assays, which may be a result of the presence of worms in the initially acidic soils (Berthelot et al., 2008; Ma et al., 2002). These changes in the pH could affect the mobility and availability of contaminants in the soil.

<center>Table 3 here</center>

Changes in physicochemical soil properties due to dilution affected the availability of As and metals differently (Table 3). Thus, in each series Cd, Zn and Cu showed a correlation between available and total concentration ($r^2 = 0.99$), with an increase in the available concentrations corresponding to an increase in the percent of contaminated soil. Whereas for arsenic, the percent available concentration increased with dilution, i.e., when the proportion of contaminated soil to control soil was low, the geochemical characteristics of the control soil dominated and the arsenic was more available. As a result, in S3 and S4 the available concentrations of As were independent of its total concentration, while in soil S2 the available fraction was actually reduced by the increasing proportion of contaminated soil.

Although the effect of pH on As(V) adsorption varies considerably among soils, in general As is less mobile in acidic soils due to adsorption onto iron oxide surfaces (Madejon and Lepp, 2007; Hartley et al., 2009). Therefore, increasing from acidic to neutral pH could explain the behavior of As. For metals, significant differences in element availability were not observed between the beginning and the end of the assay despite the increase of pH. However, As underwent an increase in soil availability with time corresponding to the rise of pH induced by earthworms. This effect was more pronounced in the more diluted soils. These results were important because they showed that available pollutants may be released into the environment due to significant changes in soil properties (Alexander, 2000).

<center>Table 4 here</center>

3.3. Chemical analysis of soil leachates

Soil leaching in packed columns is a reliable method to research the mobility of contaminants in soil (Garcia-Sanchez et al., 2010). Moreover, the MS-3 system used in this work allows the determination of soil contaminant mobility under realistic conditions via microcosms columns that reproduce natural conditions, including simulated rainfall events. Data of geochemical characteristics, As and metal concentrations in the soil leachates are presented in Table 4. Leachates from undiluted samples of contaminated soils presented the lowest pH and conductivity. The transfer of As and metals from soil to leachate was very low, especially in S3, where the percent concentration of trace elements in leachates varied from 0.001 to 0.7 % of soil concentration. However, due the high concentration of contaminants in soils, the levels of contaminants in leachates were still quite high in some cases. For example, 5.3 and 89.2 mg L^{-1} of Zn were measured in undiluted soils from samples S2 and S3, and 31.8 mg L^{-1} of As was measured in leachates from S2 at 12.5% of the soil concentration.

The effect of soil dilution was different on As and metal mobility. Metals showed the highest concentrations in leachates from undiluted soil samples, corresponding with the highest soil metal concentrations (both total and extractable). Conversely, As concentration in leachates from soils 2, 3 and 4 decreased with an increasing proportion of mining soil in the mixture, despite the increase in total concentrations of As in the soil. Concentrations of contaminants in leachates (from undiluted and diluted soils) were not related to the total concentrations in soil but to the extractable concentrations, which showed correlation coefficients of 0.74 (As), 0.98 (Zn), 0.97 (Cd) and 0.96 (Cu). Differences in pH between diluted and undiluted soils could explain the mobility observed as mentioned above. The amounts of As leached in columns of undiluted soils were lower than 0.005% of the total As. This low value of As soil transfer could indicate that As is nearly immobile in these soils. However, the increase of mobility with dilution indicates that changes in soil properties can increase As mobility and consequently the risk of water contamination. According to sequential extraction of soil samples of the same area, between 40 and 60 % of arsenic is associated to amorphous and crystalline hydrous oxides of Fe

and Al (Moreno et al., 2010), so that a certain fraction of arsenic can be mobilized by changes in the geochemical conditions.

Table 5 here

3.4. Ecotoxicity data

Toxicity data on soil and aquatic organisms obtained in the assessment of undiluted samples of soils using the MS-3 microcosm system are shown in Table 5. Data were expressed as a percentage of inhibition with respect to control soil. As expected, the toxic effects varied substantially depending on soil and test species. In the earthworm assay, only the soils nearest to the mine (S2 and S3) caused earthworm mortality, whereas S4 and S5 did not show significant differences with control soil. However, soil 5, which presented the lowest element concentrations, showed sublethal effects on this organism with a decrease in earthworm weight of $50 \pm 4\%$. All soils were toxic to the three tested species of plants, but effects varied with the plant species and the endpoint measured (i.e., emergence of seedling or growth). Samples 2 and 3 presented the greatest adverse effects on R. sativus germination and weight of T. aestivum and V. sativa. However, in other tests, effects on plants were independent of the concentration of As and metals in the soil. The effects of soil S5 on plants were pronounced, despite the low concentrations of contaminants in this sample. Toxicity in this sample could be related either to its geochemical soil properties and low nutrient availability or to the presence of contaminants not analyzed in this research. The effects due to differences in soil properties could be resolved with the use of a reference soil with similar properties to contaminated soils but without contaminants. However, such a soil was not available.

Effects of soil on microorganisms did not relate to element concentrations in the soil. Sample S5, sited in the most distant zone, showed similar effects to S2 and S3 on the respiration rate induced by glucose and dehydrogenase (DHA) activity, whereas S4 toxicity was lower. The effects on phosphatase were not correlated with the other effects measured in microorganisms and showed different behavior in the tested soils. Thus, soil S2 showed inhibition of enzymatic activity, soil S3 showed enzymatic activation and no significant effects were observed in soil

S5. Dehydrogenase is very sensitive to element pollution, whereas acid phosphatase is less affected by it. Moreover, other authors have found that some of these elements may exert a positive effect (e.g., hormesis) on soil microbial activity at low element concentrations [1]. This fact could explain the activation observed in soil S4. It is recognized that element toxicity is more related to the fraction of hazardous elements present in bioavailable form than to total concentration (Antunes et al., 2008; Kim et al., 2009). However, in this research, a good correspondence between toxicity profiles and available concentration was not found in the tested soils. This disagreement indicates that other factors than element concentration could be affecting toxicity. To help interpreting these results and better discern the causes of effects due to contaminants and geochemical soil properties, assays were performed at different soil dilutions.

Table 6 here

Bioassays performed on the dilution series of the original soil samples showed a dose response relationship in each series for only three groups of organisms: earthworms (mortality), microorganisms (carbon mineralization, dehydrogenase and phosphatase activity) and algae. In these cases, L(E)C50 values were determined and the data are presented in Table 6. By contrast, effects on plants and earthworm weight revealed unusual ecotoxicity curves. Thus, soil S2 showed an inhibition of earthworm weight of 57±1% at all concentrations tested, independent of soil dilution (except at 100% where all individuals died and the effects on weight could not be measured). Likewise, S2 showed wheat and radish weight inhibitions of 63 ± 5 and $60 \pm 4\%$, respectively, at all dilutions tested and S3 only showed adverse effects on radish weight at 25% of soil concentration. The unusual ecotoxicity curves in each series may be due to the behavior of arsenic in soil, which is different from cadmium, zinc or copper (Crouau and Pinelli, 2008), as shown in section 3.2. In diluted samples, the most available contaminant was As and it is the most toxic element to earthworms and plants. Consequently, higher available concentrations of this element in diluted soils than in undiluted soils might explain the unusual toxicity observed; well in agreement with extractable element concentrations. In addition, intrinsic soil properties

such as pH may have also influenced the soil toxicity because many organisms used in standardized bioassays are sensitive to acidic soils. In some cases, this sensitivity can overshadow the toxicity of the hazardous elements of concern. (Frouz et al., 2011; Chapman et al., 2012). Thus, undiluted samples of S2 and S3 caused a 100% inhibition of radish emergence, whereas in diluted samples of these soils, effects on emergence were not observed. Radishes have low tolerance to acidic soils, although they can tolerate slightly acidic soils (pH 5.5-6.8) (Environment Canada, 2005). The pH of undiluted soil samples 2 and 3 was 4.7 and 3.8, respectively, which could prevent seedling germination. Considering this fact, R. sativus, may not have been the ideal plant species for the assessment purposes of this work. In the microorganism assay, a dose-response relationship was observed between pH and the respiration rate induced by glucose and the dehydrogenase activity in each series. These results indicate that differences with respect to the control are due to contaminants in the sample rather than effects due to differences in microbial populations in the soils.

The ecotoxicity of aqueous leachates obtained from mining soils was tested using algae and daphnia. In the algae assay, leachate from undiluted samples of soils 2, 3 and 5 showed similar toxicity despite differences in the concentrations of As and metals measured in these leachates (Table 5). Algal growth is highly affected by nutrients present in the soil solution, and nutrient concentration differences could overshadow the toxicity of the samples. However, in the dose-response test, results in each series were dose-dependent and more coherent with soil concentrations than those obtained in the assay with undiluted soil samples. Thus, EC50 values varied in the order S2<S3<S4<S5 according with the element concentration in soils (Table 6).

In the daphnia assay, lethal effects on D. magna were observed in the leachates from undiluted soils S3, S2 and S5. Lethal effects observed in leachates of soil 3 could be influenced by the low pH, 3.91. Daphnia is very sensitive to pH and effects due to pH alone can occur for values outside the rage 6.0-9.0 (OECD, 2004). However, pH adjustment was not performed to avoid changes in As and metal bioavailability. Effects from diluted samples of soils were not seen except in S2 at 12.5 % of soil concentration, where 100% mortality was observed. The toxicity of this leachate corresponded to the high content of As (31.88 ± 0.07 mg L^{-1}) measured in this

sample. Again, the importance of increasing As mobility with soil dilution is seen. It is remarkable the toxicity of leachates from S5, to both algae and daphnia, despite the concentration of trace elements in this soil and its leachates was the lowest. As indicated above, in the algal assay, inhibitory effects could result from the low concentration of nutrients in the leachates from soil 5 compared to those of control soil.

Table 7 here

The toxicity of water samples taken in the streams at the mine to algae and daphnia is shown in Table 7. Toxicity to daphnia was observed in all samples, causing 100% immobilization in all individuals, even in sample A9, sited at the farthest point. Effects on algae were different depending on the sample. The worst effects were observed in samples A1 and A2 near the mine pit with a percentage of growth inhibition of 100%. Samples A3, A4 and A5 exhibited an inhibitory range between 75 ± 11 % and 94 ± 5 %, respectively, whereas sample A9 did not impair algal growth. The response to samples A1 and A2 could be due to the low pH (4.16 and 4.34) of these water samples. However, assays performed with diluted samples indicated that effects were due to contaminants in the samples rather than pH, as shown below.

According to the hazard classification system for natural waters proposed by Persoone et al. (2005), A1 and A2 should be classified as very high acute hazard because a 100% effect level was reached in both test algae and daphnia. Samples A3, A4 and A5 would be classified as high acute hazard because the 100% effect level was reached in at least one test. Likewise, although A9 was not toxic to algae, this sample caused 100% immobilization of the daphnia, and therefore it is also classified as high acute hazard.

Values of EC50 (Table 7) provided more detailed information than the results from undiluted samples. They allowed the differences in toxicity between A1, A2 and the rest of the samples to be distinguished. Samples A1 and A2 showed very high acute toxicity, corresponding with their proximity to the mine and the fact that they had not undergone dilution with other surface waters, hence the levels of As and metals measured in these waters were very high.

In summary, a notable difference between soils at nearby sites and soils from intermediate and distant sites was observed only in the earthworm toxicity test results. In the rest of the assays, the differences were not as evident. Thus, all of the soils affected plant growth and microorganism activity. Moreover, soil leachates showed transference of contaminants from soil to leachate at levels which were toxic to algae and D. magna, except soil 4, which was not toxic to daphnia. This fact indicates that the soils have the capability to contaminate nearby courses of ground and surface water via lixiviation of contaminants. Furthermore, analysis of waters on the site indicated that even streams sited farthest from the mine were toxic to one of the tested organisms. Nevertheless, according to Spanish regulations (Ministerio de Medio Ambiente, 2005), the toxicity of the soils and their leachates was lower than the threshold established to declare a soil as contaminated (i.e., EC50 < 1% test soil/total soil).

3.5. Relationship between chemical concentration and toxicity. Toxicity index

Table 8 here

The soils tested in this research had a complex mixture of contaminants with different behavior in soils. Moreover, in most cases their toxic effects did not seem directly related to the total soil concentration of toxic elements. A Toxicity Index (TI) was used to explain if the results could be justified by the contaminants present in the sample and to estimate the contribution of individual elements to the mixture's toxicity (Vaj et al., 2011). TIs were defined as the quotient between the soil contaminant concentration and the toxicity of the substance measured as L(E)C50.Thus, the toxicity index of a substance i on effect j ($TI_{i,j}$) was calculated by the following:

$$TI_{i,j}= C_i /L(E)C50$$

where C_i is the concentration of the individual chemical i in the mixture and $L(E)C50_{i,j}$ is the median lethal or effect concentration of a chemical i on effect j. L(E)C50 ecotoxicity values on

soil and aquatic organisms used for determining the TI index for As and metals were obtained from the literature (ECOTOX Database, 2013; EU, 2013). Values of the TI for soil organisms based on total soil concentration are shown in Table 8.

These data revealed that the mixture's effects were dominated by arsenic, with TI values for earthworms one order of magnitude higher than Zn and Cu, and TI values for plants one order of magnitude higher than Zn and two orders higher than Cu in most soils. Toxicity data of As for microorganisms were not found and hence the TI could not be determined for this element. The TI of Cd in soils was less than 0.2 toxic units, except for plants in soil S3. Therefore, Cd should not contribute to the toxicity of the mixture, except for the indicated exception (McLaughlin et al., 2000). Taking into account the high toxic index determined for soils near the mine, especially for their As content (Table 8), a higher toxicity in S2 to plants and in S3 to earthworms is expected. In S4, a TI higher than 1 was measured for As to earthworms and plants. However, earthworms were unaffected in this soil. In these cases, risk analysis based on geochemical data alone would overestimate the toxicity of the samples, because the observed toxicity was less than what would be expected from their soil concentration (TI). A possible explanation is that the L(E)C50 values described in the literature are based on laboratory toxicity tests, which are performed on soils freshly spiked with element salts. However, in the field, element availability decreases with time, mainly due to weathering processes (Ma et al.,2005; Song et al., 2006). Thus, the use of toxicity data obtained with spiked soils can result in an overestimation of the hazards posed by element contaminated soils (Lock and Janssen, 2003; Oorts et al., 2007). One approach to solving this problem would be to compare data from the literature with trace element concentrations in the weakly adsorbed, easily extractable fraction. Accordingly, toxicity indices were also calculated on the basis of the fraction extracted by ammonium sulfate.

These values, as well as the sum of the TIs for As and metals in soil, were lower than 1 for all soils and elements except for Zn towards plants in soil 2, where the TI was 1.6 and the sum of TI for all elements was 1.8. However, adverse effects were observed for all soil organisms, indicating that the easily extractable fraction was also unable to explain soil toxicity. Therefore,

in this research, chemical analysis based on either total or extractable concentrations of soil trace elements did not allow, in most cases, the prediction of soil toxicity for soil organisms, underscoring the importance of including ecotoxicological analyses in the evaluation of soil contamination. Moreover, an effort to obtain more relevant and realistic toxicity data must be made.

Toxicity of leachates was primarily due to Zn and then to Cu, according to the TIs. Nevertheless, at difference of soil samples, Cd also contributed to the overall toxicity of the leachates. The high TI estimated for daphnia explained the 100% mortality found in soils 2 and 3. However, in soil 4, effects on survival were very low (16 ± 2 %) despite the fact that the TI values for Zn and Cu were >1. In algae, observed effects were also lower than expected according to the TI, except for in soil 5.

<div align="center">Table 9 here</div>

Finally, in water samples taken at the site, observed toxicity could be explained by the concentration of contaminants measured. Thus, great differences were found between the TIs calculated for A1 and A2 and the rest of the samples (see Table 9). Samples A1 and A2 had the highest toxicity indices, especially for Zn and Cu, with EC50 values between 0.6 and 2.2 to daphnia and algae. Samples A3, A5 and A9 were the least toxic to daphnia, corresponding with the low TIs measured in these samples. Sample A9's TI for algae was lower than 1 and it did not show toxicity towards this organism. By contrast, TI for daphnia was 1.6 and 100% of immobilization was observed. The correlation between TIs and element concentrations was stronger in aqueous samples than in soil samples or leachates, which would indicate that the influence of intrinsic properties of the medium on contaminant availability is lower in aqueous samples than in soil samples as known.

4. Conclusions

An assessment of the mining area based on direct toxicity assays indicated that the current presence of As and metals at the site may cause injuries to soil and aquatic organisms throughout the entire research area. In more distant zones, the low risk indicated by chemical analysis did not agree with the toxicity values from the ecotoxicological assays. Of the elements examined, As was the most hazardous to soil organisms and Zn and Cu were the most hazardous to aquatic organisms. Moreover, this research demonstrated that changes in geochemical conditions can increase the availability of arsenic and consequently, the environmental risk of these soils. Changes in soil use or remediation practices might alter As mobility and its ability to transfer to other receptors such as humans, groundwater or soil biota. Nevertheless, according to current Spanish law (Ministerio de Medio Ambiente, 2005), the observed toxicity of these soils and their leachates was lower than the threshold established to declare a soil as contaminated (i.e., EC50 <1% test soil/total soil). In this research, a strong correlation between toxicity profiles and soil contaminant concentrations (based on either total concentration or extracted element concentrations) was not found. The results indicated that other factors besides total element concentration affected soil toxicity. This finding emphasizes the usefulness of assessing site-specific risk by obtaining ecotoxicological information using a variety of test organisms, covering different sensitivities and exposure routes. Direct toxicity assays allow for the toxicological assessment of complex mixtures of contaminants and have a valuable role in determining the overall toxicity of a sample. They allow considering those factors affecting toxicity: soil's geochemical properties, element availability and the synergistic and antagonistic effects of the contaminant mixture at the site.

Acknowledgements

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Figure captions:

Figure 1. Orthophoto of soils adjacent to the Mónica mine. Soil (S2, S3, S4 and S5) and water (A1, A2, A3, A4, A5 and A9) sampling points are shown in the figure.