Journal of Contaminant Hydrology 251 (2022) 104100

Contents lists available at ScienceDirect

Journal of Contaminant Hydrology

journal homepage: www.elsevier.com/locate/jconhyd

Ecotoxicological risk assessment in soils contaminated by Pb and As 20 years after a mining spill

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ARTICLE INFO

Keywords:
Toxicity risk
Metabolic quotient
Basal respiration
Exposure pathway
Bioassay
Potentially toxic element

ABSTRACT

This study evaluates the potential toxicity of the soils of the Guadiamar Green Corridor (GGC) affected by the Aznalcóllar mine spill (Andalusia, Spain), one of the most important mining accidents in Europe in recent decades. Twenty years after the accident, although the area is considered to be recovered, residual contamination in soils persists, and the bioavailability of some contaminants, such as As, is showing trends of increasing. Therefore, the potential residual toxicity in 84 soil samples was evaluated by bioassays with lettuce (*Latuca sativa* L.), earthworms (*Eisenia andrei*) and determining the microbial activity by basal respiration and metabolic quotient. The selected soils sampled along the GGC were divided into 4 types according to their physicochemical properties. In the closest part of the mine two soil types appear (SS1 and SS2), originally decarbonated and loamy, with a reduction in lettuce root elongation of 57% and 34% compared to the control, as well as a the highest metabolic quotient (23.9 and 18.1 ng C_{CO2} μg Cmicrob^{-1} h^{-1}, respectively) with the highest risk of Pb and As toxicity. While, located in the middle and final part of the affected area of the spill (SS3 and SS4), soils presented alkaline pH, finer textures and the lowest metabolic quotient (<9.5 ng C_{CO2} μg Cmicrob^{-1} h^{-1}). In addition, due to Pb and As exceeded the Guideline values established in the studied area, the human toxicity risk was determined according to US-EPA methodology. Although the total contents were higher than the Guidelines established, the obtained hazard quotients for both contaminants were less than one, so the risk for human health was discarded. However, monitoring over time of the toxicity risks of the GGC soils would be advisable, especially due to the existence of areas where residual contamination persist, and soil hazard quotient obtained for As in children was higher and close to unity.

1. Introduction

Soils may accumulate potentially toxic elements (PTEs) that affect their ecosystem functions, compromising their quality and the balance of the ecosystem's communities (Palma et al., 2019). Metal mining represents a potential pollution concern since it can cause emissions of PTEs, such as heavy metals and other associated elements like arsenic, which can be mobilized within the soil–plant-water system and dispersed in the atmosphere, and might have adverse impacts on human health of local citizens (Liu et al., 2017; Khelifi et al., 2019). Therefore, mining companies and public administrations should assess the risk of pollution associated with extractive activities and promote prevention, protection, and decontamination measures to ensure the safety of our environment (Morales et al., 2019), because they may represent potential health hazard from chronic and daily exposure to mine wastes enriched with toxic elements (Hamed et al., 2022).

There are numerous regulatory measures to limit emissions and to establish cleanup standards for PTEs in soil, which are generally based on the total soil concentration of these elements. However, total metal concentrations are poor predictors of toxicity (Lanno et al., 2004; Smolders et al., 2009), with bioavailable concentrations being more accurate to use. Bioavailable concentrations indicate the amount of PTEs that may be easier absorbed by organisms and can cause damage to the ecosystem and/or enter to the trophic chain (Van Gestel, 2008; Favas

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https://doi.org/10.1016/j.jconhyd.2022.104100
Received 11 July 2022; Received in revised form 13 October 2022; Accepted 23 October 2022
Available online 29 October 2022

et al., 2011; Niemeyer et al., 2015; Son et al., 2019). Therefore, when the guidelines are exceeded based on total soil concentrations, complementary studies are necessary for environmental and human health toxicity risk assessment (Renaud et al., 2017; Martinez et al., 2018; Fajana et al., 2020). To establish the toxicity risks in contaminated soil ecosystems, toxicity bioassays should be performed, which are effective tools to assess the actual level of contamination of soils, in addition to providing relevant and objective information to regulate the land use, as well as to plan remediation measures if needed (González et al., 2011; García-Carmona et al., 2017).

The Aznalcóllar mining accident is one of the most important mining accidents associated with metal mining worldwide (Nikolic et al., 2011). The breakage of the tailing dam caused the spill of 0.9 hm^3 of pyritic sludge and 3.6 hm^3 of acidic water into the Guadiamar river basin (Simón et al., 2001). Due to the nature of the wastes stored in the tailing dam, the acidic water and the sludge spilled into the soils of the Guadiamar river basin resulted in a severe contamination of the soils by PTEs, with significant total soil concentrations reached for As, Bi, Cd, Cu, Pb, Sb, Tl and Zn (Alastuey et al., 1999; Cabrera et al., 1999; Simón et al., 1999). Given the magnitude of the accident and the high pollution observed in the soils affected by the spill, the Regional Government of Andalusia invested an enormous amount of human, technical and economic resources for the recovery of the area, which was finally declared a Protected Site, currently known as the Guadiamar River Green Corridor (GGC) (CMA, 2003). Thanks to remediation measures applied and the effect produced by the passage of >20 years for the stabilization of the affected area, the total and bioavailable concentrations of many of the PTEs have been reduced, with the latest work in the area focusing mainly on those PTEs that persist in higher concentrations such as Pb, As, Zn, Cu and Cd (Madejón et al., 2006; Martín et al., 2015; Romero-Freire et al., 2016a). However, despite the remediation work carried out in the area between 1998 and 2001, there are still soils in which the Guideline values established for Andalusia for total Pb and As concentrations are exceeded (Pastor-Jáuregui et al., 2021).

Approximately 7% of the area affected by the spill remains contaminated, randomly distributed in heterometric patches easily

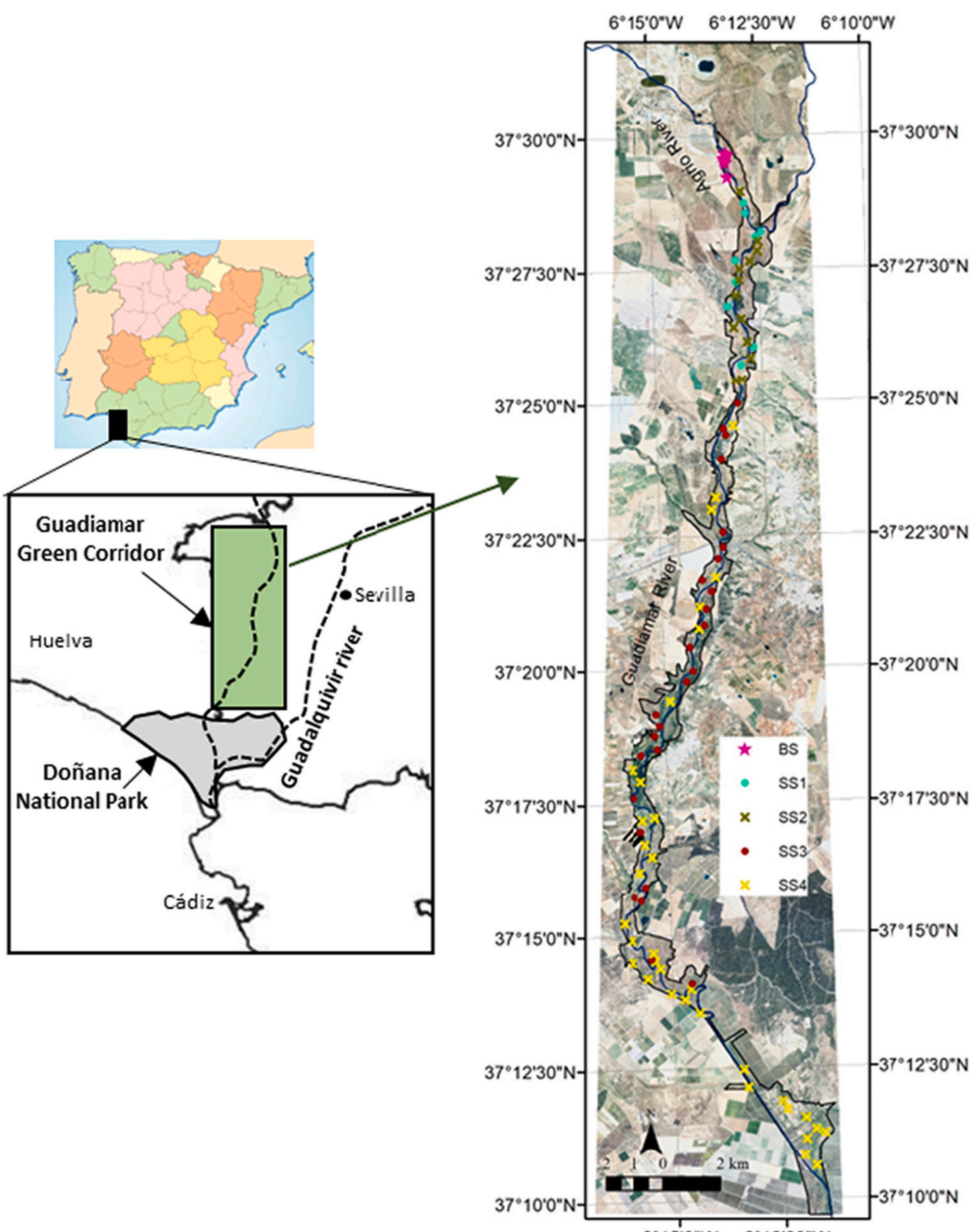

Fig. 1. Study area location and the soil sampling points along de GGC.

identifiable by the absence of vegetation, where soils are acidic, with high salinity and high total and bioavailable concentration of PTEs (Martín et al., 2015). These contaminated patches stay due to deficiencies in the removal of sludge and cleaning tasks carried out shortly after the accident. Thus, there has been a continuous oxidation of the sludge remains that persists throughout the soil profile (Simón et al., 2008). It represents a risk of spreading contamination linked to erosion and/or leaching as the soils are not stabilized by vegetation (Madejón et al., 2006).

Therefore, a long-term monitoring of the remediation of contaminated soils is necessary to evaluate the effectiveness of the measures applied and the safety of the soils. For this reason, the objective of this work is to evaluate the toxicity risks of the soils affected by the toxic spill of the Aznalcóllar mining accident, twenty years after the accident and fifteen years since the establishment of the area as recovered and declared as a protected landscape.

2. Material and methods

2.1. Site description and soil sampling

The study area corresponds to the entire Guadiamar Green Corridor (GGC) area affected by the toxic spill of the Aznalcóllar mine (Andalusia, Spain), which occurred in April 1998. It is located in the west of the province of Seville, between latitudes 37° 30′ and 37° 00′ north and longitudes 6° 10′ and 6° 20′ west, covering a 45 km long and 500 m wide strip of irregular shape on both sides of the Agrio and Guadiamar riverbeds (Fig. 1). The climate of the area is typically Mediterranean, characterized by hot, dry summers and cold, wet winters. According to the data of the agro-climatic station of the Andalusian Institute for Agricultural, Fisheries and Food Research and Training, the average annual temperature is 18.3 °C and the annual rainfall is 490 mm, with an evapotranspiration coefficient of 920 mm, considering the last 20 years 2001–2021.

A total of 84 plots (10 × 10 m) homogeneously randomly distributed along the GGC were sampled, collecting composite samples at 0–10 cm depth, to obtain a representative sample every 100 m^2.

Satellite images (Fig. SI.1) were used to identify contaminated areas where remediation efforts were insufficient and which, 20 years after the mining accident, can be identified by the absence of vegetation. According to that, in addition of the 84 sampling points, 6 patches without vegetation where pollution persists were randomly selected in the area closest to the mine, and composite samples were also taken from the top 10 cm of soil.

2.2. Soil properties and pollutant concentrations

All soil samples were bagged in the field, identified and homogenized. Once transferred to the laboratory, they were dried at room temperature and sieved (<2 mm). Soil texture was determined by the Robinson pipette method (USDA United States Department of Agriculture, 1972); calcium carbonate content ($CaCO_3$) by volumetric method (Barahona, 1984); soil pH was measured in water in a ratio 1:2.5 with a 914 pH/Conductometer Metrohm; total organic carbon (OC) was analyzed by a LECO® TruSpec CN after soil samples were acid-washed (HCl 1 mol/l for 24 h) to remove carbonates, following Ussiri and Lal (2008); soil:water extract (1:5) was prepared to determine the electrical conductivity (EC) using a Eutech CON700 conductivity-meter; field capacity was measured according to Richards (1945).

The total soil concentrations of the studied PTEs (Pb and As) were analyzed in finely ground samples by X-ray fluorescence (XRF) with a portable NITON XL3t-980 GOLDD+ analyzer (Niton, Billerica, USA). The precision and accuracy of this method was performed based on the measurement, with 6 replicates, of a certified reference material (CRM 052–050 RT-Corporation Limited, Salisbury, UK), obtaining the following results (*certified* vs. measured, mean values in mg kg^{-1} and standard error in parentheses): Pb: *82.6 (4.0)* vs. 92.6 (4.8); As: *14.6 (2.9)* vs. 15.5 (3.9). In all cases, the measured values were within the confidence interval of the certified value.

The concentration of water-soluble pollutants was determined in a 1:5 (soil:water) suspension according to Sposito et al. (1982). Bioavailable pollutant concentration was extracted using 0.05 M EDTA (pH 7) as described by Quevauviller et al. (1998). The extracted elements were measured by inductively coupled plasma mass spectrometry (ICP-MS) using a Perkin Elmer SCIEX ELAN-5000A spectrometer (Waltham, MA, USA). The precision of the method was corroborated by analysis (6 replicates) of a standard reference material (SRM 2711), obtaining the following results (certified vs. measured, mean values in mg kg^{-1} and standard error in parentheses): Pb: *1162.0 (31.0)* vs. 1138.1 (11.0); As: *105.0 (8.0)* vs. 102.4 (1.1).

2.3. Toxicity risk assessment of GGC soils

To evaluate the potential risk of environmental toxicity, for each of the 84 composite samples (SS1-SS4) and the 6 bares soils (BS), taken along the GGC, two different short-term assays were performed. 1) A bioassay of germination and root elongation was performed with *Lactuca sativa* L. (USEPA, 1996). In Petri dishes, a filter paper with 5 ml of soil:water extract (1:5) were incubated with 20 seeds of L. *sativa* L at 25 ± 1 °C, for 5 days. After that, the % of germinated seed and the length of the roots of the germinated seeds were recorded. As control, same procedure was done with distilled water in triplicate. The percentage (%) of root elongation was calculated in comparison with the control samples, from 0 (maximum toxicity) to 100 (no toxicity). 2) A second assay determined the (i) basal heterotrophic respiration rate, (ii) microbial carbon and (iii) metabolic coefficient, following adapted ISO 17155 (ISO, 2012) and OECD (2000) protocols. Around 5 g of soil, at field moisture capacity, were placed with 2 ml of KOH (0.2%) in a SY-LAB µ-Trac 4200 respirometer. Samples were incubated at 25 °C for 96 h and, after that, (i) mean basal heterotrophic respiration rate (µg CO_2 h^{-1} g_{soil}^{-1}) was obtained. Microbial biomass carbon (ii) (µg $Cmicrob_{.}$ g C_{suelo}^{-1}) was quantified using the irradiation/incubation method according to Ferreira et al. (1999). Part of the soil microorganisms were removed by electromagnetic irradiation by microwave heating and then the soil was incubated for 10 days at 28 °C and the amount of CO_2 released after incubation was measured in the same equipment. The metabolic quotient (iii) (qCO_2) was obtained as the ratio between (i) and (ii), expressed as ng C_{CO2} µg $Cmicrob^{-1}$ h^{-1}, according to Anderson and Domsch (1993). Higher values of the metabolic coefficient indicate elevated stress conditions for soil microbial communities (Nielsen and Winding, 2002).

In addition, in the 4 bare soils (BS) sampled in the areas where the pollution persists, the evaluation of toxicity was performed by a long-term toxicity test, with earthworms (*Eisenia andrei*) (OECD (Organization for Economic Cooperation and Development), 2015). To determine soil toxicity, a control soil was sampled in the vicinity of the contaminated area, but outside the boundaries of the GGC, thus ensuring that the soil was unaffected by the spill (Table SI.1). Summarizing, containers with 500 g of moistened to field capacity soil were placed with five pre-acclimatized weighed adult earthworms and incubated at 20 °C with 14 h of light per day for 4 weeks. To feed the earthworms, wet horse manure (drug-free) was added to each container and, weekly, moisture content and food requirements were monitored. After these 4 weeks, mortality rate and weight variation (average difference in the weight of earthworms at 4 weeks in relation to their initial weight) were recorded. Soils containing cocoons were incubated again for another 4 weeks at same conditions, and at the end of these 4 weeks, the containers were placed in a water bath (60 ± 2 °C) to force the juveniles to come up to the surface and to count the number of hatchlings present in each sample. The % of survival and %weight variation, compared to the beginning of the experiment, and the % of juveniles, compared to the control samples, were calculated.

2.4. Human health risk assessment

The potential human health risk for Pb and As for children and adults in the GGC was estimated based on models proposed by the USEPA (2017a) and USEPA (2017b), from the calculation of the hazard quotient (HQ), considering exposure pathways by ingestion, inhalation and dermal contact in the studied soil samples. To determine the HQ by ingestion, soil samples were digested simulating gastric digestion, according to USEPA (2017a). Summarizing, 1 g of soil dried and sieved at 150 μm, was shaken and incubated during 1 h at 37 °C with 100 ml of extraction fluid (0.4 M glycine adjusted pH 1.50). After that, supernatant was separated from the sample by filtration and analyzed for total Pb and As concentration by ICP-MS spectroscopy. The HQ by inhalation in soil samples sieved at 50 μm according to the procedure described in Islam et al. (2016) were measured. The HQ by dermal contact were calculated from the finely ground samples according to the standard procedure (USEPA (United States Environmental Protection Agency), 2004). The total soil concentration of Pb and As, for inhalation and dermal contact risk assessment, were determined by X-ray fluorescence (XRF). Finally, the human health risk assessment was estimated as the cumulative hazard index (HI) for children and for adults by dividing the average daily potential dose of the three exposure routes (mg kg^{-1} day^{-1}) by the chronic reference dose of the three routes (mg kg^{-1} day^{-1}) (USEPA, 1989). When HI is higher than 1, it is considered that there may be a risk to human health. The parameter values, the reference values and the equations used for estimating human exposure to Pb and As in this study are presented in SI.

2.5. Statistical analysis

Prior to the statistical treatment of the data, the normal distribution test (Kolmogorov-Smirnov) was performed, and the homogeneity of variances was tested using the Levene test. The data were analyzed with the comparison of means test (ANOVA) and homogeneous subsets were performed using Tukey's test, determining significant differences between parameters ($p < 0.05$). If normality and/or homogeneity of variances were not met, nonparametric tests (Kruskal Wallis and Mann-Whitney U) were performed. Based on the analytical data of the edaphic properties (pH, EC, $CaCO_3$ and CO), a division by soil type was carried out by means of a hierarchical cluster analysis. To determine the relationship between the different study variables, a Principal Component (PCA) Analysis was performed. All statistical analyses were carried out using SPSS v. 23.0 software (SPSS Inc., USA).

3. Results and discussion

3.1. Soil properties

The hierarchical cluster analysis (Fig. SI.2), performed based on soil properties (pH, CE, $CaCO_3$ and OC) and total Pb and As soil concentration, defined 4 soil types (SS1-SS4) that are heterogeneously geographically distributed in two sectors (Fig. 1 and Fig. SI.2). Soils SS1 ($n = 9$) and SS2 ($n = 11$) are located in the upper part of the GGC closest to the mine and are loamy textured soils, not or slightly carbonated, neutral or slightly acidic pH and show some salinity (Table 1). In contrast, soils SS3 ($n = 25$) and SS4 ($n = 39$) are randomly located in the middle and lower sector of the GGC and are alkaline, carbonated, and fine-textured soils. Two decades after the accident, and after the cleaning and soil amendment work, completed in 2001, the soils show similar properties to the soils existing before the accident, described by Simón et al. (1999) as acid soils, not or slightly carbonated and with loam to sandy loam texture in the sector near the mine. This coincides with soils SS1 and SS2, while in the middle and lower part of the GGC the soils were neutral to slightly alkaline with carbonate contents above 5% and textures varying between clay loam, loam, and silty clay.

Bare soils (BS) are extremely acidic and showed low pH and high EC, properties that, along with the presence of pollutants, are a clear consequence of the continued contamination process and/or as a result of the rapid and deficient first extensive cleanup operation that mixed the tailings with the soil in depth (Simón et al., 2008; García-Carmona et al., 2019a). Low carbonate contents are influenced by the acidic pH of the BS. Whereas, the topsoil removed during the clean-up in 1999 and the absence of vegetation over time in the BS is reflected in the low OC values observed.

Table 1
Main soil properties for the 4 different soil types (SS1-SS4) and the bare soils (BS).

Soil type	Texture	pH	EC (dS m^{-1})	$CaCO_3$ (%)	OC (%)
SS1 (n = 9)	Loam	5.88 b *(1.80)*	2.73 a *(2.30)*	0.83 b *(1.26)*	1.55 a *(0.45)*
SS2 (n = 11)	Loam	7.69 a *(0.65)*	1.08 b *(0.63)*	5.52 b *(3.07)*	1.69 a *(0.68)*
SS3 (n = 25)	Clay loam	8.38 a *(0.22)*	0.59 b *(0.38)*	15.14 a *(7.99)*	1.85 a *(0.66)*
SS4 (n = 39)	Silty clay	8.08 a *(0.36)*	0.82 b *(0.69)*	12.81 a *(5.32)*	1.96 a *(0.41)*
BS (*n* = 6)	Loam	3.53 *(0.22)*	3.05 *(0,93)*	0.74 *(0.31)*	0.61 *(0.12)*

Lowercase letters indicate significant differences between subsectors (Tukey HDS test $p < 0.05$).

The soils affected by the spill greatly increased in acidity, in many cases reaching values below pH = 4 (Aguilar et al., 2004) due to the acidity of the water (pH ≈ 5) (Cabrera et al., 1999) and of the pyrite sludge which, given its heterogeneity, ranged from pH = 4.1 to pH = 5.1 (Simón et al., 1999). The acidification of soils was aggravated by the oxidation of the sludge at the surface, and even continued after the sludge removal tasks, by sludge debris trapped in the soil cracks (Dorronsoro et al., 2002). The oxidation of the sulphides involves oxidation, hydrolysis, and hydration processes that Stumm and Morgan (1981) summarized in Eq. (1).

$$FeS_2 + 3.75O_2 + 3.5H_2O \rightarrow Fe(OH)_3 + 2H_2SO_4 \quad (1)$$

This reaction starts with the release of Fe^{2+} and under oxidizing conditions is converted into Fe^{3+}. When soil pH is above 4.5, then Fe^{3+} precipitate as iron hydroxide and the pH becomes more acidic (Eq. (2)).

$$Fe^{3+} + 3H_2O \rightarrow Fe(OH)_3 + 3H^{+} \quad (2)$$

However, when soil pH remains under 4.5, the Fe^{3+} can oxidize the pyrite. This reaction is faster and can generate more acidity (Eq. (3)).

$$FeS_2 + 14Fe^{3+} + 8H_2O \rightarrow 15Fe^{2+} + 2SO_4^{2-} + 16H^{+} \quad (3)$$

The continued oxidation of the sulfides contained in the sludge significantly increased the soluble sulfate content of the soil, leading to a sharp increase in soil salinity, which over time has been significantly reduced, especially in the most carbonated area, which coincides with the SS3 and SS4 soils.

Despite the positive evolution of the pH and salinity of the GGC soils, acidic soils with high salinity (SS1) are still detected, located in the area closest to the mine. These results agree with Aguilar et al. (2007), Simón et al. (2008) and Martín et al. (2015), which indicated the persistence of residual contamination in punctual areas of the corridor.

The carbonate content was significantly higher in SS3 and SS4, compared to SS1 and SS2, even though, with the liming measures, the doses of carbonates applied in the upper part of the GGC were higher, 60–90 t ha^{-1} (SS1 and SS2) compared to 20 t ha^{-1} applied in other areas. Soils SS1 and SS2, closer to the mine and originally decarbonated, received a greater contribution of sludge after the accident and much of the added carbonate was consumed in the neutralization of acidity.

In general, the recovery of the soils allowed the development of dense vegetation along the GGC, which contributes a significant amount of organic matter to the soil, reaching average contents higher than those reported by Simón et al. (1999) in the agricultural soils of the area before the accident, and without significant differences in the OC content for the 4 studied soil types.

3.2. Concentration of Pb and as in studied soil samples

After the Aznalcóllar mining accident, the soils were seriously contaminated by heavy metals and associated elements, the main soil contaminants spread in the area were As, Cd, Cu, Sb, Pb, Tl, and Zn (Simón et al., 1999). Two decades after the accident, Pb and As still show total soil concentrations above the guideline values established by the Regional Government of Andalusia (Pastor-Jáuregui et al., 2020). Fig. 2 shows the Pb and As total concentrations in the four soil types considered in the GGC and for the studied bare soils (BS). In the SS1 and BS total Pb exceeded in >89% of the studied sampling points the Pb guideline value (275 mg kg^{-1}). Whilst, in the SS2, SS3 and SS4 <10% of the studied sampling points showed values higher. For the case of As, the established guideline value (36 mg kg^{-1}) was exceeding in all sampling points of SS1 and BS, in more than the 70% of sampling points of SS2 and SS4 and in 44% of the sampling points of SS3. In SS1 soils (n = 9), the persistence of higher total Pb and As concentrations coincide with more acidic soils with higher salinity, which Martín et al. (2008) and Otero et al. (2012) relate to more contaminated soils due to the formation of soluble sulfates by the oxidation of the sludge. SS2 soils (n = 11), although sharing their geographic distribution in the GGC, show significantly lower total Pb and As concentrations similar to those reported by Romero-Freire et al. (2016a), Sierra et al. (2019) and García-Carmona et al. (2019b) in soils that are considered recovered within the same area of the GGC. Soils SS3 (n = 25) and SS4 (n = 39) show, in general lower total Pb and As concentrations than SS2, but are only statistically significant with respect to SS1 (Table 2). Since Pb and As are elements considered not very mobile (García-Carmona et al., 2019a), there is no expectation that the total concentration of these elements will decrease over time. In addition, the average annual rainfall in the area is 490 mm over the last 20 years, with a trend towards greater aridity, so the risk of Pb and As leaching is limited. On the other hand, the natural vegetation in the area is mainly herbaceous, so that Pb and As assimilated by plants is recycled back to the soil.

According to the water-soluble concentration (PbS and AsS), we observed that values did not show significant differences for the case of Pb, while the AsS was higher in the SS4 section (Table 2). The presence

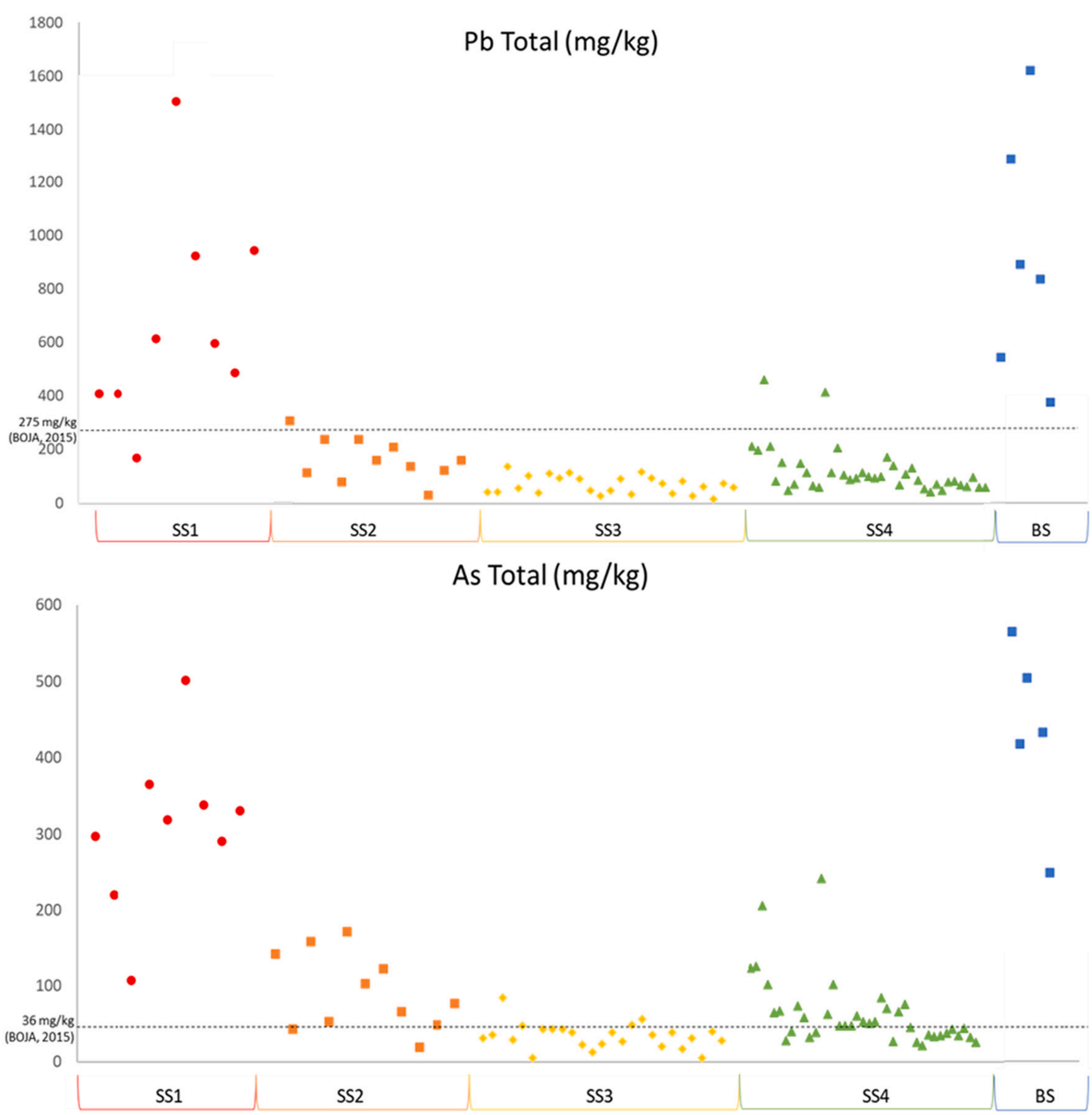

Fig. 2. Total Pb and As content for the 84 samples that contribute to form the four different soil types (SS1-SS4) and the 6 samples of the selected bare soil (BS). Dashed dotted lines represent the Pb and As guideline values established by the Regional Government of Andalusia (BOJA, 2015).

Table 2
The total soil concentration (PbT and AsT), the concentration of water-soluble elements (PbS and AsS) and the bioaccessibility of the studied elements (PbE and AsE) for the different soil types (SS1-SS4) and the bare soil (BS). Average, minimum and maximum values for each soil type.

	PbT		min	max	PbS		min	max	PbE		min	max
	mg kg^{-1}											
SS1	673	b	170	1504	0.03		<0.01	0.14	0.55	a	0.01	1.59
SS2	166	a	31	310	0.02		<0.01	0.03	1.26	b	0.32	2.77
SS3	71	a	18	138	0.03		<0.01	0.11	1.31	b	0.12	3.55
SS4	122	a	43	461	0.05		0.01	0.44	2.17	c	0.93	6.01
BS	925	c	377	1618	0.01		<0.01	0.02	0.48	a	0.18	1.19
	AsT		min	max	AsS		min	max	AsE		min	max
	mg kg^{-1}											
SS1	307	c	107	501	0.06	ab	0.01	0.31	0.10	a	0.02	0.22
SS2	91	b	19	170	0.05	ab	0.02	0.14	0.13	a	0.03	0.26
SS3	34	a	5	84	0.05	a	0.01	0.12	0.07	a	0.02	0.27
SS4	63	ab	21	242	0.10	b	0.01	0.26	0.08	a	0.04	0.17
BS	466	d	249	626	0.07	ab	0.01	0.23	5.58	b	0.08	28.86

Lowercase letters indicate significant differences between soils (Tukey test, $p < 0.05$).

of calcium carbonate in all soil samples can produce the precipitation of Pb at acidic pHs, which can support the low Pb solubility observed in the studied soil samples (Simón et al., 2005; García-Carmona et al., 2017). Lead is known as a very immobile element in soils, with mineral content and type, pH and organic matter, being the most important factors determining Pb sorption in soils (Kabata-Pendias, 2010; Romero-Freire et al., 2015a; García-Carmona et al., 2017). The observed properties of the different sectors (Table 1) can be the responsible of the low water soluble Pb concentration.

The water soluble As concentration has increased considerably in 2018 in SS4 (0.10 mg kg-1) with respect to the concentrations reported by Martín et al. (2015) and Romero-Freire et al. (2016a) in soils recovered from the middle and final part of GGC analyzed in 2013 (0.01 and 0.07 mg kg^{-1}, respectively). Even authors such as Sierra et al. (2019) or Paniagua-López et al. (2021) report soluble As concentrations in soils recovered from the GGC of 0.15 and 0.24 mg kg^{-1}, respectively, when in 2004 the water soluble As concentration reported by Martín Peinado (2001) were 0.006 mg kg^{-1} in the middle and final part of the GGC. The increase in water solubility of As in SS4 may be related to the naturalization of the soils in the middle and final part of the GGC, where the recovery of the vegetation was faster after the remediation measures applied until 2001. The SS4 soils are the ones that presented the highest organic matter content, although without reaching significant differences. However, the rapid development of vegetation has given rise to a supply of organic matter that promote interactions between the organic matter and As forms, partially explaining the generally greater mobility of As in this natural environment. Wang and Mulligan (2006), report that soil organic matter can increase As water solubility through competition for available adsorption sites, formation of aqueous complexes, and/or changes in the redox potential of site surfaces and As redox speciation. Another factor that determines the mobility of As is its affinity for Fe oxy-hydroxides since under oxic conditions, arsenic exists mainly in As(V) oxidation state as the arsenate oxyanion ($HAsO_4^{2-}$, $H_2AsO_4^-$) (Al-Abed et al., 2007). This adsorption affinity is higher for As (V) at lower pH values, ranging from 4 to 7 (Pierce and Moore, 1982) and in this sense, Simón et al. (2010) indicates the importance of not exceeding pH values >6.5 when remediation tasks in As contaminated soils are carried out, since it can lead to an increase in its solubility.

The bioaccessible Pb content (PbE) showed interesting results, because although lower total Pb content was observed far from the upper part of the GGC closest to the mine, the PbE higher values were observed in the SS4 > SS3, SS2, with significant differences. García-Carmona et al. (2019a) reported EDTA extractable Pb concentrations in the GGC recovered soils even higher than ours and correlated with the higher carbonate content. In this sense, Simón et al. (2005) reported that Pb co-precipitate with Fe coating the surface of carbonates that were used as an amendment in GGC soils and the quantity precipitated decreased at pH < 6.5. Castro-Larragoitia et al. (2013) and Armienta et al. (2016) also reported that calcium carbonate soils have a neutralization potential that influences the availability of Pb. That is why SS2-SS4 soils, due to its carbonate content and alkaline pH, showed low water-soluble Pb concentration while EDTA extractable Pb is high due to the strong chelating ability of the EDTA extracting Pb from carbonate which is difficult to release owing to the stronger ionic bonding (Wang et al., 2021). In the case of AsE, there were not significant differences between soil types, and only in the BS high contents of AsE were observed.

3.3. *Toxicity assessment and soil properties*

The performance of bioassays in contaminated areas allows estimating the real ecotoxicological risk of soils under natural conditions (Fernández et al., 2006). Ecotoxicity tests are key tools in the study of the fate and bioavailability and to assess the potential ecotoxicological effects of metals as well as their possible transfer to the other compartments such as groundwater, or even their transfer to the food chain. The use of biological tests is essential for determining the potential ecological risk of soil contamination to organisms and ecosystems. Depending on the potentially toxic element (PTEs) present in the soil, it may produce toxicity in one specific organism and not in others (Matejczyk et al., 2011; Baderna et al., 2015). For this reason, in soils with multi-elemental contamination it is difficult to attribute toxic effects to one specific element due to possible synergies or antagonisms between them (Spurgeon and Hopkin, 1995). This justifies the need to use bioassays with different organisms to obtain a reliable ecotoxicological risk assessment (Romero-Freire et al., 2016a; García-Carmona et al., 2019a). Moreover, it is recommendable to use different bioassays with both, liquid and solid phase, to assess soil toxicity in a more reliable way, due to organisms can interact with soil particles and liquid soil components (Farré and Barceló, 2003; Martín et al., 2010). For the evaluation of the ecotoxicological risk of the GGC soils, bioassays were performed in liquid phase with *Lactuca sativa* L. and in solid phase by determining the rate of heterotrophic respiration and establishing the stress degree of soil microorganisms through the metabolic quotient (qCO_2), which expresses the ratio between respiration rate and microbial biomass of the soil (Table 3).

The liquid-phase bioassay with *Lactuca sativa* L. has been recommended by many international organizations for the determination of ecological effects of toxic substances and for toxicity testing (Escoto et al., 2007; Lyu et al., 2018) as it is simple, fast, reliable, inexpensive and does not require expensive equipment. The germination rate of lettuce seeds from SS1 soils was significantly lower than that of the other

Table 3
Mean values (standard deviation) of the results of bioassays with *Lactuca sativa* L. and microbial activity, for soil types (SS1-SS4).

Soil Type	*Lactuca sativa* L.		Microbial activity		
	Germination (%)	Root elongation (%)	Respiration rate (μg CO_2 h^{-1} g_{soil}^{-1})	Microbial biomass (μg C_{microb} g C_{soil}^{-1})	qCO_2 (ng C_{CO2} μg C_{microb}^{-1} h^{-1})
SS1	85.00 b *(32.11)*	43.17 c *(26.13)*	8.06 a *(5.76)*	798.26 a *(392.56)*	23.87 a *(12.05)*
SS2	99.09 a *(2.02)*	66.29 b *(24.37)*	7.86 a *(3.97)*	730.53 a *(434.17)*	18.12 ab *(9.61)*
SS3	97.40 a *(3.57)*	96.98 a *(8.75)*	7.53 a *(2.71)*	1561.36 b *(894.43)*	9.42 b *(4.09)*
SS4	97.18 a *(3.40)*	99.41 a *(3.68)*	5.64 a *(2.15)*	1426.71 b *(621.02)*	5.38 b *(3.71)*

Lowercase letters indicate significant differences between soil types (Kruskal Wallis and Mann-Whitney U, $p < 0.05$).

soil types considered, while root elongation was significantly lower in SS1 and SS2 soil types, with respect to SS3 and SS4 types that did not present toxicity in this bioassay. Root elongation was therefore a more sensitive measure of toxicity than germination rate, results that agreed with those reported in the literature (Bagur-González et al., 2011). Our results for soils SS1 and SS2 were consistent with those reported by Romero-Freire et al. (2016a), from studies performed in the same area, with a percentage of root elongation in soils close to the mine slightly below 60%, whereas, in soils in the middle and distal third of the GGC an elongation of 70% was reported, compared to values close to 100% obtained in soils SS3 and SS4, which can indicate a reduction in toxicity over time.

Heterotrophic soil respiration is related to soil fertility and soil quality (ISO, 2012; Niemeyer et al., 2012). Contamination may quantitatively and qualitatively affect soil microorganisms (Stefanowicz et al., 2008; Shukurov et al., 2014) and the processes in which they are involved, such as their role in soil nutrient cycling and in the processes of mineralization and synthesis of organic compounds (Moreno et al., 2009; Nwachukwu and Pulford, 2011). Decreased microbial activity due to the presence of contaminants in the soil causes a reduction in the amount of carbon dioxide produced, so it can be used as an indicator of stress on microbial communities (Dai et al., 2004; Azarbad et al., 2013). Basal heterotrophic respiration data measured in the GGC did not show significant differences between the different soil types considered. In fact, there is controversy over the use of respiration rate as an indicator of contamination. Wakelin et al. (2010) and Zornoza et al. (2015) found no correlation between respiration rate and potential toxicity, while Dinesh et al. (2012) and Romero-Freire et al. (2016b) reported a positive correlation between respiration and degree of contamination. In contrast, SS1 soils showed the highest heterotrophic respiration rate and the greatest total As and Pb soil concentrations. However, the heterotrophic respiration rate alone was not conclusive for the evaluation of toxicity in the GGC soils analyzed, since they showed different degrees of contamination that resulted in a high dispersion of respiration rates.

Microbial biomass is another biological indicator that provides information on soil quality and its response to the incorporation of contaminants or to the application of amendment measures, since microorganisms respond faster to these changes than to changes in soil physicochemical properties (Nannipieri et al., 2017; Oijagbe et al., 2019). As a consequence of the exposure of soil microbiota to potential toxic elements, the microbial biomass can be reduced due to cell death caused by the alteration of essential functions or by an unaffordable increase in vital energy cost (Akmal and Jianming, 2009; Oijagbe et al., 2019). Soils in the SS3 and SS4 showed a significantly higher microbial biomass content than SS1 and SS2 soils, nearly twice their value. These results agree with those obtained from the lettuce bioassay, showing signs of toxicity in SS1 and SS2 soils.

The metabolic quotient (qCO_2) is, perhaps, the most widely used index to evaluate the efficiency of energy use by soil microorganisms. It is usually correlated with an increase in biodiversity and ecosystem maturity and has been used to compare the impact that seasonal changes, management systems, addition of metals, agrochemicals and xenobiotics have on soil microorganisms (Paolini, 2018). The qCO_2 allows diagnosing the efficiency of microbial biomass in soil carbon use in terms of respiration expenditure (Anderson and Domsch, 1990). According to this index, in SS1 microorganisms would be subject to higher stress compared to the other soil types, coinciding with more limiting edaphic conditions in terms of salinity and acidity, and higher total soil concentration of Pb and As. Under unfavorable conditions, microorganisms require more energy to maintain biomass and therefore qCO_2 increases and carbon is lost as CO_2 (Insam and Domsch, 1988), which may justify the higher respiration rate and lower microbial biomass content in SS1 soils where qCO_2 is significantly higher. This circumstance may be indicative of a change in the structure and diversity of microbial populations in contaminated soils with respect to natural soils, since microorganisms are able to develop resistance and resilience to certain PTEs (Allison and Martiny, 2008; Hänsch and Emmerling, 2010). In this sense, Paniagua-López et al. (2021) described differences between contaminated and recovered soils in the same area in terms of gene copy number, microbial biodiversity (Shanon Index) and bacterial community structure at the genus level.

The principal components analysis of the 84 soil samples along the GGC includes the soil properties analyzed, the total, water-soluble and bioavailable Pb and As concentration, and the bioassays, grouped into two components that explain 49% of the variance (Fig. 3). The total soil concentration of Pb and As is directly related to the soils where the salinity and acidity are higher and the soils are not or slightly carbonated. This trend is strongly marked by the SS1 soils, which are the most contaminated and in which a higher qCO_2 is recorded. Furthermore, PCA component 1 groups the heterotrophic respiration rate, the microbial biomass, the root elongation with the pH and the carbonate content of the soil, which are higher in soils with lower total concentration of Pb and As. PCA component 2 inversely relates the water-soluble concentrations of Pb and As with the germination rate of the lettuce seed, while the EDTA extractable concentration of Pb and As, considered to be bioavailable in the long-term (Labanowski et al., 2008; Hurdebise et al., 2015) is not grouped with any of the variables considered.

The root elongation bioassay, compared to the metabolic coefficient, has been shown to be more sensitive to contamination, possibly due to the limitation of basal respiration as an indicator of soil toxicity. In fact, the sensitivity of the lettuce seed root elongation bioassay is widely recognized in the literature as an effective tool for ecotoxicological risk assessment (Martín et al., 2010; Lors et al., 2011; Romero-Freire et al., 2014; Romero-Freire et al., 2015b; García-Carmona et al., 2019b; Hong et al., 2021).

From the bioassays it can be concluded that the SS1 soils are the ones that present a greater potential risk of toxicity for the ecosystem due to the high content of Pb and As, related to the soil acidity and the absence or low content of carbonates. However, carbonated soils, especially SS4, in which the recovery of the vegetation was denser and faster, can promote the remobilization of As due to the increase in the soil organic matter, and this increase in the As solubility can be encouraged by the moderately alkaline soil pH. Therefore, monitoring over time would be advisable to assess the evolution of the ecotoxicological risk of the GGC soils.

3.4. Ecotoxicological risk assessment of bare soils

In the six contaminated areas selected in this work (bare soils, BS), the average pH was 3.5 and the electrical conductivity was 3.1 dS m^{-1} (Table 1). In these BS, total Pb and As concentration were higher than the other sampling points (Table 2), and >10 times higher than adjacent

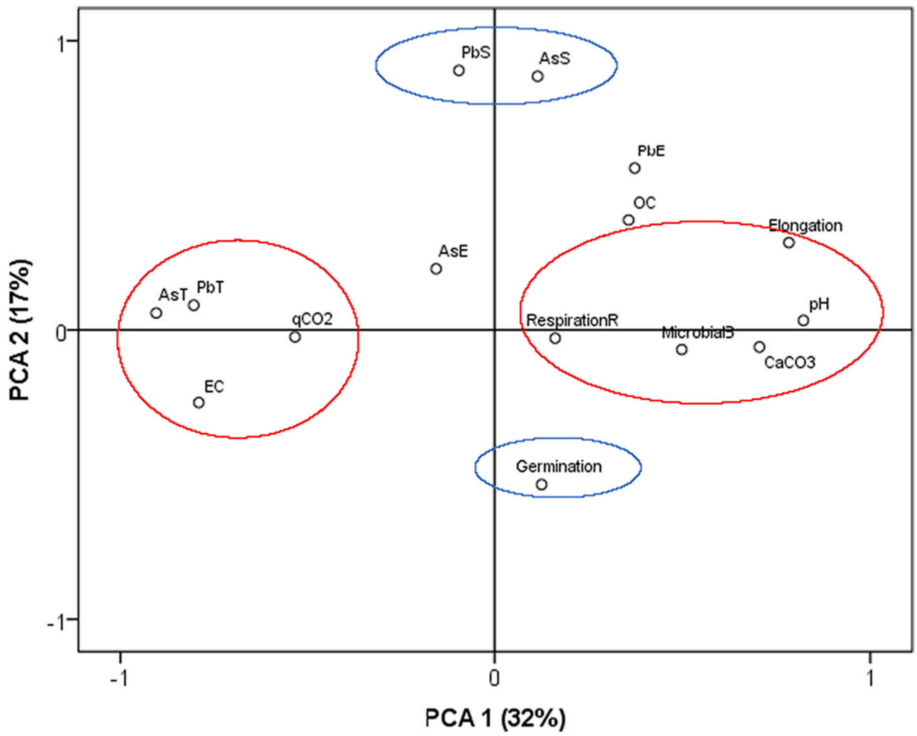

Fig. 3. Principal components analysis (PCA) with the total, soluble and bioavailable Pb and As concentrations and the toxicity bioassay results for the 84 soil samples studied in the GGC.

soils that were not affected by the toxic spill. Lead and As average content in uncontaminated soils of the area were 81.6 and 19.5 mg kg^{-1}, respectively.

Martín et al. (2015) have previously described the existence of areas where soil contamination persists, of variable size and randomly dispersed in the upper part of the GGC and identifiable by the absence of vegetation, which is hindered by the high concentration of contaminants, as well as by the strong acidity and salinity. The Pb and As concentration measured were similar to those reported by Paniagua-López et al. (2021), Sierra et al. (2019) and García-Carmona et al. (2019a), who also analyzed contaminated soils without vegetation located in the upper part of GGC. Due to the specific physicochemical conditions and the potential toxicity of the bare soils, it is necessary to perform a deeper ecotoxicological risk assessment using not only short-term test but also long-term test to ensure the determination of risk for Pb and As pollution. For this reason, the 6 bare soils were studied in comparison to uncontaminated soils near the area, but not affected by the spill, and 2 short-term test and 1 long-term test were performed.

Basal heterotrophic respiration and root elongation bioassays confirmed toxic effect in bare soils, with respiration rate and lettuce seed response being reduced by 6 and 9 times, respectively, compared to the selected soil control (Table 4). Other studied in contaminated soils, belonging also to the GGC, without vegetation Romero-Freire et al. (2016a) reported respiration rates lower than 0.5 μg CO_2 h^{-1} g^{-1} while in a similar studied Paniagua-López et al. (2021) recorded values close to 1 μg CO_2 h^{-1} g^{-1}, in the range of our results. Thus, the heterotrophic respiration rate was shown to be a sensitive bioassay when contamination levels are high or edaphic conditions are extreme. The results of the reduction in lettuce seed root elongation were also consistent with the literature, which reports a reduction of around 90% with respect to the control (Romero-Freire et al., 2016a; García-Carmona et al., 2017; Paniagua-López et al., 2021).

Due to the toxicity detected in the BS, highlighted by the absence of vegetation and the results obtained with the bioassays with L. *sativa* and heterotrophic respiration, the long-term toxicity bioassay with earthworm (*E. andrei*) was conducted to include an invertebrate species to complete the toxicity risk assessment. Romero-Freire et al. (2015a) indicated that these organisms are more susceptible to metal contamination than other soil invertebrates, making them suitable organisms to be used as bioindicators to determine the toxicity of chemicals in soil, and hence *E. andrei* has been adopted as a standard organism for ecotoxicological testing by the European Union (EEC (European Economic Community), 1984). In addition, they grow easily under laboratory conditions, have a high reproduction rate, are sensitive to numerous contaminants and their size allows for easy handling (Nahmani et al., 2007a) thus making them a species of choice for ecotoxicological studies (EEC (European Economic Community), 1984; ISO, 2008; OECD (Organization for Economic Cooperation and Development), 2015).

Table 4
Mean values (standard deviation) of pH, salinity and bioassays results performed on the bare soils (BS) and the selected control in an uncontaminated area.

	pH	EC	Basal respiration	*Lactuca sativa* L.	*Eisenia andrei*		
		(dS m^{-1})	(μg CO_2 h^{-1} g^{-1})	Elongation	Survival	Weight variation	Juveniles
				(%)	(%)	(%)	(%)
Bare soil (BS)	3.53 *(0.22)*	3.05 *(0.93)*	0.87 *(0.35)*	11.1 *(27.2)*	100 *(0.0)*	−13.13 *(11.71)*	2.46 *(4.26)*
Uncontaminated soil (Control)	6.84 *(1.16)*	0.41 *(0.08)*	5.51 *(1.95)*	100.0 *(0.0)*	100 *(0.0)*	47.13 *(19.56)*	100.00 *(25.29)*

Exposure of the earthworm to a PTE can be either by ingestion of the contaminated soil or by dermal contact, either route of exposure can cause death, weight loss or impairment of their reproductive capacity (Hobbelen et al., 2006; Li et al., 2008; Kılıç, 2011; Leveque et al., 2013).

The earthworm mortality rate measured in contaminated and uncontaminated soils was not a good indicator of toxicity since earthworms survived in BS and control soils (Table 4). These results agree with Nahmani et al. (2007b) which indicated the low sensitivity of this parameter in toxicity bioassays with earthworms in soils. However, it was observed that in BS the weight of earthworms, after 4 weeks of exposition, decreased (−13%), with a considerable difference when compared to the control soil (US), were earthworm gain weight (+47%). Nevertheless, we cannot be sure that this difference was exclusively due to the Pb and As concentration of the contaminated soil, since extreme soil conditions, such as the very acidic pH of contaminated soils can also influence earthworm development (García-Gómez et al., 2014; Aziz et al., 2019; Lorente-Casalini et al., 2021). The reproductive capacity expressed as the percentage of juveniles with respect to those counted in the selected control soil proved to be a good indicator of toxicity, since BS showed a very marked reduction in the juvenile population. This parameter has shown great sensitivity in bioassays with different earthworm species in As contaminated soils (García-Gómez et al., 2014; Romero-Freire et al., 2015a; Romero-Freire et al., 2017).

According to the toxicity observed in the short-term test and in the reproduction of the earthworm in the long-term test, there is some indicatives of the risk that pose the BS, therefore it will be necessary further studies. In this sense, as a recreative area, the human toxicity risk assessment should be necessary for ensure the safety of the GGC for the users.

3.5. Human health risk assessment of GGC soils

Soils contaminated with PTEs may pose a risk to human health by direct ingestion of soils (Luo et al., 2011; Okorie et al., 2011), inhalation of particles (Laidlaw and Filippelli, 2008; Schmidt, 2010) or dermal contact (Siciliano et al., 2009), especially in residential areas or parks (Ljung et al., 2007; Luo et al., 2012a; (Luo et al., 2012b). The soils of GGC are currently intended for recreational use, being an area for children and adults to enjoy walking or playing sports in nature. However, there are areas of the corridor where the Guideline values established by the Regional Government of Andalucía for PTEs are exceeded, as is the case of Pb and As (275 and 36 mg kg-1, respectively), so an assessment of the risks to human health is necessary to ensure the safety of recreational use of the GGC. Both Pb and As are considered non-essential elements that can pose a risk to human health when their concentration and bioavailability is high in soils (Kabata-Pendias, 2010; Nagajyoti et al., 2010), especially in the case of prolonged exposures over time as they can trigger cardiovascular, nervous system, blood, liver, and bone diseases (Bhattacharya et al., 2007; Nriagu et al., 2007; Liu et al., 2017). These elements can be transferred to water, soil and plants and reach humans through the trophic chain or by direct ingestion, posing a threat to human health (Bi et al., 2006). Therefore, it is necessary to study the transfer of heavy metals from soil to humans, both for human health risk assessment and pollution control (Liu et al., 2017).

To determine the risk of human toxicity from exposure to GGC soils, individual hazard quotients, for ingestion, inhalation and dermal exposure, for Pb and As have been determined in all studied samples ($n = 90$) (see SI for more information). The cumulative hazard index (HI) from exposure to GGC soils was calculated as the sum of the estimated risks for each of the exposure pathways depending on the exposed individual, child or adult. According to the methodology proposed by the USEPA, HI values <1 indicate that there is no potential risk of chronic toxicity from exposure to contaminated soils. Our results for adults showed HI values really lower than the unit both for Pb and As (HI < 0.05) (data not shown). Therefore, from the results obtained for adults we can consider that the GGC soils do not represent a potential risk. In the case of the HI calculated for children, Pb showed HI also lower than one, however we observed a potential risk assessment for the case of As in children (Fig. 4). In bare soils, 50% of the studies sites showed HI values higher than the unit, whereas one studied site was also near the unit. The HI for the soils belonged to the different soil types studied (SS1-SS4) did not show values higher than the unit, however, HI where higher as near as the SS was to the mining dam (Fig. 4).

Therefore, from the results obtained we can consider that the GGC soils do not represent a potential risk to human health for the recreational use to which it is currently dedicated, despite exceeding the Guideline values established by current regional legislation. And only risk for children has been reported in the half part of the bare soils studied. Pecina et al. (2021) advise against the development of recreational areas in heavily contaminated areas that have been reclaimed, so a monitoring over time of the toxicity risks of these soils would be advisable, especially due to the existence of areas of soils where residual contamination persist and where the hazard quotient obtained for As in children were higher and close to unity.

4. Conclusions

Twenty years after the Aznalcóllar mine spill, one of the most important mining accidents in Europe, in soils belonged to the affected area, currently converted in a recreational area, total soil concentrations of Pb and As still exceeded the regional guideline values. This study did a long-term monitoring of the area in order to validate the state of soils after the remediation and to ensure the safety of the recreational use. Our results indicate that two decades after the accident, most soils showed similar properties to the soils existing before the accident, but there is still acidic soil with high salinity near the mine and randomly distributed as patches of bare soils with high pollution. Soils closed to the mine present a greater potential risk of toxicity for the ecosystem, however there is also remobilization of As in soils distributed far away of the mine encouraged by the alkaline soil pH, and, therefore, monitoring over time would be necessary. Although, currently the human health risk assessment done in the most polluted soils of the region consider that the GGC soils do not represent a potential risk to human health for the recreational use, but we observed a potential risk for children due to arsenic. The establishment of soil properties over time, with the remobilization of As indicates that, although the area is considered recovered, the monitoring over time should be done for ensuring human safety because the elevated content in Pb and As can be a chemical time bomb if the current soil properties changed. In areas that remain contaminated and where vegetation does not grow, it would be advisable to carry out remediation work to phytostabilize the area, limiting the possible dispersion of Pb and As to the rest of the ecosystem. To this end, organic amendments and liming should be applied to bare soils to correct their organic matter deficit and acidity, together with the addition of iron-rich amendments to prevent the increase in As solubility as the pH of these soils rises.

Credit authorship contribution statement

R P-J: Conceptualization, Methodology, Soil Sampling, Formal analysis, Writing – original draft, Writing – review & editing. M P-L: Conceptualization, Investigation, Methodology, Soil Sampling, Formal analysis, Writing – original draft. A R-F: Investigation, Methodology, Formal analysis, Writing – review & editing. FJ M-G: Conceptualization, Methodology, Soil Sampling, Formal analysis. FJ M-P: Soil Sampling, Writing – review & editing, Project administration, Funding acquisition. M S-A: Conceptualization, Investigation, Methodology, Soil Sampling, Data curation, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial

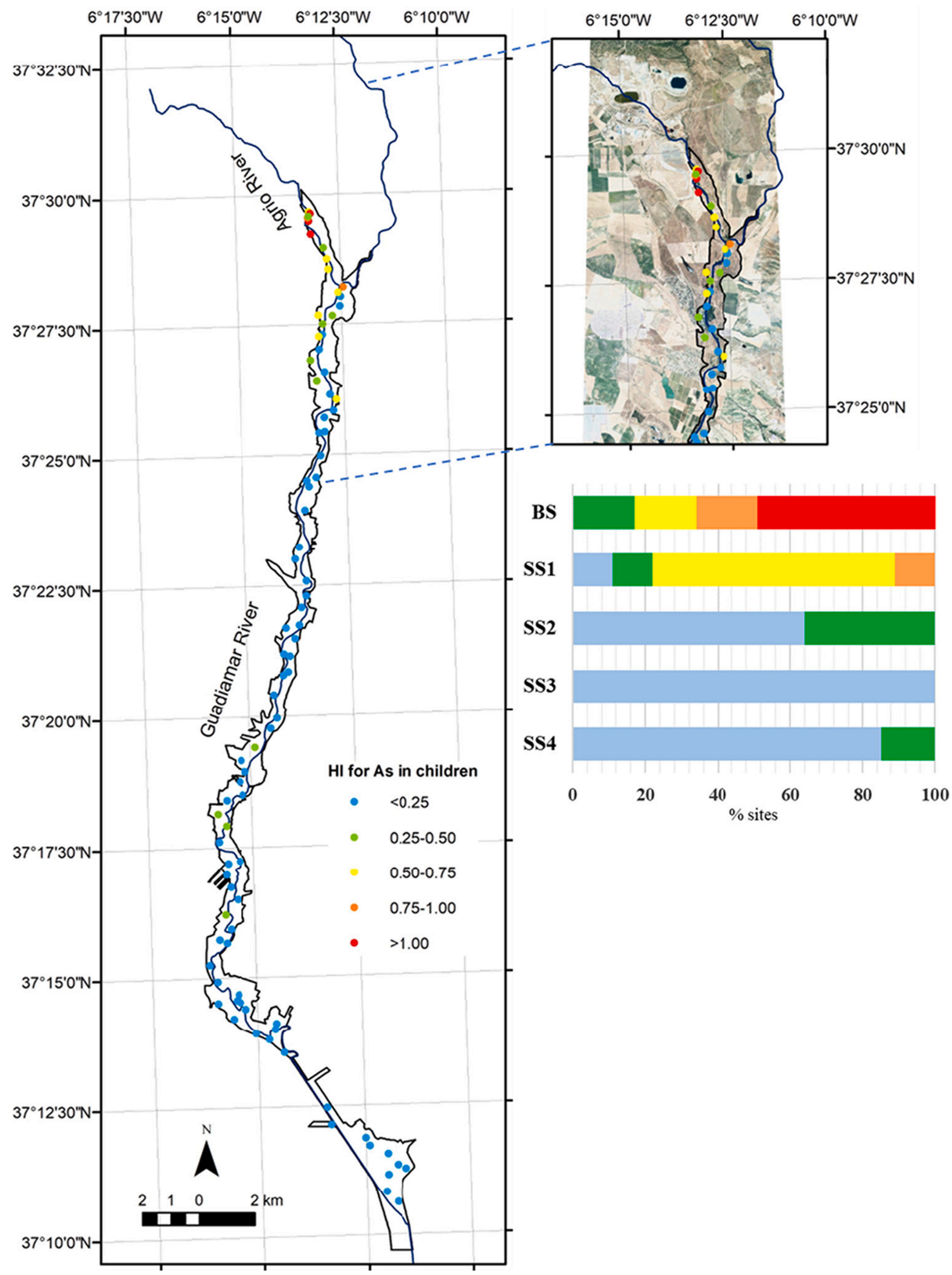

Fig. 4. Cumulative HI calculated for As in children. a) Geographical distribution of the cumulative HI along the GGC, and, b) percentage of soils belonged to each studied sector (bare soils, BS and different soil types, SS1-SS4). Values higher than 1 indicate risk.

interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Acknowledgements

The first author acknowledges to Universidad Nacional Agraria La Molina (Peru) for the support in the doctoral secondment carried out at the University of Granada (Spain). This work was supported by the Research Project RTI 2018-094327-B-I00, Grant FPU-18/02901 (Spanish Ministry of Science, Innovation and Universities) and the Research Groups RNM-269 and RNM-101 (Junta de Andalucía, Spain).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jconhyd.2022.104100.

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