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Assessment of the environmental impact of an abandoned mine using an integrative approach: A case-study of the "Las Musas" mine (Extremadura, Spain)

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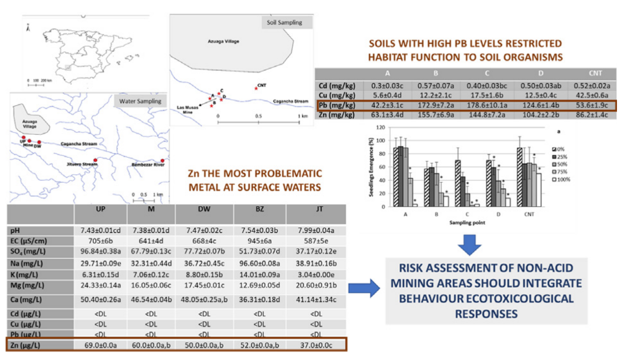
HIGHLIGHTS

- Environmental impact of an old abandoned Pb-Zn mine on soil and water was assessed.
- Soils still have high Pb levels and restricted habitat function to soil organisms.
- Water pollution with metals is low and mixed with other anthropogenic pollutants.
- Environmental risk is limited in the area of the study.
- Risk assessment of non-acid mining areas should integrate toxicological behavior responses.

GRAPHICAL ABSTRACT



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ABSTRACT

The mine abandonment is generally associated with the release of potentially toxic metals into the environment, which may depend on metals speciation, soil properties and climate conditions. The goal of the present work was to assess the environmental impact of the abandoned Pb-Zn mine "Las Musas" (Spain) using an integrative approach. The impact on soils and surface waters was performed using: chemical parameters, quantification of potentially toxic metals (Cd, Cu, Pb and Zn), and ecotoxicological responses using lethal and sub-lethal bioassays with organisms' representative of different trophic level ((soil: *Eisenia fetida* (mortality and reproduction test); *Latuca sativa* and *Lollium perenne* (seedling emergence); and water: *Vibrio fischeri* (luminescence inhibition), *Daphnia magna* (immobility and reproduction test), *Thamnocephalus platyurus* (mortality), *Pseudokirchneriella subcapitata* (growth inhibition)). The results showed soils with neutral to slight alkaline pH (7.64–8.18), low electric conductivity (125–953 μS/cm) and low organic matter levels (0.20–1.85%). For most of the soil samples, Pb was the only metal which surpassed the limit proposed by the Canadian soil quality guidelines, with values ranging from 42.2 to 181.4 mg/kg. The ecotoxicological results showed that the soils with the highest levels of Pb induced a decrease on *E. fetida* reproduction and on L. *sativa* germination, indicating negative impacts on

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the habitat function. The analysis of the surface waters showed levels of Zn surpassing the legal limit adopted from the Water Framework Directive (37.0 to 69.0 μg/L). The ecotoxicological results highlight the importance of bioassays that evaluate the behavior of species, when assessing the risk of mining areas with non-acid soils and waters with high nutrients/organic matter concentrations and low concentrations of potentially toxic metals. The results indicated a moderate environmental risk from potentially toxic metals, at the areas analyzed around the Azuaga mine.

1. Introduction

One of the main deleterious effects of a mine abandonment is usually associated with the rejection of large volumes of wastes, which can have a negative impact on the environment, mainly as a consequence of the spread and accumulation of potentially toxic metals (Moreno-Jimenez et al., 2011; Romero-Baena et al., 2018). Soils may represent the main reservoir for these hazardous elements, which will usually affect their retention function and can contribute to the contamination of surface and groundwater, compromising their quality and the balance of the ecosystem's communities (Pereira et al., 2008; Mighanetara et al., 2009; Beane et al., 2016;). Indeed, studies have reported deleterious effect of toxic metals on the environment, aquatic organisms, and plants in many parts of the world (Alvarenga et al., 2013; Liu et al., 2013; Yanqun et al., 2004). Furthermore, the dispersion of these wastes can reach agricultural or urban soils and expose humans to toxic metals, either directly, by suspended dust particles in the air, or indirectly, by transfer into the food chain (Alvarenga et al., 2014; Mighanetara et al., 2009; Ngole-Jeme and Fantke, 2017).

The environmental risks posed by metal-contaminated sites were traditionally assessed through chemical analysis (Bori et al., 2017). Nevertheless, it was concluded that this approach do not provide enough information about: (i) the toxicity of hazardous substances in soil; (ii) the synergic and antagonist effects between contaminants; (iii) the interactions among harmful substances, soil matrix and terrestrial/aquatic organisms; and sometimes, (iv) may overestimate the risk (Alvarenga et al., 2012; Bes et al., 2014).

Recent researches highlighted the importance of using a tool-box comprising multiple lines of evidence for soil risk assessment framework (Antunes et al., 2013). In fact, the TRIAD approach, first proposed by Chapman (1990) for the sediment compartment, was adopted for the risk assessment of contaminated soils (Weeks and Comber, 2005; Jensen and Mesman, 2006). The ecotoxicological LoE must integrate terrestrial bioassays, which assess the habitat function of the soil, being a complementary tool to report the interactions among ecosystem communities and the soil compartment (Alvarenga et al., 2012; Bori et al., 2016). At the same time, the use of aquatic bioassays will allow to assess the impact of soil composition on runoff and receiving waters (Rocha et al., 2011). This approach includes information from three Lines of Evidence (LoE): (i) chemical (support parameters, quantification of hazardous substances); (ii) ecological (communities' diversity); and (iii) ecotoxicological (Antunes et al., 2013).

The Mediterranean region presents a historical mining activity since pre-Roman times, but most of the mines are presently abandoned (Doumas et al., 2018). Abandoned mines represent "hot spots" for local populations, because they are usually adjacent to villages and are surrounded by farmland. The local population is subjected to exposure to potential toxic metals through the air, water, soil, and the food chain. Furthermore, Spain had an intensive mining activity, particularly in the eastern Pyrenees, the Beatic Cordillera, and in the regions of Andalucia and Extremadura (Doumas et al., 2018).

Bearing this in mind, the aim of this study was to characterize the environmental risk of the abandoned mine "Las Musas", located at the Azuaga district (Extremadura, Spain), one of the most important mining areas of Zn/Pb exploitation in Spain. To do so, physicochemical parameters, metal quantification and a set of ecotoxicological responses were assessed in soils and waters from the area. The bioassays were selected taking into consideration: (i) the group of organisms commonly used/sensitive to assess the environmental impact of abandoned mines; (ii) different times of exposure, to assess acute and chronic responses during the entire life-cycle of the organism; and (iii) previous studies have already used the same bioassays, which may be an easier way for comparing results (Alvarenga et al., 2012; García-Gómez et al., 2014; Oberholster et al., 2013; Bori et al., 2017).

Further, this study is integrated in a research line about the quality of soils, rivers/streams in the Mediterranean areas, with a strong mining influence, that intends to deliver a tool-box, which can be used to respond to the main alterations that influence their ecological status and may compromise public health.

2. Material and methods

2.1. Characterization of the study area and sampling sites

"Las Musas" mining area (331,000 m^2), is located near the village of Azuaga (Badajoz province, SW Spain) (38°15′30″ N and 1°59′20″ W) (see Fig. 1). This mine belongs to the Azuaga mining district, which was one of the most important mining areas of Zn/Pb exploitation in Spain. The mineralization in this site is associated to Early-Middle Cambrian. The mineral association is mainly composed of blende (ZnS), pyrite (FeS_2), galena (PbS), and chalcopyrite ($CuFeS_2$), with quartz and carbonate gangues. Furthermore, the materials of interlocking of the fault filling extension of the mineralization are mainly secondary Pb carbonates (Zalduegui et al., 2007). "Las Musas" mine was active since the end of the XIX century until about the middle of the XX century. This mine exploitation generated a volume of wastes of about 100,000 t (Gumiel, 2001). "Las Musas" mine is abandoned, and its environmental restoration has been neglected, due to financial limitations, despite its proximity to the village. It is integrated in a protected area, named "Parque Periurbano of Conservación y Ocio da Serra de Azuaga" (Decree Law of 113/2002 of 10 September), constituting a special zone of conservation, due to its biodiversity. Previous reports indicated high bioaccumulation of Pb in wildlife animals (hunting animals) (Garcia-Fernandez and Soler, 2006), and in native plants of the area (Gala, 2014).

Azuaga is integrated in the Guadalquivir basin (Fig. 1) that presents a Mediterranean climate, characterized by dry and hot summers, as well as mild and wet winters. With an annual precipitation between 500 and 800 mm, the scarce rainfalls are concentrated during the October–April period.

Bémbezar river and its tributaries, Cagancha and Jituero streams, are located near the mine and the Azuaga village and belong to the Guadalquivir hydrographic basin. The Bembézar basin is dominated by Mediterranean vegetation and subjected to extensive livestock breeding (e.g., sheep, goat, pig), with some agricultural and forest areas (Gala, 2014; Novo et al., 1994). The Bembézar spring is near Azuaga, it crosses the Hornachuelos village (Córdoba, SW Spain) and receives waters from other tributary streams, forming the Bembézar reservoir. This river runs along the Natural Park of "Sierra de Hornachuelos", a zone of special conservation, declared as Site of Community Importance (Real Decreto 136, 2004).

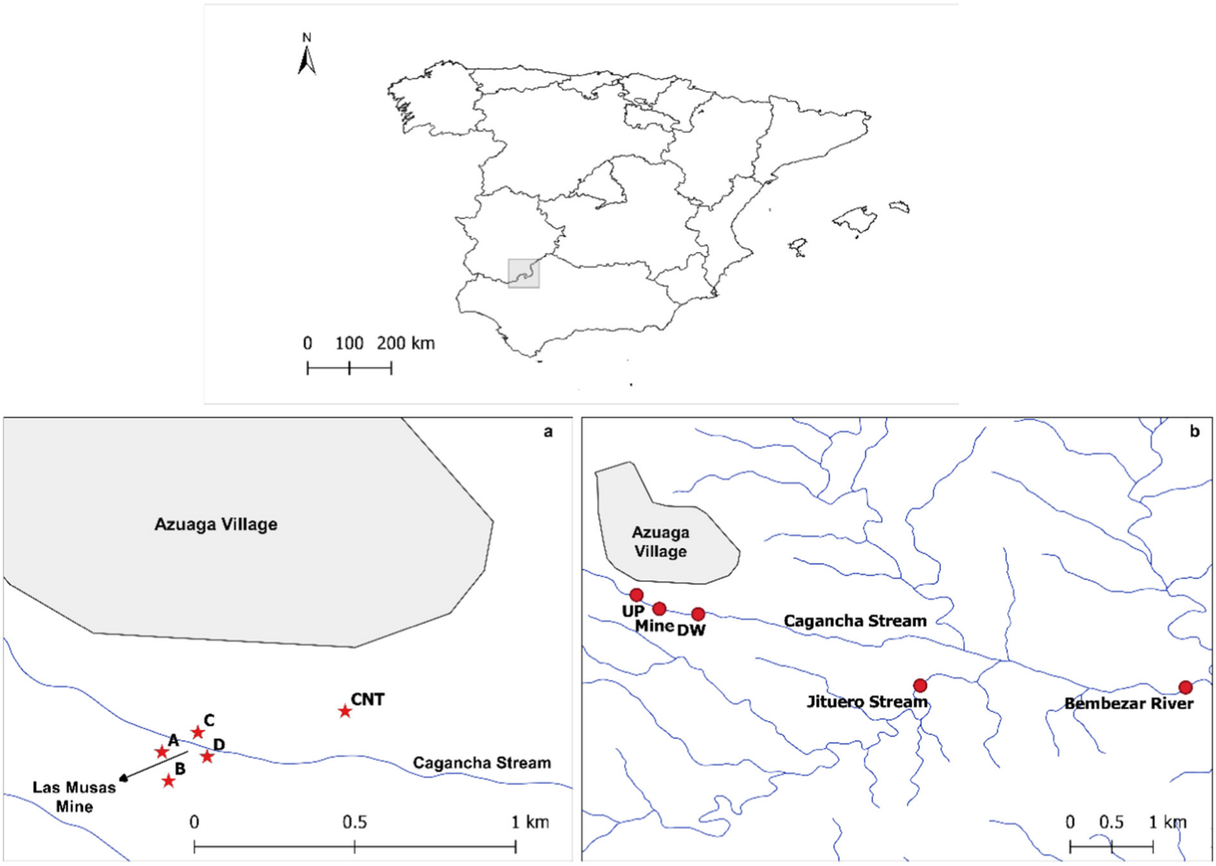

Fig. 1. Location of soils sampling points (a): A (38°15′4.13″ N and 5°40′43.39″ W), B (38°14′58.47″ N and 5°40′41.95″ W), C (38°15′4.68″ N and 5°40′38.20″ W) and D (38°15′2.48″ N and 5°40′37.24″ W), and CNT (38°15′7.40″ N and 5°40′19.33″ W); and water sampling points (b): Upstream mine (UP: 38°15′10.2″N and 5°40′51.7″W), Mine (38°15′03.3″N and 5°40′40.4″ W), Downstream mine (DW: 38°15′01.4″N and 5°40′20.7″W) at Cagancha stream, Bembézar river (38°14′21.8″N and 5°36′22.6″W) and Jituero stream (38°14′25.1″N and 5°38′30.9″W).

Soils were collected at five sampling sites, during May of 2015: (i) four sampling sites around the mine, collected at a distance between 3 and 292 m of the mine tailings, in the four cardinal directions, North, South, East, and West (A (38°15′4.13″ N and 5°40′43.39″ W), B (38°14′58.47″ N and 5°40′41.95″ W), C (38°15′4.68″ N and 5°40′ 38.20″ W) and D (38°15′2.48″ N and 5°40′37.24″ W)); and (ii) one sampling site near the village, considered the control soil, CNT (38°15′7.40″ N and 5°40′19.33″ W) (Fig. 1a). The distance between soil sampling points was chosen taking into consideration the results from Gala (2014), that delimited an area of 100–200 m from the mine tailing, as that with higher concentrations of Pb.

Water samples were collected at five locations (at the same period that soils) belonging to the Guadalquivir basin, to have a representative characterization of the aquatic systems in this area: (i) three sampling locations at Cagancha stream (watercourse that crosses the "Las Musas" mine, and it is a tributary to the Bembézar river): Upstream mine (UP; 38°15′10.2″ N and 5°40′51.7″ W), Mine (M; 38°15′03.3″ N and 5°40′40.4″ W), and Downstream mine (DW; 38°15′01.4″ N and 5°40′20.7″ W); one location at the Jituero stream (another tributary to the Bembézar river: JT; 38°14′25.1″ N and 5°38′30.9″ W); and one location at Bembézar river (BZ; 38°14′21.8″ N and 5°36′22.6″W) (Fig. 1b).

2.2. Soils collection and analysis

Soil samples were collected from the 20-cm topsoil, air-dried and sieved through a 2-mm non-metallic sieve. Soil physicochemical analysis were performed using well described methodologies (Alvarenga et al., 2008): particle-size distribution was determined by the pipet method (Gee and Bauder, 1986); soil pH (H_2O) was determined in a soil to deionized water suspension of 1:2.5 (w/v); electrical conductivity (EC; μS/cm) was determined in a soil to deionized water suspension of 1:5 (w/v); total nitrogen was analyzed by the Kjeldahl method ($N_{Kjeldahl}$; % w/w); total oxidizable organic carbon was determined according to Walkley and Black (1934), and converted to organic matter content (OM; % w/w) multiplying by a factor of 1.72; and extractable P and K (mg/kg) were determined using the Egner-Riehm method (Riehm, 1958). All results were reported in a dry weight basis (dw).

Total metal concentrations (Cd, Cr, Cu, Ni, Pb and Zn) were determined by flame atomic absorption spectrometry after digestion of the samples with aqua regia according to ISO 11466 (1995), using a Varian apparatus (SpectrAA 220FS). Three independent replicates were performed for each sample and blanks were measured in parallel. The choice of these elements was based on the type of mineral that was explored (Zn/Pb) and on the geological characteristics of the minerals, obtained in previous results from our group (not published), and in some works developed near the mining area (Garcia-Fernandez and Soler, 2006; Gala, 2014).

2.3. Water samples collection and analysis

Water samples were collected at a 50 cm depth (when possible) and transported to the laboratory in coolers at 4 °C, to be preserved and stored, following the requisites for water conservation for each parameter, until the analysis was performed (maximum storage time: 1 week) (APHA, 1998).

In the laboratory, the water chemical parameters were determined using officially recommended methods of analysis (APHA, 1998), which were reported by Palma et al. (2010). The parameters analyzed in the water samples were: pH, electrical conductivity (EC; μS/cm), biochemical oxygen demand (BOD_5; mg/L), chemical oxygen demand (COD; mg/L), total phosphorus (TP; mg/L), total nitrogen (TN; mg/L), nitrate (NO_3-N; mg/L), nitrite (NO_2-N; mg/L), ammonium (NH_4-N; mg/L), chlorides (Cl; mg/L), sulphate (SO_4; mg/L), potassium (K; mg/L), magnesium (Mg; mg/L) and calcium (Ca; mg/L).

For the quantification of metallic elements, the water samples were digested with aqua regia, and Cd, Cu, Pb and Zn were assessed by flame atomic absorption spectrometry according to ISO 11466 (1995).

2.4. Ecotoxicological assessment

Table 1 summarizes the description of the ecotoxicological assays performed, in both matrices.

2.4.1. Terrestrial bioassays

Soil ecotoxicity evaluation was performed using three different tests: *E. fetida* mortality (OECD 207, 1984), *E. fetida* reproduction (OECD 222, 2004) and seedling emergence of L. *sativa* and L. *perenne* (ISO 17126, 2005), using the whole soil and its dilutions with an artificial soil. The artificial soil was prepared according to OECD 207 (1984) (10% sphagnum peat, 20% kaolin clay, 70% industrial sand, w/w dw, pH adjusted to 6.0 ± 0.5 with calcium carbonate). This artificial soil was also used as the control in these tests.

2.4.2. E. fetida bioassays

The mortality toxicity tests using earthworm *E. fetida* were conducted according to the standard protocol OECD 207 (1984), with some modifications (Conder et al., 2001). For each soil and its dilutions, 100 g of sample were moistened to approximately 70% water holding capacity (WHC), in perforated plastic boxes, 24 h prior to the addition of five earthworms (three replicates per concentration). Mature earthworms (clitellate worms), weighing approximately 0.2 to 0.4 g, were obtained from in-house cultures. The test was conducted in environmental chambers maintained at 20 ± 2 °C, with a photoperiod of 16 h light and 8 h darkness. Earthworm mortality and biomass were monitored daily. Mean percent cumulative mortality (%) was outputted.

The reproduction test was performed according to the OECD guideline "Earthworm Reproduction Test" (OECD 222, 2004). For each test, 500 ± 5 g dw of soils, and if its dilutions (25, 50, 75 and 100% w/w), were placed in each container (four replicates per concentration). Mixtures were moistened to reach 60% of the WHC, which was monitored during the test period. Ten earthworms, which have been conditioned for 24 h in the artificial soil and then washed quickly before use, were placed on the soil surface. The containers were kept at 20 ± 2 °C and a photoperiod of 16:8-h light/dark. After 4 weeks of exposure, the adults were removed from the soil and effects on reproduction assessed after a further 4 weeks, by counting the number of offspring present in the soil. The reproductive output of the worms exposed to the soil was compared with that of artificial soil to determine the no observed effect concentration (NOEC) and/or the EC_{50} value (concentration, % w/w, which promoted a reduction on 50% of the observed endpoint).

2.4.2.1. Seedling emergence bioassays. Seedling emergence of L. *sativa* L. and *L. perenne L.* were performed according to ISO 17126 (2005). For each test, 400 ± 5 g dw of the soil, or dilutions of it with artificial soil (25, 50, 75% w/w), was placed in each container (plastic, 10 cm length × 10 cm width). Mixtures were moistened to reach 60% of the WHC. Artificial soil was used as the control, and four replicates per concentration were prepared. Forty seeds of each plant were homogeneously spread on the surface of the soil and covered with a thin layer of dry sand. Containers were incubated for 48 h in the dark, at 20 ± 2 °C, and then subjected to a photoperiod of 16:8-h light/dark, at the same temperature, for a further 72 h. At the end of the test (5 d), the number of germinated seeds was counted and compared with that of the artificial soil, to determine the percentage of seedling emergence and, if possible, the EC_{50} value.

2.4.3. Aquatic toxicity tests

The toxicity of the water samples was tested through aquatic bioassays. When required, dilutions were prepared mixing water samples with the corresponding test medium. Toxicity results were expressed as the percentage of water sample in test medium (%; v/v) which promoted a reduction in 50% of the endpoint measured (EC_{50}). Organisms from the species *P. subcapitata* and *D. magna* were cultured and maintained, at Laboratory of Ecotoxicology of the Polytechnic Institute of Beja, (Portugal), according to the recommended protocols (ISO/DIS 8692, 2002; OECD 201, 2002; ASTM, 1998). Temperature, pH, dissolved oxygen and electrical conductivity (EC) of the samples were measured and were in accordance with the standard protocols used. A reference test with potassium dichromate ($K_2Cr_2O_7$) was conducted as a positive control.

2.4.3.1. Luminescent bacteria bioassay. Luminotox® was used to evaluate the inhibition on the luminescence of the marine bacteria *V. fischeri* (NRRL B-11177), according to the protocol "DR LANGE luminescent bacteria test" following ISO 11348-2 (1998). Tests were carried out using the following dilutions (100, 50, 25, 12.5, 6.25 and 3.125% v/v). Two replicates per treatment were used. The inhibition of the bacteria natural light emission was measured against a non-toxic control (2% w/v NaCl solution), at a temperature of 15 ± 0.5 °C. Tests were carried out with a LUMIStox 300 equipment. For each sample, luminescence decrease was measured before and after the desired incubation period (30 min) and EC_{50} (% v/v; the concentration of each sample that reduced 50% of the bacterial luminescence) was determined.

2.4.3.2. Algal bioassay. The potential inhibitory effect of the water samples on the growth of the unicellular algae *P. subcapitata* was assessed based on the protocol of OECD 201 (2002). The assay was initiated with 5×10^4 cells/mL of *P. subcapitata*, in the log exponential growth phase, and was carried out using six replicates per water sample concentration, and eight replicates for the control group (MBL medium without vitamins). Twenty-four-well plates containing 900 μL of sterilized MBL medium with the tested solutions (100, 75, 50 and 25% v/v) of the water samples diluted with MBL medium) and 100 μL of the

Table 1
Summary of bioassays used at ecotoxicological assessment of both matrices (soil and water) of the study.

	Specie	Control medium	Temperature (°C)	pH	Photoperiod ((light/dark (h)))	Exposure time	Endpoint
Soil samples	*Eisenia fetida*	Artificial soil[a]	20 ± 2.0	6.0 ± 0.5	16 h/8 h	7 days	Mortality
	Eisenia fetida	Artificial soil	20 ± 2.0	6.0 ± 0.5	16 h/8 h	56 days	Number of offspring
	Latuca sativa	Artificial soil	20 ± 2.0	5.0–7.0	16 h/8 h	5 days	Seedling emergence
	Lollium perenne	Artificial soil	20 ± 2.0	5.0–7.0	16 h/8 h	5 days	Seedling emergence
Water samples	*Vibrio fischeri*	NaCl (2%)	15 ± 0.5	7.0 ± 0.2	–	30 min.	Luminescent inhibition
	Daphnia magna	ASTM	20 ± 2.0	6.0–9.0	16 h/8 h	48 h	Immobility
	Daphnia magna	ASTM	20 ± 2.0	6.0–9.0	16 h/8 h	21 days	Number of offspring
	Thamnocephalus platyurus	Synthetic freshwater	25 ± 0.5	7.0–8.0	Continuous dark	24 h	Mortality
	Pseudokirchneriella subcapitata	MBL	24 ± 2.0	8.1 ± 0.2	Continuous light	72 h	Growth inhibition

[a] Artificial soil: 10% sphagnum peat, 20% kaolin clay, 70% industrial sand, w/w dw, pH adjusted to 6.0 ± 0.5 with calcium carbonate.

inoculum were incubated in an orbital shaker (100 rpm) under the conditions previously referred. After 72 h, the average specific growth rate was calculated as the logarithmic increase in cell concentration from the equation:

$$\mu_{i-j} = (\ln B_j - \ln B_i)/(t_j - t_i)$$

where: $\mu_{i\text{-}j}$ is the average specific growth rate from time i to j (per day); B_i is the cell concentration at time i (initial time of the exposure), and B_j is the cell concentration at time j (final time of the exposure).

The percent inhibition of the growth rate for each sample replicate was calculated from equation:

$$\%I = (\mu_c - \mu_T)/(\mu c) \times 100$$

where: %I, is the percent inhibition in average specific growth rate; μ_C is the mean value for average specific growth rate (μ) in the control group; μ_T is the average specific growth rate for the treatment replicate.

2.4.3.3. T. platyurus bioassay. The effect of the water samples on the mortality of *T. platyurus* was evaluated in accordance with the standard operational procedure provided from the THAMNOTOXKIT FTM kit (Persoone, 1999). The solutions to be tested were obtained by dilution of the samples with synthetic freshwater included in the test kit (100, 75, 50 and 25% v/v), also used as a nontoxic control. Larvae of shrimp *T. platyurus* (<24 h), obtained by the hatching of the cysts, were incubated in 24-well plates, ten animals per well (1 mL test dilution), three replicates for each of the five concentrations and the control group, at 25 °C for 24 h, in the dark. The number of dead shrimps after 24 h contact for each test solution was used as the selected endpoint.

2.4.3.4. D. magna bioassays. The *D. magna* acute bioassay was performed according to the ISO 6341 (1996) guideline. Five neonates (<24 h old) were exposed to 25 mL of the water samples and their dilutions with ASTM medium (100, 75, 50 and 25% v/v; ASTM, 1998), for a period of 48 h. Tests were conducted in environmental chambers at 20 ± 2 °C. A 16 h light and 8 h dark cycle was used. The endpoint used for the toxicological evaluation was the immobility of the daphnids.

Chronic toxicity tests were performed according to the Guideline for Testing of Chemicals N° 211: *Daphnia magna* reproduction test (OECD, 2012), to evaluate the potential effects of the water samples on the reproduction of this species. The chronic assay was initiated with the 3rd brood of offspring (<24 h old), from a single clone derived from a healthy parent stock culture. Daphnids were exposed to water samples diluted with ASTM solution (12.5, 25, 50, 75 and 100% v/v) (10 replicates per concentration containing one *D. magna* neonate). All assays had a negative control with ASTM. Test solutions were renewed three times a week and, at the same time, the organisms were fed with *Pseudokirchenериella subcapitata* (3.0×10^5 cells/ml/daphnia) and Marinure extract®. All experiments were conducted for 21 d, in a chamber with a constant temperature, 20 ± 1 °C, and a photoperiod of 16 h light and 8 h dark. During the assay the number of living offspring produced per female was recorded for each broad. After the exposure period, the total number of viable offspring produced by each female was used to estimate the reproduction rate.

2.5. Statistical analysis

All physicochemical data were checked for homogeneity of variance and normality (Kolmogorov–Smirnov test) and, when possible, subjected to one-way ANOVA. Whenever significant differences were found ($P \leq 0.05$) a post hoc Tukey's HSD test was used to further elucidate differences among sampling locations ($P \leq 0.05$).

The EC_{50} values for *T. platyurus* and *D. magna* mortality/immobility were calculated using the Probit analysis (Finney, 1971). In the *V. fischeri* bioluminescence inhibition test, the EC_{50} values were determined using LUMISsoft 4 Software™. Data on *P. subcapitata* growth and on *D. magna* reproduction, were checked for normality by the Kolmogorov–Smirnov test and variance homogeneity (Levene's tests). As the ANOVA assumptions were not met, data were analyzed non-parametrically using Kruskal–Wallis ANOVA by ranks test. When significant differences were found ($P \leq 0.05$), a post-hoc Dunnet's test was used to compare sampling stations with the control with a *P*-value of 0.05 as the minimum significant level (Zar, 1996). All statistical analyses were performed with the STATISTICA 7.0 (Software™ Inc., PA, USA, 2004).

3. Results and discussion

3.1. Soil general physicochemical characteristics and potentially toxic metals assessment

The studied soils presented marked variability in the physicochemical parameters obtained at different sites, with statistically significant differences among them ($P \leq 0.05$; Tukey's HSD test; Table 2). The pH ranged from neutral, in CNT, to slightly alkaline, in the soils around the mine (7.64–8.18). This gradient of pH had already been reported by Gala (2014), which identified values of pH around 6.5 to 8, in a total of 60 soil samples analyzed around the "Las Musas" mine, and reported a positive correlation with the percentage of carbonates, identifying the higher concentration of carbonates near the mine tailings, and the lower ones near the Azuaga village. Electrical conductivity is low, in all the studied points, with values from 12.7 μS/cm (CNT) to 690 μS/cm (3B), which indicated that the soils present low salinity. Comparing with the EC values obtained by Gala (2014), ranging from 125 to 953 μS/cm, a decrease of soils salinity was observed. The high pH values and the overall low EC were essentially due to the limestone environment (Azharia et al., 2017). In general, soils are poor in OM (<1.8% w/w; USEPA, 2004), classified as mineral soils. Similar physicochemical proprieties of mining soils, in the Mediterranean region, were observed by Azharia et al. (2017) in a study developed in an abandoned Pb/Zn-mining area at the Zeïda mining district (Maroccos) and by Bori et al. (2017) at the abandoned F–Ba–Pb–Zn Osor mining area (Catalonia, Spain).

Relatively to the nutrients, the results highlighted soils poor in $N_{Kjeldahl}$, being the higher values quantified in the locations with higher organic matter (CNT; C). Concerning to the other macronutrients, the soils presented medium (50 to 100 mg/kg) to high (100 to 200 mg/kg) amounts of the Egner-Rhiem extractable of P and K.

Total concentrations of potentially toxic metals presented a significant variability among sampling locations (Table 2). Among the metals assessed, Pb was the metal found with higher concentrations, followed by Zn, mainly at the B, C and D soils.

The concentrations observed for Pb were lower than the results generally reported in abandoned mines of Pb-Zn (940 up to >5000 mg/kg; Bori et al., 2017; Azharia et al., 2017). Furthermore, Gala (2014) reported concentrations of Pb higher than those found in the present study (82 to 3275 mg/kg), at similar distances from the mine tailings (3–292 m), directions (A, B, C, D) and depths (0–20 cm). Despite these facts, and taking into consideration, the possible migration of the metal to other abiotic and biotic compartments, the soils heterogeneity may be the main responsible for the differences found among concentrations, which are very pronounced in samples collected in the same area. Further, the pattern of the area of higher concentrations of Pb (area B) was like the results of Gala (2014). Nevertheless, the levels detected are still higher than the limit proposed by the Canadian soil quality guidelines for agricultural (70 mg/kg) and residential use (140 mg/kg) (CCME, 2018).

On the contrary, the concentrations of Zn are much more variable among the study sites, with levels 10,000 times lower than those found on the Osor mine district (Bori et al., 2017), and similar to those found at Zeïda district (Morocco) (Azharia et al., 2017). Copper contents

Table 2
Soils physicochemical characteristics and potentially toxic metals (mean ± SD; $n = 3$), determined in the four sampling locations (around of the Las Musas mine: A; B; D; C) and at the control location (CNT) near the village of Azuaga (Extremadura; Spain).

	A	B	C	D	CNT	Canadian soil quality guidelines for agriculture/residential use (*)
pH (1:2.5)	8.18 ± 0.07a	7.64 ± 0.03c	7.87 ± 0.02a	8.11 ± 0.03b	6.67 ± 0.09d	
EC (μS/cm)	65.50 ± 4.76c	690.00 ± 13.23a	147.97 ± 4.46c	75.87 ± 3.06b	12.70 ± 0.24d	
OM (%; w/w dw)	0.2 ± 0.02d	0.4 ± 0.00b	1.9 ± 0.02b	0.4 ± 0.01a	0.7 ± 0.02c	
$N_{Kjeldahl}$ (%; w/w dw)	0.01 ± 0.00d	0.02 ± 0.00c	0.08 ± 0.00c	0.02 ± 0.00b	0.10 ± 0.01a	
Extractable P (mg P_2O_5/kg dw)	81 ± 2d	111 ± 5c	127 ± 4b	242 ± 6a	48 ± 5e	
Extractable K (mg K_2O/kg dw)	68 ± 6c	79 ± 6c	104 ± 4b	96 ± 4b	124 ± 3a	
Texture (*)	Loamy clay	Loamy sand	Loamy clay	Loamy clay	Loamy clay	
Cd (mg/kg)	0.3 ± 0.03c	0.57 ± 0.07a	0.40 ± 0.03bc	0.50 ± 0.03ab	0.52 ± 0.02a	1.4/10
Cu (mg/kg)	5.6 ± 0.4d	12.2 ± 2.1c	17.5 ± 1.6b	12.5 ± 0.4c	42.5 ± 0.6a	63/63
Pb (mg/kg)	42.2 ± 3.1c	172.9 ± 7.2a	178.6 ± 10.1a	124.6 ± 1.4b	53.6 ± 1.9c	70/140
Zn (mg/kg)	63.1 ± 3.4d	155.7 ± 6.9a	144.8 ± 7.2a	104.2 ± 2.2b	86.2 ± 1.4c	250/250

Values in a row marked with the same letter are not significantly different (Tukey's HSD test, $P > 0.05$).
(*) Information from Gala, 2014.
DL(Pb) = 15.2 mg/kg; DL(Cd) = 0.3 mg/kg; DL(Zn) = 1.5 mg/kg; DL(Cu) = 1.5 mg/kg.
Values in a row marked with the same letter are not significantly different (Tukey's HSD test, $P > 0.05$).
(*) CCME (2018): https://www.ccme.ca/en/resources/canadian_environmental_quality_guidelines/.

were higher at C and CNT soils, presenting values in the same range to those obtained by Azharia et al. (2017) with mean values of about 26 mg/kg ($n = 20$), and by Bori et al. (2017), with values ranging from 11 to 47 mg/kg. Cadmium presented higher concentrations at B and CNT soils, lower than the stipulated limit by the Canadian soil quality guidelines for agricultural uses (1.4 mg/kg).

3.2. Water general physicochemical characteristics and potentially toxic metals assessment

The physicochemical properties of water samples of the Azuaga mining area are shown in Table 3. As for soils, the water samples presented statistically significant differences among locations for the analyzed parameters ($P < 0.05$; Tukey's HSD test).

The pH results showed values close to neutrality (UP, M and DW) and slightly alkaline (BZ and JT). The values of pH obtained at BZ are like those previously reported by Novo et al. (1994). In line with our results, Bori et al. (2017) have also reported neutral to slightly alkaline waters close to a Pb/Zn mining area at Catalonia region (Spain). This gradient of pH values is characteristic of non-acidic mines, which principal drainage problems are not associated with pyrite oxidation (Tiwary, 2001).

Regarding the conductivity of the water samples, the highest value was determined at BZ (954 μS/cm), followed by the samples from the Cagancha stream (UP, M and DW) that are probably suffering the influence of salts from the mine wastes, mainly of sulphate salts (Jiménez et al., 2009). These values of conductivity are in the same range of those observed by Allert et al. (2009), Ramani et al. (2014) and Bori et al. (2017) in rivers near to Pb/Zn mines.

In general, nutrient parameters, mainly NH_4-N, NO_3-N, TN and TP concentrations in surface waters around mining sites (Cagancha stream) were significantly higher than at Jituero stream. Some authors reported high concentrations of different nitrogen forms at surface waters near mining areas, considering nitrate as one of the major anions that constitute drainage waters, which might be, together with the sulphates, representative of the extension of the mine impact (Allert et al., 2009; Tiwary, 2001). Bembézar showed the highest amount of nutrients, mainly TP = 1.20 mg/L and NH_4-N = 4.20 mg/L, probably due to the influence of the drainage waters from “Las Musas”, together with the contamination from domestic wastewater discharges and agriculture activities located along the river. In fact, when we analyzed the results obtained by Novo et al. (1994), which quantified values of TP below 0.06 μg/L and NH_4-N below 0.1 μg/L, we verified that the amounts of these parameters have been increasing since 1994. These

Table 3
Water physicochemical supporting parameters (mean ± SD; $n = 3$) and potentially toxic metals, determined in the five sampling locations (Bembézar river and its tributaries; Azuaga, Extremadura).

	UP	M	DW	BZ	JT	Max.
pH	7.43 ± 0.01 cd	7.38 ± 0.01d	7.47 ± 0.02c	7.54 ± 0.03b	7.99 ± 0.04a	6.5–8.7$^{(i)}$
EC (μS/cm)	705 ± 6b	641 ± 4d	668 ± 4c	945 ± 6a	587 ± 5e	–
TN (mg/L)	1.80 ± 0.78c	20.71 ± 0.78a	1.35 ± 0.00d	5.40 ± 1.35b	3.60 ± 0.78b,c	–
NO_3-N (mg/L)	9.7 ± 0.3a,b	9.8 ± 0.7a,b	4.9 ± 0.3c	12.3 ± 2.4a	0.96 ± 0.0d	≤20 mg NO_3-N/L$^{(i)}$
NH_4-N (mg/L)	0.19 ± 0.03c	1.39 ± 0.10b	0.16 ± 0.04c	4.20 ± 0.00a	0.03 ± 0.01c	≤0.3 mg NH_4-N/L$^{(i)}$
TP (mg/L)	0.78 ± 0.00a	0.23 ± 0.11a	0.55 ± 0.33a	1.20 ± 0.39a	<DL	≤0.2 mg TP/L $^{(i)}$
BOD_5 (mg/L O_2)	11	49	21	10	35	–
COD (mg/L O_2)	44.86 ± 0.00d	56.82 ± 2.59c	68.79 ± 2.59b	101.68 ± 2.59a	38.88 ± 2.59d	–
SO_4 (mg/L)	96.84 ± 0.38a	67.79 ± 0.13c	77.72 ± 0.07b	51.73 ± 0.07d	37.17 ± 0.12e	–
Na (mg/L)	29.71 ± 0.09e	32.31 ± 0.44d	36.72 ± 0.45c	96.60 ± 0.08a	38.91 ± 0.16b	–
K (mg/L)	6.31 ± 0.15d	7.06 ± 0.12c	8.80 ± 0.15b	14.01 ± 0.09a	3.04 ± 0.00e	–
Mg (mg/L)	24.33 ± 0.14a	16.05 ± 0.06c	17.45 ± 0.01c	12.69 ± 0.05d	20.60 ± 0.91b	–
Ca (mg/L)	50.40 ± 0.26a	46.54 ± 0.04b	48.05 ± 0.25a,b	36.31 ± 0.18d	41.14 ± 1.34c	–
Cd (μg/L)	<DL	<DL	<DL	<DL	<DL	0.45$^{(ii)}$
Cu (μg/L)	<DL	<DL	<DL	<DL	<DL	5$^{(iii)}$
Pb (μg/L)	<DL	<DL	<DL	<DL	<DL	14 $^{(ii)}$
Zn (μg/L)	69.0 ± 0.0a	60.0 ± 0.0a,b	50.0 ± 0.0a,b	52.0 ± 0.0a,b	37.0 ± 0.0c	30$^{(iii)}$

UP: (upstream); M: (mine); DW: (downstream); BZ: (Bembézar river); JT: (Jituero stream).
DL: detection limit: DL(TP) = 0.003 mg/L; DL(Cd) = 8 μg/L; DL(Cu) = 17 μg/L; DL(Pb) = 170 μg/L.
Values in a row marked with the same letter are not significantly different (Tukey's HSD test, $P > 0.05$).
(i) maximum concentration for the support parameters for good ecological status (Real Decreto 817/2015 adapted from ECC, 2000); (ii) maximum allowable concentration of hazardous substances in inland surface waters (ECC, 2013); (iii) maximum concentration for the annual average of preferential substances in surface waters (Real Decreto 817/2015).

concentrations lead to the decrease of ecological status of Bembézar river and Caganch stream, classifying it as "less than good", being another factor that can be compromising the balance of the ecosystem communities.

The organic descriptor BOD_5 reached the highest concentrations in the Mine (49 mg/L) that, together with the concentrations of COD, highlighted a possible organic contamination with untreated wastewaters to the Caganch stream.

Mining activities can lead to an increase in the concentrations of major ions (SO_4^{2-}, Na^+, K^+, Mg^{2+} and Ca^{2+}) in freshwaters, which can be toxic to aquatic organisms (Ingersoll et al., 1992; USEPA, 2009), making important the assessment of these ions. The concentrations of SO_4^{2-}, Ca^{2+}, Mg^{2+} were significantly higher in the samples around the mine, relatively to Bembézar and Jituero water samples, highlighting the impact of the mine wastes on these streams. In fact, sulphate is considered a useful indicator of mine drainage contamination, because it remains elevated even at neutral pH values, since sulphate ions are not easily adsorbed and thus migrate further than metals; its migration can represent the largest range of mine-tailing impact (Gray, 1996). Sodium, Mg^{2+} and Ca^{2+} concentrations did not exceed the freshwater-screening benchmarks defined by the USEPA (2016).

Among the metals assessed, only Zn was detectable, achieving the highest concentrations in the surface waters near the mine. Generally, metal concentrations detected on the water samples are in same range of those found in waters near non-acidic mines (Allert et al., 2009), but much lower than those found in mines affected by acid mine drainage. The speciation and solubility of metals in water depends, among other physicochemical parameters, on pH and on dissolved organic matter. Thus, Bird et al. (2010), indicated that a pH value above 6, as measured in the sampling points of this study, promotes the sorption of metals onto the particulate (including organic material) and sediment phases. This might be a possible explanation for the lowest concentrations of metals observed in the water samples collected in the mining area. Furthermore, the low salinity of the soils may be another factor to increment the adsorption of the metals, once when the concentrations of the major cations are low, like Na^+ and K^+, they may be replaced by trace metals in the adsorption sites (Bori et al., 2017).

Despite Pb being the metal with the higher concentrations in the soil samples, it was always found below the analytical detection limit on the surface waters. The contamination of the surface waters with Zn was much more pronounced than with Pb, even though Zn and Pb are present in similar amounts in Pb/Zn mines (Barnes, 1979). This could be explained by the greater solubility of Zn salts compared with Pb salts (Barnes, 1979), as well as, by the fact that Pb is readily adsorbed by Al and Fe oxides in sediment (Lee et al., 2002), thus resulting in a much quicker decrease of dissolved Pb than Zn concentration (Zhang et al., 2004). Therefore, Pb is present to a lesser degree in the dissolved phase in water, which is why Zn is a better indicator of the effects of mining works on receiving waters (Alderton et al., 2005). Further, previous studies on bioavailability assessment of metals in sediments, by sequential extraction procedures like BCR, indicated that the predominant fraction where metals were found was correlated with the textural and geological characteristics of the matrix, as well as with time of the collection (Palma et al., 2015). Therefore, results showed that, at the dry period, which corresponds to the period when our samples were collected, the major amounts of Pb were found in the reducible fraction (55 to 88%), indicating its reaction with Fe-Mn-oxyhydroxides, the main chemical form controlling its mobility in the environment. Consequently, metal concentrations were very low in the water column. Other studies reported that Pb, together with Cu and Cd, are up to 5 times enriched in the sediments than in the water column (Ciazela et al., 2018).

Zinc surpassed the legal limit for annual average to continental waters, for preferential substances, stipulated by the Real Decreto 817/2015, adapted from the Water Framework Directive (ECC, 2000). Despite, Zn did not surpass the limit value recommended for the aquatic life criteria for freshwater chronic exposure (Zn = 120 μg/L; USEPA, 2016). This apparent ambiguity in the results is concerning, since Zn presents a higher bioconcentration ability, with a high bioconcentration factor (BCF; Zn_{BCF} = 74,200; Liu et al., 2018), which increases the ecological and public health risk, due to the possibility of its transference throughout the trophic chain.

3.3. Ecotoxicological assessment

Terrestrial and aquatic ecotoxicological bioassays were successfully applied to soil and water samples. All ecotoxicological tests reported fulfilled the validity requirements established by their respective guidelines. Marked differences in sensitivity were observed depending on test endpoints and organisms.

3.3.1. Terrestrial bioassays

For the terrestrial bioassays with earthworms, lethality test was not sensitive enough to detect toxicity for the studied soils, which may be correlated with the avoidance strategy adopted by the earthworms: they arrange themselves in a cluster, near the wall of the box, avoiding contact with the soil (Alvarenga et al., 2012), as well as, with the low acute toxicity of the soil samples. The low sensitivity of this terrestrial bioassay was already reported, in other ecotoxicological studies with mine contaminated soils (Alvarenga et al., 2013; Bori et al., 2017). On the contrary, reproduction test was sensitive enough to detect toxicity in some of the studied soils (Fig. 2). Soil B was the only identified as toxic, in the chronic exposure of earthworms, being the NOEC (no observable effect concentration) lower than 25% (w/w) of soil. Despite the similarity of the metal concentrations of the soils collected at the sites B, C and D, the soil B presented low concentrations of organic matter and is mostly constituted by sand (particles >0.063 mm), factors that may influence the low adsorption of the metals to the soil, turning them more available. This increment in metal availability, together with the highest EC value, may be the reasons for the toxicity observed. Results from terrestrial bioassays with earthworms agreed with previous studies, that highlighted the higher sensitivity of sublethal endpoints in the ecotoxicological evaluation of metal contaminated soils (Alvarenga et al., 2012; Bori et al., 2016).

Percentages of seedling germination on the tested soils (Fig. 3), highlighted that L. *sativa* was more sensitive to these soils than L. *perenne*. In fact, the germination of L. *perenne* was not inhibited in any of the tested soils. The resistance of this specie to toxicity of non-acid mine soils, was already observed by Bori et al. (2017), leading to the conclusion that it is a not good specie/bioassay to be integrated in a tool-box for the risk assessment of soils contaminated with wastes from non-acid mines. On the other hand, all soils induced a decrease of the percentage of seedling germination of L. *sativa*, being soil C

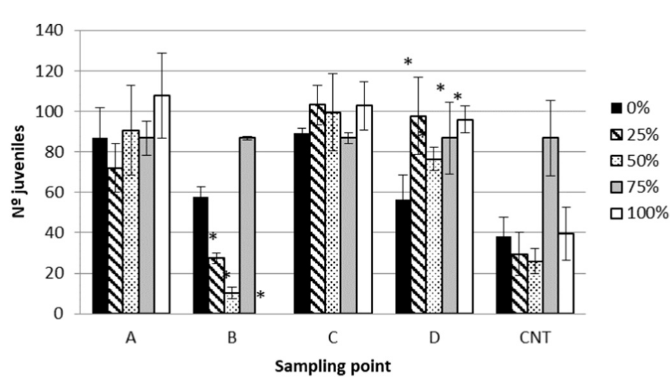

Fig. 2. *E. fetida* reproduction bioassay, total juvenile produced at each concentration (0, 25, 50, 75, 100% w/w) during the 21 days of exposure (mean ± standard deviation, n = 4). Bars marked with an asterisk represent values significantly different from control group in artificial soil (*P < 0.05, Dunnett's test with a control).

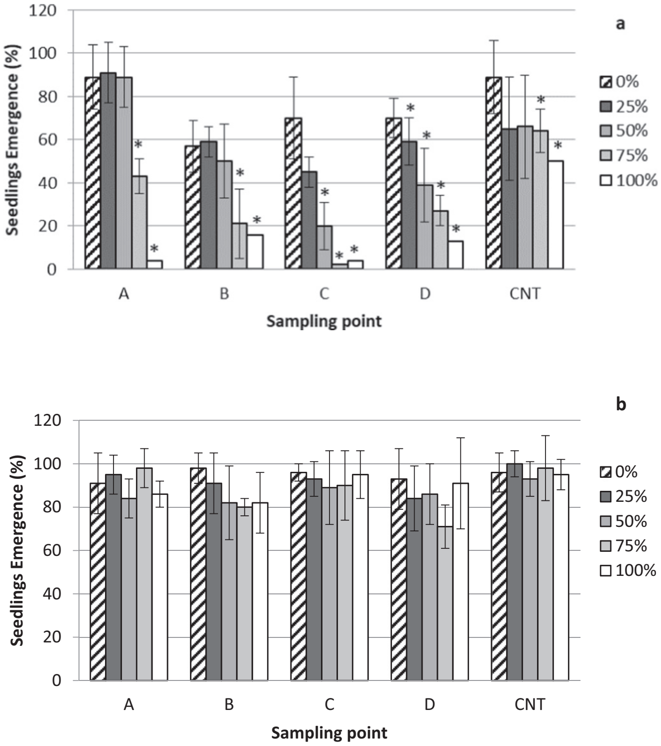

Fig. 3. *Latuca sativa* (a) and L. *perenne* (b) percentage of seedling emergence (%) at each concentration (0, 25, 50, 75, 100% w/w), after 5 d of exposure. Mean ± standard deviation ($n = 4$), *$P < 0.05$, Dunnett's post-hoc comparison test with the control.

(NOEC = 25% (w/w) and D (NOEC <25% (w/w)) the most toxic, and CNT the least toxic, with a NOEC of 75% (w/w)). The CNT soil presented the lowest concentrations for Pb and Zn, and, for the nutritional conditions, presented the higher concentrations for $N_{Kjeldahl}$ and extractable K, justifying, in this way the results observed. Once again, the results showed that the contamination with metals contributed to the toxic responses of the specie, but, in the case of plant species, the nutritional status of the soil seemed to exert a preponderant effect on the response observed, as be already reported by Alvarenga et al. (2013).

Despite the differences observed in the toxicity responses, the habitat function of the soils may be compromised, once at least one specie was sensitive to all of them.

3.3.2. Aquatic bioassays

The toxicity of water samples towards aquatic organisms, obtained for the acute exposure bioassays, evidenced that *V. fischeri* was the most sensitive species, detecting toxicity in all the samples from Cagancha stream ((30 min-EC_{50} (% v/v; $n = 2$; mean ± standard deviation): UP (46.30 ± 0.16); M (67.66 ± 0.32); DW (88.15 ± 0.27))); and Bembézar river (54.84 ± 0.33). Upstream induced the greatest luminescence inhibition of the species, site that presented the highest concentrations of Zn. These results are contrary to those from studies that reported the less sensitivity of this species to waters from acid-mine areas (Alvarenga et al., 2008; Bori et al., 2016, 2017). Thus, in cases that, due to the low pH of the waters, is necessary to do its correction for values near neutrality, this species maybe not be a good choice, once the pH correction may mask the toxic effects induced by the metals, especially when their concentrations are not very high.

The toxic effects towards *V. fischeri* were probably correlated with Zn concentrations, as was reported by Baran et al. (2014), in water extracts of soils from a Pb/Zn mine in Poland. Nevertheless, the concentration of Zn presented by the samples was not enough, by itself, to explain the toxicity observed, once the Zn-EC_{50} to *V. fischerie* is between 4.64 and 13.40 mg/L (Teodorovic et al., 2009), concentrations several orders of magnitude greater than those found in our study area. Consequently, the results highlighted that the luminescence inhibition probably occurred due to the mixture of Zn and other parameters, such as NO_3-N. In fact, the concentrations of NO_3-N surpassed the maximum level recommended for protecting the most sensitive species in freshwater systems (2.0 mg/L; Kincheloe et al., 1979).

Results from the acute bioassays showed that, none of the water samples caused mortality or immobilization towards *T. platyurus* and *D. magna*, respectively. Lari et al. (2017), showed that Zn is the metal, among Zn, Cd, Cu and Ni, less toxic towards *D. magna*. Moreover, its lethal bioassay is less sensitive than the sub-lethal tests, such as the feeding bioassay. Further, Zn concentrations detected in the water samples were several times lower than those that promote acute toxicity to this crustacean (EC_{50}: 0.18–0.56 mg/L; Teodorovic et al., 2009; De Schamphelaere et al., 2014).

Relatively to the results obtained with *T. platyurus*, they are in contrast with previous results that highlighted the sensitivity of this crustacean to low levels of potentially toxic metals in surface waters and sediment pore-water samples, being considered as a good alternative to *D. magna*, when integrating primary consumers in toolboxes for environmental risk assessment (Palma et al., 2014; Palma et al., 2016).

Considering the results of chronic exposure, in general, the samples induced an increase on the growth of *P. subcapitata* (Fig. 4), as well as an increase on the reproduction rate of *D. magna* (Fig. 5).

Relatively to *P. subcapitata*, the only sample that induced a slight decrease of its growth rate (20%) was Jituero. Comparing with the other sampling sites, Jituero showed a concentration of TP lower than the detection limit (<0.003 mg/L) which could be a limiting factor for algal growth. According to Reynolds (1984), a concentration <0.005 mg/L of TP may limit the algal biomass. Studies reported the increment of toxic effect (algae growth inhibition) induced by potentially toxic metals in the presence of low concentrations of TP, probably due to the reduction of metal detoxification processes (Chia et al., 2017). Furthermore, Gao et al. (2016), in a study that assessed the effects of the correlation Zn-TP in *P. subcapitata* growth, reported that the first stress factor for the growth of the algae is the TP concentration, and the second one is the Zn concentration, which means that, if TP concentration in the water is not limiting, the possible toxic effect induced by Zn may be masked. This fact justifies the observed results in the rest of the samples, with high amount of Zn and TP.

The observed significant increase on the number of offspring per female, relatively to the control (Dunnet post-hoc test, $P \leq 0.05$), might be explained by the high concentrations of nutrients (especially TN and TP) and organic matter content in the samples, which might have improved the metabolism and, consequently, the reproduction of daphnids. This kind of pattern was already reported by Alexander et al. (2013), Ieromina et al. (2014) and Palma et al. (2016), who observed a better performance in the reproduction parameters of the *D. magna* when the organisms were exposed to surface waters with higher levels of nutrients and organic matter, and if the concentrations of the hazardous substances are low.

4. Conclusions

"Las Musas" mine exhibits non-acid wastes with non-acid drainage waters, which limits metals mobility, being an important factor to the lower environmental risk observed at the site. The application of chemical and ecotoxicological LoE to soils and waters from the "Las Musas" mining area, allowed the determination of the real impact of the mine wastes in this region and the selection of the best bioassays to use in the risk assessment of abandoned mines with the same geological characteristics.

Hence, the soils from this mining area still have concentrations of Pb above those allowed for agricultural use, supporting the results obtained for the bioassays with L. *sativa* and the earthworm reproduction. These ecotoxicological results may, at medium-long term, affect the balance of the ecosystem, once these species integrate a trophic chain and

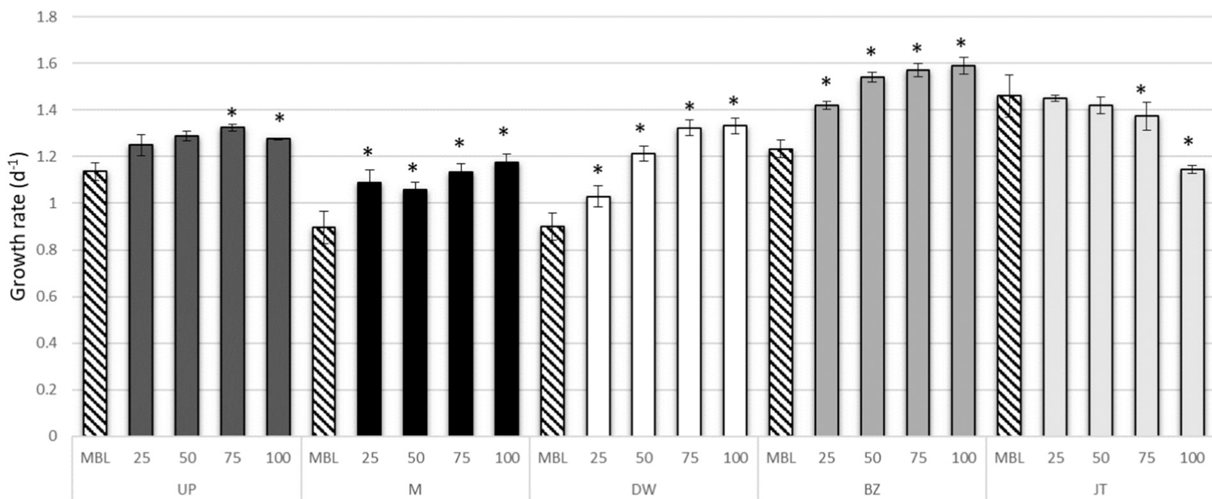

Fig. 4. Growth rate (d-1) of algae *P. subcapitata* after 72-h exposed to the different water samples tested ((Upstream mine (UP), Mine (M), Downstream mine (DW), Bembézar (BZ) and Jituero (JT))) (100% undiluted sample, 75%, 50% and 25% v/v dilutions with MBL medium). Mean ± standard deviation ($n = 6$), $^*P < 0.05$, Dunnett's post hoc comparison test with the control MBL.

the negative effect in one of them, a producer or a decomposer, will induce changes in all the chain.

Further, the results highlighted that the assessment of the terrestrial habitat function of these soils must integrate species that respond to soil contamination, as well as to the nutritional status of the soil.

As for the surface waters, the impact is not so clear and is apparently mixed with the responses to pollutants coming from other anthropogenic sources, e.g., the discharge of untreated wastewaters. Zinc was quantified in the waters at levels that deserve some attention, when considering the ecosystem balance. Despite that fact, the bioassays applied, except *V. fischerie* luminescence inhibition, did not reveal toxic responses. Indeed, *V. fischeri* bioassay showed good sensitiveness to metal contamination, as well as, to the ecological classification, highlighting the usefulness of its inclusion in a tool-box to test mining sites with non-acid wastes.

Therefore, in mining areas where the contamination by metals have decreased over time, with high concentrations of organic matter, and pH near the neutrality, the best ecotoxicological option is to choose species with responses less influenced by nutrients and organic matter, integrating behavioral endpoints, as feeding inhibition and avoidance habitat.

The results highlighted the linearity of the responses among the two groups of biological methodologies. Nevertheless, further studies including more sampling campaigns, the assessment of sediment matrices at the dry phase, and the use of a higher number of bioindicators would

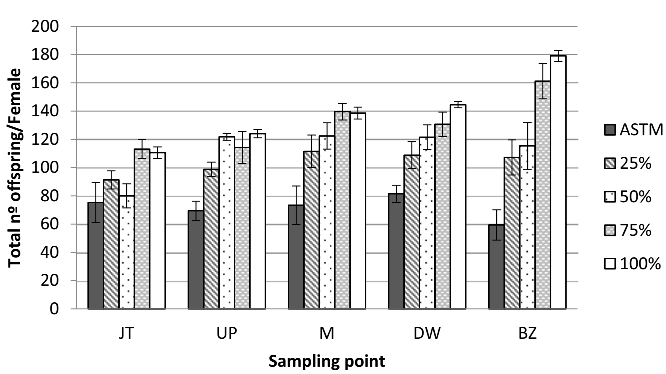

Fig. 5. Total number of offspring produced per female, after 21 d of exposure to the different water samples tested ((Upstream mine (UP), Mine (M), Downstream mine (DW), Bembézar (BZ) and Jituero (JT))) (100% undiluted sample, 75%, 50% and 25% v/v dilutions with ASTM medium). Mean ± standard deviation ($n = 10$), $^*P < 0.05$, Dunnett's post-hoc comparison test with the control ASTM.

be needed, to generalize our findings and to establish thresholds easy to apply and understand in programs of management and restoration of this type of ecosystem.

In view of the results from our study, the abandoned mining area of "Las Musas" is considered to have, nowadays, a moderate environmental threat. Nevertheless, as the results obtained were from a confined zone, further studies are needed, to determine the real environmental and health risk impacts in a larger area surrounding the mine tailings, through studies of distribution and mobilization of the potentially toxic trace metals, complemented with ecotoxicological studies.

Conflicts of interest statement

The authors declare that there are no conflicts of interest.

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