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Assessing the ecotoxicological effects of long-term contaminated mine soils on plants and earthworms. Relevance of soil (total and available) and body concentrations

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Abstract

The interactions and relevance of the soil (total and available) concentrations, accumulation, and acute toxicity of several essential and non-essential trace elements were investigated to determine their importance in environmental soil assessment. Three plant species (*T. aestivum, R. sativum,* and *V. sativa*) and *E. fetida* were simultaneously exposed for 21 days to long-term contaminated soils collected from the surroundings of an abandoned pyrite mine. The soils presented different levels of As and metals, mainly Zn and Cu, and were tested at different soil concentrations (12.5, 25, 50, and 100% of contaminated soil/soil (w/w)) to increase the range of total and available soil concentrations necessary for the study. The total concentrations in the soils (of both As and metals) were better predictors of earthworm uptake than were the available concentrations. In plants, the accumulation of metals was related to the available concentrations of Zn and Cu, which could indicate that plants and earthworms accumulate elements from different pools of soil contaminants. Moreover, Zn and Cu, which are essential elements, showed controlled uptake at low concentrations. The external metal concentrations predicted earthworm mortality, whereas in plants, the effects on growth were correlated to the As and metal contents in the plants. In general, the bioaccumulation factors were lower at higher exposure levels, which implies the existence of auto-regulation in the uptake of both essential and non-essential elements by plants and earthworms.

Keywords

trace-elements-contaminated soils; plants; earthworms; availability; accumulation; toxicity

1. Introduction

Environmental contamination by heavy metals is a well-known problem worldwide. These elements pose a serious threat to ecosystems and human health (Lee et al., 2006; Garcia-Gómez et al., 2014; Li et al., 2014). Some metals, such as zinc or copper, may have beneficial effects on terrestrial organisms at lower concentrations. However, at sufficiently high levels, all metals are potentially toxic to various ecological receptors. Metals and metalloids cannot be degraded and thus accumulate in soils and trophic chains for a long time. The accumulation of trace elements in earthworms and plants is very important for ecological risk assessment because a high concentration of metals in these organisms results in serious risk of secondary poisoning of vertebrates and predators due to biomagnification. The protection of ecosystems requires an understanding of the possible adverse effects of contaminants in organisms living in the soil. Different studies have demonstrated that biological effects are not related to the total concentration of a contaminant in the soil but rather to the fraction that is bioavailable for organisms (Alexander, 2000). However, the measurement of the bioavailable concentration is not simple because this concentration is highly dependent on the physicochemical characteristics of the soil and the physiological characteristics of the specific organism (McLaughlin et al, 2000; Mourier, 2011; Pauget et al, 2012; García-Salgado et al., 2012). Thus, the final effects of contaminants in organisms are the result of an overall process that involves a physicochemical desorption process, a physiologically uptake process, and redistribution within the organism's organs (Peijgenburg, 1999; Semenzin et al, 2007). The bioaccumulation of contaminants can also serve as a measure of the bioavailability of metals in the soil and vice versa (Van Gestel et al., 2002). Body residue is considered a better surrogate for exposure, and this measure, rather than the exposure concentration based on the total metal concentration, can be used to predict toxicity in risk assessments (Neuhauser et al., 1995; Smith et al., 2012). This approach assumes that a certain concentration of a chemical in a living receptor is associated with an adverse biological effect (Pentinnen et al., 2011). However, uptake does not necessary mean toxicity because an organism may sequester metals and thus avoid their physiological effects (Fairbrother et al., 2007). Consequently, in some species, external metal concentrations may directly determine the toxic effects rather than internal concentrations. Therefore, more information is necessary to establish relationships among soil availability, tissue accumulation, and toxicity in order to obtain a complete view of the effects of contaminated soils in the environment.

Most studies on metal availability and accumulation in organisms have been conducted using soils that are freshly spiked with chemicals; however, these soils have limited environmental significance for various reasons, including the fact that the system-soil-matrix contaminants are far from equilibrium. Moreover, these studies have focused on the responses to single chemicals, even though few sites in nature are polluted with only one chemical. Experiments conducted with long-term contaminated soils containing chemical mixtures are important for the development of ecologically relevant criteria because these more realistically resemble polluted environments.

The purpose of this research study was to investigate the interactions and relevance of soil availability, accumulation, and acute toxicity for several essential and non-essential trace elements in order to perform an environmental soil assessment. To tackle this objective, long-term contaminated soils from the surroundings of an abandoned pyrite mine in Bustarviejo (Madrid, Spain) were collected. The soils presented different levels of As and metals, mainly Zn and Cu (Moreno-Jimenez et al., 2011). The study was performed with organisms belonging to two taxonomic groups (earthworms and plants), which were selected based on their environmental relevance. These organisms are widely used as bioindicators of soil contamination because they are common and abundant in most terrestrial environments and are fundamental for soil structure and functionality. Moreover, toxicity tests for these organisms are well defined, and both organisms are important from the point of view of accumulation because they are the first link in the food chain. The aim can be divided into three main sub-aims: i) to determine the accumulation in earthworms and plants after exposure to naturally aged multi-contaminated soils with a wide range of metal exposure concentrations, ii) to relate the external soil concentrations (total and available) with the tissue concentration and effects, and iii) to investigate to what extent the toxic effect of the metals can be predicted from the body concentrations.

2. Materials and methods

2.1. Site description

Our research site was the area surrounding the Mónica pyrite mine, which is near the village of Bustarviejo (Sierra de Guadarrama, Madrid, Spain). Mining activities were conducted in this are from 1427 to 1980, and a group of galleries and pyritic dumps remain. The site extends across an area of 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. A shrubland (higher sites) and a woodland (lower sites) have developed in this area. High levels of arsenic and heavy metals have been previously detected in this area by Moreno Jiménez et al. (2010).

2.2. Soil samples

Four soil samples were selected according to the level of As and metals in the soils. Control soil was collected from a field located near Madrid (Spain). This soil was also used to prepare the dilution series of the contaminated soils. The control and contaminated soils were collected from the top soil layer (0-20 cm), air-dried, and sieved (2 mm mesh). Table 1 details the main physicochemical characteristics and chemical concentrations (total and available) of the control and test soils.

The contaminated soils were diluted with the control soil at four dilutions (100, 50, 25, and 12.5% test soil, w/w). The dilutions of polluted soil with control soil were prepared on a dry-weight (d.w.) basis and were obtained by mechanically mixing the soils in a B50 Solid V-mixer (Lleal, S.A.). The mixed soils were allowed to equilibrate during 24 hours.

Table 1 here.

2.3. Ecotoxicity assay

The control and test soils were placed in 15-cm-high x 15-cm-diameter methacrylate columns (2.0 kg soil d.w. per column). Water was added to bring the soil to its water holding capacity. Three replicates of each treatment were examined. Adult earthworms (*Eisenia fetida*) with a wet weight between 300 and 600 mg were washed with distilled water and maintained for 24 hours on moist filter paper to depurate the gut content. Ten adult earthworms per column were then placed on each soil surface. Seven seeds of three plant species (wheat (*Triticum aestivum*), radish (*Raphanus sativus*), and vetch (*Vicia sativa*)) were sown in three sectors into the soil in each microcosm. Twenty one seeds of the three plant species (seven per species) were sown in sectors The seeds were obtained from the Spanish National Centre for Seeds and Vegetal Varieties (Madrid). The columns were incubated in a climate room (20 ± 2ºC) and illuminated with fluorescent bulbs (18 W) with a photoperiod of 16 h of daylight and 8 h of darkness; the light intensity was 3000-4000 lux. The columns were watered five days a week with 50 mL of dechlorinated water. After 21 days, the surviving earthworms were counted, washed with distilled water, maintained for 24 hours on moist filter paper, and weighed. The earthworms were then frozen at -20ºC for 24 hours, lyophilized (Telstar Cryodos), and analyzed to determine their total As and metal contents, as described in the Chemical Analyses section. Because dead worms decompose quickly, the body residues cannot be measured. The seedling emergence and above-ground biomass production, which was measured as the wet mass of the shoots, were recorded. The plant material was washed thoroughly in tap water and then in distilled water and dried at 60ºC for 24 hours for chemical analysis.

2.4. Chemical analyses

After the soils were dried, sieved, and homogenized, the dichromate-oxidizable organic matter (OM) and pH of a 1:2.5 (soil:water) suspension were measured according to the protocols of the Spanish Ministry of Agriculture (MAPA, 1994). The pseudo-total concentrations of the elements were assayed after HNO_3:H_2O_2 digestion in an autoclave (Wenzel et al., 2001). The 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 in 20 mL of 0.1 M $(NH_4)_2SO_4$ for 4 h; the suspension was then filtered, and the filtrate was analyzed (Vázquez et al., 2008).
The lyophilized earthworms were ground to a fine powder with an agate pestle and mortar. All of the earthworms belonging to the same column were treated and analyzed together. For the acid mineralization of the organism tissues, 10 mL of Milli-Q water, 3 mL of HNO_3, and 2 mL of H_2O_2 were added to 0.5 g (d.w.) of tissue, and digestion was performed at 1500 Pa and 125ºC in an autoclave (Lozano-Rodríguez et al., 1995). The extract was filtered and diluted with water to final volume of 25 mL. The plant material was mineralized following the protocol described by Moreno Jiménez et al. (2010).

The As and metal concentrations in the samples of soil (total and extractable), plants, and earthworm extracts were analyzed by atomic absorption spectrometry (Perkin Elmer Analyst 800 for Zn and Cu) or atomic fluorescence (PSAnalytical, As). Three analytical replicates were measured per sample.

2.5. Statistical analysis

The statistically significant differences in the chemical and toxicological data were established by analysis of variance (ANOVA) with Fisher’s least significant difference procedure (LSD, $P<0.05$).

Regression analysis was used to investigate the relationships between the body metal concentrations (earthworms and plants) and the total and available soil concentrations of trace elements. This analysis was applied to pooled data from all test soils and dilutions. The concentrations were processed after being log-transformed to normalize the distribution of the data. The data from the control and contaminated soils were very different and may deviate the analysis. For this reason, the data from the control soil were omitted in the regression analysis.

Only those significant data for which the fit of the model exceeded an R^2 value of 50 are mentioned.

The possible relationships between the soil concentrations (total and available) and the organism concentration or residue of trace elements with toxicity were investigated by probit analysis. Whenever possible, the L(E)C50 and L(E)R50 values were calculated. These values were defined as the soil concentration or residue levels of the element that cause 50% mortality or effect in the organisms.

The bioconcentration factors (BCF) for the accumulation of trace elements in earthworms and plants were calculated as the ratio of the measured concentrations of the chemical in the body to that in the soil; both of these measures were related to the dried weight.

All of the analyses were run using the software package STATGRAPHICS (Version 5.0).

3. Results and Discussion

The present study attempted to interrelate the concentration (total and available) of As, Zn, and Cu in an aged soil and the accumulation and toxicity of these elements in earthworms and plants. We designed our study to include soils with a wide range of metal concentrations. Thus, the natural soils selected from a derelict mining area were diluted to 12.5, 25, 50, and 100% (w/w) concentrations of the contaminated soils. The dilution of aged soils with a natural non-contaminated soil allowed the study of earthworm and plant bioaccumulation and toxicity under many different conditions of pH and with different levels of total and available element concentrations. Because all these factors potentially influence metal bioavailability, there should be substantial differences in the body concentrations and toxicities between soils.

3.1. Soil pH and total and available concentrations

Table 1 shows the physicochemical parameters and element concentrations in soils from the mining area and their respective dilutions as well as the control soil. The non-diluted soils, particularly soils 2 (pH=4.7) and 3 (pH=3.8), had a low pH, indicating the acidic nature of the contaminated soil, which is usual in arsenopyrite mining sites. The soil dilutions with control soil increased the pH and organic matter content, obtaining mixtures with pH values ranging from 3.8 to 7.4. The differences in the organic matter content among the soils were lower and ranged from 0.4 to 1.9%.

The soils comprised a wide range of trace element total concentrations: the As, Zn, and Cu levels ranged from 5.5, 47, and 3.3 mg kg^{-1} to 9760, 10185, and 1997 mg kg^{-1}, respectively. 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 metal analysis (Adriano, 2001; Berthelot et al., 2008; Marabottini et al., 2013). The available concentrations of As, Zn, and Cu were very low, ranging from 0.18, 0.79, and 0.03 mg kg^{-1} to 8.4, 172, and 21.1 mg kg^{-1}, respectively. These levels were less than 1% of the total soil concentrations, similarly to results obtained by Pignattelli et al. (2012) in mining sites. However, some exceptions existed, e.g., the non-diluted samples of soils 2 and 4 exhibited Zn percentages that reached 8% and 6%, respectively, and the non-diluted soil 2 presented a ratio of available to total Cu concentration of 4%.

3.2. Concentration of heavy elements in organisms

Earthworms and plants accumulated elements to varying degrees depending on the soils and elements (Table 2). The earthworms accumulated higher internal metal concentrations of As and metals than plants during a similar exposure interval. In general, the arsenic concentrations measured in organisms (earthworms and plants) exposed to contaminated soils increased significantly compared to those of the control group. The analysis of the zinc and copper concentrations revealed differences of the control soil only with the soils containing the highest element concentrations.

Table 2 here

The comparison of the contaminated soils showed that the organisms exposed to the most polluted soils (soils 2 and 3) accumulated the highest levels of As, whereas the Zn and Cu concentrations exhibited slight differences between soil 2 and soils 4 and 5. This finding may be explained by the lower difference in the concentrations of Zn and Cu compared with that of As between these soils. It is remarkable that the lowest tissue Cu concentration was measured in plants exposed to diluted samples of soil 2, even though the highest Cu concentrations were found in these soils compared with the corresponding diluted samples of soils 4 and 5.

3.3. Bioconcentration factor

The bioconcentration factors showed large variability among the soils and were higher in organisms exposed to the least contaminated soils. In general, the BCF for plants and earthworms were lower than 1, with the exception of those exposed to control soil and soil 5. This should limit the risk of the transfer of contaminants along the food chain. However, due to the high element soil concentrations, high element levels were detected inside earthworms and plants.

Regarding the elements, the comparison of the average value of $BCF_{earthworm}$ showed similar values for As (0.89±0.87), Zn (0.72±0.66), and Cu (0.73±0.86). However, for plants, differences were observed in the different elements, indicating that the absorption capacity depends on the elements and plant species. The lowest BCF values were obtained for As in the three plant species wheat (0.092±0.077), radish (0.26±0.26), and vetch (0.051±0.054), according with the values measured by Anawar et al. (2006). Moreover, these results are consistent with the literature (Smith et al., 2008), which shows that radish exhibits a certain ability to transport arsenic to aerial tissues

3.4. Toxicity to earthworms and plants

The toxicity data on plants and earthworms are expressed as a percentage of inhibition with respect to the control soil and are shown in Table 3. As expected, the toxic effects varied substantially depending on the soil and test species. In the earthworms, only the samples from the most contaminated soils (soils 2 and 3) caused significant earthworm mortality. The earthworm body weight decreased substantially after exposure to samples of soil 2 and undiluted samples of soil 5. The changes in weight may bias the element concentration in the earthworms. Thus, weight loss may indirectly result in a relative higher body concentration of metals or the uptake can be directly affected due to changes in earthworm behavior. However, in this work, the changes in weight did not appear to affect the observations.

Table 3 here

Most soils were toxic to the three plant species. Plant growth appeared to be a more sensitive endpoint than the emergence of seedlings and exhibited less variability. The effects on *T. aestivum* germination were observed only with the non-diluted samples of the contaminated soils, regardless of the element concentrations in these soils. *R. sativus* and *V. sativa* germination was affected only by undiluted soil 2 (most likely due to the low pH) and the diluted and undiluted samples from soil 5, even though the lowest concentrations of elements were measured in this soil. In contrast, the effects on plants growth were observed in most soils with an inhibition range of 13 to 91%.

3.5. Correlation studies

3.5.1. Relationships between total and available concentrations

The logarithmic-based statistical correlation between the total and available concentrations showed that the element availability was apparently independent of the respective total content in the soil ($R^2<50$). This finding may be due to changes in the soil pH and, in minor extension in organic matter, when soils are mixed with non-contaminated soils. The lack of a correlation between the total and available concentrations allowed the independent study of the influence of both variables on the accumulation and toxicity of the elements.

The addition of pH as a predictive parameter in the regression slightly improved the regression analysis but only for the description of the available concentration of As, which is shown by the following multiple regression equation:

$$\log [As]_{Av} = -4.1 + 0.97 \log[As]_T + 0.28 \text{ pH}$$

where $[As]_{Av}$ is the available soil concentration and $[As]_T$ is the total soil concentration of arsenic. Although the correlation factor was low ($R^2=55$), the significance was lower than 0.001. The soil pH is a positive term in the equation; thus, an increase in the pH will increase the available arsenic concentration, which is agreement with the chemical characteristics of this element (Moreno-Jiménez et al., 2012).

3.5.2. Relationships between element accumulation in earthworms and soil concentration

To determine whether it is possible to establish a relationship between the external concentration (total or available) and the element accumulation in earthworms and plants after exposure to mining soils, both parameters were compared in a logarithmic basis. The monovariate regression formulas describing these quantitative relationships are given in Table 4.

Table 4 here

Differences between essential and non-essential elements were observed. For example, As showed a strong link between the earthworm body burden and the soil concentration (Figure 1) with a continuous increase in the concentration in earthworms with an increase in the total As soil concentration (R^2=97; P<0.001). Based on the high regression coefficient and significance of the model, this model can be used to predict the amount of this element in the total body tissue of earthworms exposed to similarly contaminated sites.

Figure 1 here

Zn and Cu, which are essential elements, showed a regulation in metal uptake at the lower concentrations tested in this study. The zinc concentration in *Eisenia fetida* was maintained between 100 and 200 mg kg^{-1} in organisms exposed to soil concentrations between 50 and 1000 mg kg^{-1} soil, similarly to the results found by other authors (Lock and Janssen, 2001; Smith et al., 2010). Likewise, the concentrations of Cu in earthworms exposed to soils ranging from 3 to 380 mg kg^{-1} showed tissue concentrations lower than 56 mg kg^{-1} (Smith et al., 2012). At concentrations higher than these soil concentrations, the log-transformed internal earthworm concentrations of Zn and Cu increased linearly with an increase in the log-transformed soil concentrations of Zn (R^2=57, P=0.02) and Cu (R^2=56, P=0.03), reaching values up to 1440 mg Zn kg^{-1} and 862 mg Cu kg^{-1} in earthworms tissues. The steady-state levels of Cu (60 mg kg^{-1}) and Zn (200 mg kg^{-1}) observed in *E. fetida* in the present study corresponded well with those reported in previous studies, reinforcing the hypothesis that *E. fetida* is able to regulate essential metals, such as Cu and Zn, in its body until a certain level (Peijnenburg et al., 1999; Lock and Janssen, 2001; Smith et al., 2010).

The available concentration did not explain the variation in the As and metal concentrations in earthworms. In contrast to the findings obtained with the total concentration, no range in the soil concentration of essential metals at which the accumulation was regulated was observed. The accumulation in earthworms did not show any relationship with the pH, which is in agreement with the results reported by Ma (2004), who found that the soil pH is of only minor importance with respect to the accumulation of Cu and Zn in earthworms.

3.5.3. Relationships between element accumulation in plants and soil concentration

The data showed a complex interaction between the element soil concentration and the plant accumulation, which depended on the species and mainly on the metal involved. Thus, the total soil concentration cannot explain the accumulation of any of the elements, except As, observed in plants (Figure 2a).

When the tissue plant concentration data were compared with the available concentration (and both were expressed as logarithmic data), a correlation of arsenic was only found for radish, which shows that *V. sativa* and *T. aestivum* exhibit a stronger excluder behavior than *R. sativus*; this finding was also reflected in the BCF. The accumulation of Zn was regulated and was best described by the available rather than the total concentration; this finding was obtained for the three plant species (Figure 2b). Increases in the plant tissue concentrations were only observed with the highest Zn available soil concentration.

Figure 2 here

The Cu concentration in plants could only be well predicted based on the available soil concentration for wheat, although this correlation exhibited a low coefficient of regression (R^2=58). The small range of available Cu concentrations, which was lower than that obtained for As and Zn, could explain the low power of the relationships found for all regressions. Pignattelli et al. (2012) also found differences in the influence of the available and total concentrations on the plant accumulation between As and metals (e.g., Zn and Cu), which was attributed to the difference in the uptake mechanisms of arsenate (phosphate transporters) and metals.

The results of the correlation analysis showed that the pH is not a determining factor for the accumulation of any of the tested elements in plants, similar to results obtained for earthworms.

3.5.4. Comparison and relationships between metal concentration in earthworms and plants

As shown in the previous section, in earthworms, a strong dependence of body concentrations on the total element (As, Zn and Cu) levels in soil was observed. In contrast, although the As accumulation in plants was determined by the total concentration, the extractable Zn and Cu concentrations were the most representative of the fraction accumulated in plants. The influence of the total and available concentrations on organism accumulation has been discussed in different articles, and the results are contradictory. Some studies revealed a dependence of the metal burden on worms (Peijnenburg, 1999; Janssen, 1997) and plants (Smith et al., 2012) with the total concentrations, whereas others (García-Salgado et al., 2012) found a dependence on the extractable concentrations. Pignattelli (2012) found a correlation between the As content in plants with the total and available concentrations, whereas the Zn and Cu accumulation was irrespective of both external concentrations.

The differences observed between earthworms and plants in this study may be explained by the fact that uptake is not only controlled by the element distribution in the soil but also by the exposure pathways. Plants essentially take up elements from the soil via the soil solution

(McLaughlin et al., 2000), and hence, metal burdens are determined primarily from the available soil metal levels. Earthworms can be exposed to contaminants present in the soil by two major routes: ingestion of soil particles and dermal exposure to the soil solution. Although the exposure via soil pore water is considered the most important (Van Straalen and Van Gestel, 1993; Vijver et al., 2003), the correlation found between the total concentrations and the body concentrations pointed toward the hypothesis that, due to soil ingestion, earthworms may access less labile metal pools than plants (Nannoni et al., 2011). In contrast, Scott-Fordsmand et al. (2004) found that earthworms and plants access the same fraction of soil Zn; however, a wider range of soil concentrations was used in our study.

To aid the understanding of these differences, the accumulation data in earthworms and plants species (*T. aestivum*, *R. sativus*, and *V. sativa*) were correlated by regression (Table 5). Plants and earthworms were exposed simultaneously in the same soil and under the same conditions; therefore, the differences in accumulation depended on species-relative factors. For As, good correlations were obtained between the internal concentrations in earthworms and plants, indicating that the soil concentration of this element rather than species-related factors determined the metal uptake in the organism. In contrast, the zinc and copper accumulation in earthworms and plants followed a different pattern, and a correlation could not be established. The differences between the accumulation of Zn and Cu in earthworms and plants could be due to either differences in the regulation mechanisms between these organisms or differences in the uptake pathways. Both As and metals showed good correlations in the comparison of the tissue concentrations between the three plant species with the exception of Cu in wheat and vetch. This finding indicates that the uptake pathway and regulation mechanisms are similar in these plant species.

Table 5 here

3.5.5. Relationships between bioconcentration factor and soil concentration

The variations in BCF for earthworms and plants with variations in the soil element concentrations are shown in Figure 3. In the earthworms, the value of $BCF_{earthworm}$ decreased with increasing soil concentration, as was also observed by other authors (Lock and Janssen, 2001; Bade et al., 2012). This decrease was higher at lower concentrations. Thus, the curve of BCF versus log-transformed total concentration was fit to a logarithmic-x model for As (R^2=95, P<0.001), Zn (R^2=77, P<0.001), and Cu (R^2=75, P<0.001).

Figure 3 here

In the case of plants, a decrease in BCF with an increase in concentration was also observed, although regression equations were obtained only for the essential metals: zinc (R^2=67 for wheat and R^2=58 for vetch, P<0.001) and copper (R^2=80 for wheat, R^2=88 for radish and R^2=66 for vetch, P<0.001). In the case of arsenic, the lowest contaminated soil (soil 5) showed a deviation in the three plant species and cannot be fit to the general logarithmic curve, even though it also followed the same tendency (Figure 3b). When the data from soil 5 were omitted, good correlations were found with values of R^2=87 for wheat, R^2=65 for radish, and R^2=81 for vetch (P<0.001). Soil 5 is a soil with low nutrient availability and presented very low available As. Due to the limiting conditions of this soil for the support of plant development, the As uptake and transport to shoots may be favored, as has been observed in phosphorous-deficient conditions in several plant species (Esteban et al., 2003; Geng et al., 2006; Abbas et al., 2008). Uptake regulation was shown for higher As and metal (Cu and Zn) concentrations in the soils.

The higher bioconcentration factors in the non-contaminated compared with the contaminated soils as well as the decreasing in the BCF with increasing total soil metal concentrations indicate the presence of regulation mechanisms in plants and earthworms for both essential and non-essential elements. The existence of regulation mechanisms is widely accepted for essential metals, such as Zn and Cu. However, the uptake and elimination of non-essential elements, such as arsenic, may also be partially regulated (Langdon et al., 2003). The regulation found in earthworms for As agrees with the data presented by Geiszinger et al. (1998). These authors found values of BCF higher than 0.64 in uncontaminated sites and values of 0.1-0.22 in contaminated sites. In our study, the contaminated soils showed values close to 0.11, whereas soil 5, which presented a low As concentration (18.3±0.9), exhibited a BCF value of 2.0. The influence of the soil concentration on the BCF value indicated that this factor is not a good indicator of potential environmental risks. In soils with low contamination, organisms concentrate metals to obtain higher BCF values than those found in contaminated soils. Therefore, the use of BCF data obtained in non-contaminated soils for risk assessment would overestimate the risk of secondary poisoning in contaminated soils.

3.5.6. Relationships between total and available soil concentrations, body accumulation, and toxicity

To determine the importance of accumulation and total and available concentrations on the ecotoxicological effects, the toxicity to earthworms and plants was correlated with the soil (total and available) concentrations and the elements accumulated (body concentrations and total residues in the organism). Earthworm growth and seedling emergence were less sensitive endpoints than earthworm survival and plant growth, respectively. Therefore, the first endpoints were not taken into account in the correlation study. In all cases, the possible relationships were investigated by testing the goodness-of-fit of a probit dose-effect model, which was applied to the pooled data from all test soils and dilutions. The regression formulas describing the regression equations are given in Table 6. Only those models with acceptable statistical parameters were included. For almost all regressions, the explanatory power remained quite low, and only a limited number of statistically significant models for predicting toxicity values could be developed. The regression coefficients in the models were low, but all were significant at the 99.9% confidence level.

Table 6 here

Based on the results, it appears that the total metal or available element soil concentrations better explained the toxicity observed in earthworms than the body accumulation (Smith et al., 2012). Regressions were only performed using the toxicity data from soils that exhibited significant differences with the control soil (soils 2 and 3). Monovariate analyses showed a dose-response relationship between the surviving worms, the total As concentration, and the available fraction of Cu. A multivariate correlation including all of the elements showed a multiple dependency of the earthworm mortality on the available concentrations of As and Cu. Conversely, a clear dose-related response was lacking when lethality was plotted against the element accumulated (body concentration or total residues) (Alvarenga et al., 2013). Threshold body concentrations of the chemicals that were correlated with the onset of a toxic response were not found. The importance of the relation between earthworm toxicity and As soil concentration agreed with data obtained in a previous study performed in this site (Garcia-Gómez et al., 2014). In this work, the toxicity index (TI) for trace elements was determined to estimate the contribution of the individual chemicals to the mixture's toxicity (Vaj et al., 2011). The TI was calculated as the quotient between the soil contaminant concentration and the toxicity of the substance and was measured as L(E)C50. These data revealed that the mixture's effects were dominated by arsenic because the TI values for As were one order of magnitude higher than those for Zn and Cu.

The earthworm tissue concentrations were not predictive of the toxicity of the elements to invertebrates, even though some authors hypothesize that the body concentration is a better measure of toxicity that the conventionally used exposure concentration (Penttinem et al., 2011). Models based on body accumulation assume that the internal concentrations represent the concentration at the site or the toxic action. However, elements may be sequestered in organelles in a biologically inactive form; thus, the body burden of a metal may increase without an observable adverse response. Moreover, different authors (Lock and Janssen, 2001; Faibrother et al., 2007) have affirmed that the absolute level of metal accumulation is not as important as the rate of uptake. In the present work, the body accumulation was measured in living organisms, and hence, the effects on earthworms were associated with mortality ranging from 0 to 40%. The insufficiency in our data could explain the lack of a dose response when the percentage of lethality was plotted against the element body accumulation. Therefore, the influence of the tissue concentrations on earthworm toxicity was not conclusively demonstrated in this study.

Conversely, in plants, the total tissue content was the best predictor of the effects on growth, which is in opposition to the results found by Smith et al. (2012). However, the exact measure was dependent on the plant species and element. Thus, the analysis of plant toxicity revealed no significant relationships between any of the plants, with the exception of vetch, and the external concentrations (total or available) in soil. In this plant (vetch), a multiple correlation was obtained with the available concentration of the three elements, although the monovariate correlations were not significant to explain the toxicity to this plant species. The higher influence of the available concentration compared with the total concentration was in accordance with the exposure of plants to the contaminants present in the soluble fraction.

In contrast, some consistent relations were observed when the total residues in plants were used as the independent variable. The effects on the three plant species were dependent on the levels of the total As and metal contents in the plants (i.e., plant residue) and not on the plant concentration. The increase in the biological burdens of As or Zn was related with higher toxicity. However, a negative correlation was found between growth and Cu levels in plants. This finding may be explained because Cu is an essential micronutrient for plant nutrition and deficiency effects could be mistaken for toxic responses. Levels between 5 to 20 mg kg^{-1} plant are considered adequate for normal growth, whereas levels higher than 20 mg kg^{-1} are considered toxic (Adriano, 2001). The lowest levels of Cu in plant tissue were measured in plants exposed to dilutions of soil 2 and to non-diluted samples of soil 5, and high toxicity was observed in these soils, indicating that the Cu concentration in these soils may be lower than the nutritional requirements for these plant species.

To elucidate the influence of the pH on earthworm survival and plant growth, the regression model containing the soil metal concentrations as a single independent variable was compared with the results of a new extended analysis that also contained the pH (equations not shown). The results of the multivariate analysis showed that pH is only of minor importance with respect to the toxicity. The addition of pH as a predictive parameter in the regression resulted only in a slight increase in the R^2 value and did not contribute strongly to the explanation of the variance in the toxicity of the chemicals in earthworms and plants. In all cases, the correlation between pH and plant growth was negative, indicating the higher toxicity of acidic soils compared with neutral and weakly basic soils.

In this study, we assumed that no interactions took place in the derivation of the mixture toxicity and that the elements in the mixture acted independently. However, interactions may play a major role in the uptake and metabolism of elements, and antagonism and/or synergism phenomena are important in determining the toxicity to organisms of soils containing several contaminants (Fairbrother et al., 2007). The interaction among multiple factors acting simultaneously may explain the poor correlations obtained when considering a single factor.

4. Conclusions

Our study showed that bioaccumulation is a complex process that cannot be predicted by measuring the available fraction of contaminants alone. The body concentrations depend on the physiological characteristics of an organism (plants or worms), not only on its regulation mechanisms but also on its exposure routes. Thus, the total As and metal concentrations in soils were better predictors of earthworm uptake than were the available concentrations. This finding suggests that earthworms could be able to uptake elements from several soil fractions and not only soluble elements. In plants, the accumulation of metals was related to the available concentrations of Zn and Cu, in accordance with the exposure of plants to the contaminants through the soluble fraction in soils. Moreover, the metal concentrations of essential elements in earthworm and plant tissues cannot be used as a measure of soil availability because an auto-regulation of the accumulation of Zn and Cu was observed until a critical threshold soil concentration was reached.

In this study, the external metal concentrations predicted toxic effects rather than tissue concentrations in earthworms. This finding suggests that the toxic effects in earthworms are not a direct function of the excess accumulation in the body. However, the data were limited, and more studies are necessary to confirm this finding. In plants, the tissue residue contributed the most to the explanation of the effects on growth and thus appears to have an internal poisoning effect. The effects were more related to the total uptake rather than the concentration in the plant.

In general, the bioaccumulation factors were lower at higher exposure levels, which implies the auto-regulation ability for both essential and non-essential elements. These results reinforce the conclusion that BCF is a poor indicator of potential environmental risks.

The outcomes of this study showed that univocal analyses based on chemical or biological data (bioaccumulation or toxicity) are insufficient for an accurate evaluation of soil contamination in long-term contaminated soils.

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Notes

The authors declare no competing financial interest.

The authors declare that there are no known conflicts of interest associated with this publication.

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Table 1. Physicochemical parameters and concentrations (total and available) of As and metals measured in the control soil and soils collected from the mine surroundings. The contaminated soils were diluted to 12.5, 25, 50, and 100% of the mine soil concentration.

Table 2. Tissue concentrations in earthworms and plants exposed to control and contaminated soils for 21 days.

Table 3. Toxicity data for earthworms and plants exposed to control and contaminated soils for 21 days. The toxicity data are expressed as a percent inhibition compared with the control soil.

Table 4. Monovariate regression formulas describing the quantitative relationships between the log-transformed body concentrations in *Eisenia fetida* (Ef), *T. aestivum* (Ta), *R. sativus* (Rs), and *V. sativa* (Vs) after exposure to mining soils and the log-transformed total (T) or available (Av) element contents.

Table 5. R-squared and P values for the simple regressions between the logarithmic As and metal concentrations in earthworms and plant species.

* indicates the significance levels ($P<0.001$ ***) of the regressions.

Table 6. Results of the monovariate and multivariate probit analyses of the relationship between the toxicity of soils to earthworms and plants with the total soil concentration (T), the available soil concentration (Av), and the residue levels (R). The monovariate analysis was performed for each trace element, and the multivariate analysis included all of the elements.

Figure 1. Interrelationships between the steady-state concentrations of As, Zn, and Cu in earthworms after 21 days of exposure and the total element concentrations in soil.

Figure 2. Plot of the log-transformed tissue plant concentrations as a function of the soil (total or available) concentrations. a) As plant concentration versus total soil concentration; b) Zn plant concentration versus available soil concentration.

Figure 3. Decrease in the BCF value for arsenic and metals (a) in *E. fetida*, b) *T. aestivum*, c) *R. sativus*, and d) *V. sativa* exposed to contaminated soils (test soils and dilutions). The data are the mean of three repetitions.