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Andrés Rodríguez-Seijo: Investigation, Data curation and formal analysis, Resources, Writing - original draft, Writing - review & editing.

Joana Lourenço: Investigation, Data curation and formal analysis, Resources, Writing - original draft, Writing - review & editing.

Daniel Arenas-Lago: Investigation, Resources, Writing - original draft, Writing - review & editing.

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Ruth Pereira: Conceptualization, Resources, Supervision, Writing - original draft, Writing - review & editing, Funding acquisition.

Chemical availability versus bioavailability of potentially toxic elements in mining and quarry soils

Andrés Rodríguez-Seijo[1,2], Joana Lourenço[3], Daniel Arenas-Lago[4], Sónia Mendo[3], Flora A. Vega[4], Ruth Pereira[5]

[1]CIIMAR - Interdisciplinary Centre of Marine and Environmental Research, University of Porto, Matosinhos, 4450-208, Portugal

[2]Department of Biology, Faculty of Sciences, University of Porto, 4169-007 Porto, Portugal

[3]Department of Biology & CESAM, University of Aveiro, 3810-193, Aveiro, Portugal

[4] Department of Plant Biology and Soil Science. Área de Edafoloxía e Química Agrícola. Facultade de Ciencias. University of Vigo. As Lagoas 1, 32004 Ourense, Spain.

[5]GreenUPorto – Sustainable Agrifood Production Research Centre & Department of Biology, Faculty of Sciences, University of Porto, 4169-007, Porto, Portugal

Corresponding author:

Andrés Rodríguez-Seijo

[1]CIIMAR - Interdisciplinary Centre of Marine and Environmental Research, University of Porto, Matosinhos, 4450-208, Portugal

[2]Department of Biology, Faculty of Sciences, University of Porto, 4169-007 Porto, Portugal

E-mail: andres.seijo@fc.up.pt

Abstract

Abandoned mining and quarry areas are sources of potentially toxic elements (PTEs), through lixiviates or transfer processes of bioavailable fractions from mining wastes and tailings. In this study, earthworms (*Eisenia fetida* Savigny, 1826) were exposed for 28 days to two mining soils from a lead/zinc mine and two quarry soils from an old serpentine quarry. Despite their pseudo total metal contents, a previous characterization of these soils pointed out for a low chemical availability of PTEs. Therefore, a multibiomarker approach was used and the response of *E. fetida* to soils was assessed through the analysis of neurotoxic, oxidative stress, energy metabolism and DNA damage biomarkers (acetylcholinesterase, catalase, glutathione-s-transferase, lactate dehydrogenase, lipid peroxidation and DNA strand breaks). Metal bioaccumulation was also assessed to evaluate bioavailability and organism's exposure. Results showed that high contents of PTEs were recorded in the whole body of earthworms exposed to lead/zinc mine. However, the bioaccumulation factors for worms exposed to soils from both sampling sites were < 1 due to the high PTEs contents in soils. Earthworms exposed to both types of soils displayed neurotoxic and energy metabolism effects. However, significant levels of oxidative stress and DNA damage were recorded only for earthworms exposed to lead/zinc mine soils. This study demonstrated that despite the low availability of PTEs showed by previous sequential chemical extractions, the results obtained from the direct toxicity assessment performed in this study, highlight the importance of a multibiomarker approach using soil organisms to provide a better evaluation of soils pollution.

Keywords: mild extractions; comet assay; metals; neurotoxicity; oxidative stress; risk assessment

Abbreviations: **AChE**, Acetylcholinesterase; **BAF**, Bioaccumulation factor; **CAT**, catalase; **GST**, glutathione-S-transferase; **LDH**, Lactate dehydrogenase; **LPO,** Lipid peroxidation**; NW**, Northwest; **OM**, organic matter; **PTEs**, Potentially Toxic Elements; **TBARS**, thiobarbituric acid reactive substances.

1. Introduction

Mining and quarrying activities are the third source of Potentially Toxic Elements (PTEs) in European soils. High amounts of waste materials are deposited after mine processing, often without any environmental mitigation actions (Panagos et al. 2013; Arenas-Lago et al. , 2018). These tailings are usually exposed to weathering conditions, which can accelerate meteorization processes, and become a source of PTEs to the surrounding ecosystems. The new soils, which are a consequence of the alteration of original soils by mining activities, are classified as spolic technosols according to WRB classification (IUSS Working Group WRB 2015) and have adverse characteristics to microorganisms, animals and plants development, such as low content of organic matter, extreme pH values or unfavourable structure and texture (Arenas-Lago et al. 2018).

The bioavailability of PTEs to ecosystems and soil organisms can be determined by indirect (e.g., single or sequential extractions) or direct measures (e.g., bioaccumulation of PTEs in plant and soil organisms) (Lanno et al. 2004). In addition, the assessment of biological effects through organism's stress responses to PTEs improves our knowledge on toxicant's bioavailability and mode of action (Lourenço et al. 2011; Arenas-Lago et al. 2018; Mkhinini et al. 2019).

In previous studies, different measurements of PTE's availability and of bioavailability were done for soils from an abandoned lead/zinc mine (contaminated by Cd, Pb and Zn) (Arenas-Lago et al. 2014; Lago-Vila 2017) and from an abandoned serpentine quarry (contaminated by Cr, Co and Ni) (Arenas-Lago et al. 2016; Lago-Vila et al. 2015; 2017). This was made through selective and sequential extractions, and through the assessment of accumulated metals by plants species growing spontaneously in the study areas (*Cytisus scoparius* and *Festuca rubra* for lead/zinc mine and quarry area, respectively). A low availability of studied PTEs (up to 20% of pseudo total concentrations) was recorded, with some exceptions, for all the soils. In both cases, Fe/Mn oxides and Mg silicates had a strong influence on the retention of studied elements (Arenas-Lago et al. 2014; 2016), and this was likely responsible by the lower uptake by native plants (Lago-Vila et al. 2015; 2017). However, the bioavailability and toxicity for soil invertebrates were not evaluated for these soils. Earthworms are excellent model organisms to assess the bioavailability and toxicity of PTEs as they are directly exposed through both ingestion of soil particles and absorption by dermal contact (Becquer et al. 2005; Sizmur et al. 2009). Thus, in this study earthworms (*Eisenia fetida* Savigny, 1826) were exposed for 28 days to soils from an abandoned lead/zinc mine and a serpentine quarry, with different physicochemical characteristics and PTEs contents.

After exposure, the bioaccumulation of PTEs was determined through the quantification of earthworm's body burdens. PTE's toxicity was determined through the evaluation of changes in the activity of severalbiomarkers of neurotoxicity [acetylcholinesterase (AChE)], oxidative stress [catalase (CAT)], biotransformation and oxidative stress [glutathione-S-transferases (GST)], energy metabolism[lactate dehydrogenase (LDH)], and lipid peroxidation [thiobarbituric acid reactive substances-TBARS (LPO)]. The analysis of DNA damages also was measured by the alkaline comet assay. These biomarkers are sensitive, time- and cost-effective and have been used in a wide range of scenarios, species and toxicity assessment approaches (e.g. Cataldo et al. 2011; Colacevich et al. 2011; Lourenço et al. 2011; Bessa et al. 2016; Boughattas et al. 2016; Correia et al. 2017; Rodríguez-Seijo et al. 2018).

Thus, the main objectives of this study were: i) to assess the bioavailability of PTEs on both soils, through the assessment of their bioaccumulation on earthworms and also their potential to induce toxicity; ii) to compare the results recorded for direct bioavailability measurements (with earthworms as bioindicators) with those obtained for the chemical availability by a mild extraction and by a sequential chemical extractions (Arenas-Lago et al. 2014, 2016).

2. Materials and methods

2.1. Study sites and soil properties

This study was carried out with four contaminated soil samples from Galicia (NW Spain): i) two soils from an old serpentine quarry (Penas Albas, Moeche, NW Spain) (Soils S1 and S2), and ii) two soils from an abandoned lead/zinc mine (Rubiais mine, NW Spain) (Soils S3 and S4) (Fig. 1). The selected soils from each area have different physicochemical characteristics, pseudo total contents of studied PTEs (Co, Cr, Ni, Cd, Pb and Zn), and degrees of plant cover. The physical and chemical properties (soil pH, total Kjeldahl-N, organic matter content and effective cation exchange capacity) of these lead/zinc mine soils and quarry areas, were already described in previous studies (Rodríguez-Seijo et al. 2014) (Lago-Vila et al. 2015) (Table 1). An OECD artificial soil with 5% of organic matter (pH 6.25 ± 0.18), adjusted with $CaCO_3$, was used as a control soil for the ecotoxicological assays.

2.2. Determination of pseudo total and available PTEs content in soil samples

Pseudo total PTEs contents were extracted from 0.2 g of soil by acid digestion (*aqua regia* procedure) with a mixture of HNO_3 and HCl (1:3 v/v) in a microwave oven (Ethos 1; Milestone) (experimental

conditions: 9 bar, 190°C and 45 min) (Lago-Vila et al. 2017). A single $CaCl_2$ extraction was performed to determine the available content of PTEs in the studied soils (0.01 M $CaCl_2$ acidified with HCl 0.1 M, 1:10 w/v soil to extractant ratio, 2 h shaking) (Houba et al. 2000) (Table 1). In both cases, the concentration of PTEs in the extracts was determined by ICP-OES (Perkin Elmer Optima 4300 DV) at CACTI-Universidade de Vigo (Vigo, Spain).

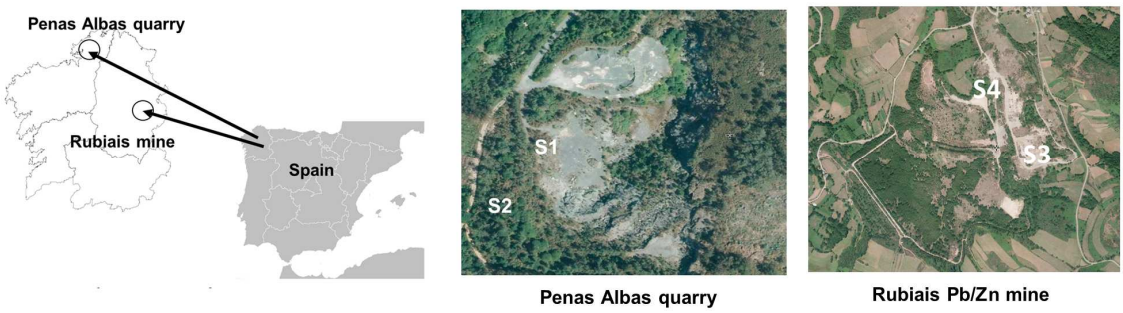


Fig. 1. Location of study areas. Penas Albas quarry area (S1 and S2) and Rubiais Pb/Zn mine (S3 and S4) (Source: SIGPAC 2015).

2.3. Earthworms analysis

2.3.1. Experimental procedure

Earthworms (*E. fetida*) were selected for this study and obtained from a laboratory culture kept under environmentally controlled conditions (photoperiod $16h^{L}$:$8h^{D}$; temperature 20 ± 2 ˚C). The organisms from the culture are fed with defaunated horse manure and oatmeal once a week and grown in plastic boxes in a medium composed of sphagnum peat, horse manure, and deionised water.

Ten clitelated adult earthworms (weight ranging between 300 and 600 mg) were added to each soil sample and control-CTL replicates (four replicates by soil sample), after being acclimatized to OECD artificial soil for 24h. Soils water holding capacity (WHC) was previously adjusted to 40% of their WHC*max* (OECD 1984, ISO 2008). Plastic containers were kept at 20 ± 2ºC with a light cycle of 16/8 h light/dark for 28 days. Dry and defaunated horse manure (± 5 g) was added every week during the test period, as well as deionised water, whenever necessary, to maintain a constant soil water content.

After 28 days of exposure, the earthworms from each test vessel were removed, rinsed with deionised water and, to allow total clearance of the gut content, left to depurate for 24 h in a plastic container with Milli-Q water moistened filter paper. Survival and weight change were assessed at the end of the test period.

2.3.2. Potentially Toxic Elements exposure and accumulation by earthworms

Earthworms were thawed at room temperature and dried at 60ºC until constant weight. Hence, a pool of two worms from each replicate (4 replicates per soil tested) was digested with a mixture of 1 ml H_2O_2 (30%) and 3 ml (HNO_3) (70%) (Ultra-pure reagents) (Rodríguez-Seijo et al. 2017). The solution was filtered and diluted to 50 ml with Milli-Q water. Sample blanks were obtained following the same procedure but without the biological sample. All samples were analysed using an ICP-OES. The Cd, Co, Cr, Ni, Pb and Zn concentrations were expressed as mg kg^{-1} dry weight.

The bioaccumulation factor (BAF) of studied PTEs was calculated according to BAF = *Cb/Cs*, where *Cb* is the concentration of a given element in earthworms, while *Cs* is the concentration of the element in the soil sample (OECD, 2010).

2.3.3. Neurotoxicity and oxidative stress biomarkers

The neurotoxicity and oxidative stress responses were assessed through the determination of the activity of specific enzymes (Sanchez-Hernandez 2006; Lionetto et al. 2012). Briefly, the activity of acetylcholinesterase (AChE), catalase (CAT), glutathione -S-transferases (GST), lactate dehydrogenase (LDH) were determined according to the methodologies proposed by Ellman et al. (1961), Aebi (1984), Habig (1974) and Vassault (1983), respectively. Lipid peroxidation (LPO) was assessed through the quantification of thiobarbituric acid reactive substances (TBARS) according to Buege and Aust (1978). Details about the methodology used for the enzymatic assays was well described by previous papers published by our research group (e.g. Correia et al. 2017; Rodríguez-Seijo et al. 2018).

Three earthworms from each replicate, randomly selected and previously depurated (24 h), were pooled and homogenised in an ice-cold phosphate buffer (50 mM, pH = 7.0 with 0.1% Triton X-100) using a tissue homogeniser (T 10 basic ULTRA-TURRAX®). The homogenates were then centrifuged (3000rpm for 15 min, at 4 °C). Finally, the obtained supernatant was separated and used for biochemical analyses. The same procedure was followed for all the experimental replicates. All biomarkers were measured in triplicate for each replicate using a spectrophotometer equipped with a microplate reader (Thermo Scientific™ Multiskan™ GO UV/Vis microplate spectrophotometer). For all assays, the protein concentration of the samples was determined by Bradford's method (Bradford, 1976), adapted to the microplate reader and measured spectrophotometrically at 595 nm in triplicate (Thermo Scientific™ Multiskan™ Go UV/Vis microplate spectrophotometer). Results were expressed as nmol min^{-1} mg^{-1}

protein (AChE, CAT and GST), µmol min^{-1} mg^{-1} protein (LDH) and nmol MDA equivalents by mg^{-1} of protein (LPO).

2.3.4. Coelomocytes extrusion and DNA damage quantification

DNA damage quantification was performed in earthworm coelomocytes (five worms from each replicate), obtained following the methodology described by Lourenço et al. (2012) and Correia et al. (2017). Finally, DNA damage was visually scored, thought the observation of one-hundred nucleoids, randomly selected, and graded into five classes (García and Mandina 2005; Correia et al. 2017): from 0 to 4, being 0 a nucleoid without damage and 4 a nucleoid with almost all the DNA in the tail (most damaged cells). The results were reported as arbitrary units, calculated by multiplying the number of observed comets (0−100) by the comet classification (0−4).

2.4. Statistical analyses

All the statistical analyses were performed with IBM SPSS Statistics v23.0 software. To assess significant differences among earthworms exposed to the different soils for the measured parameters (AChE, CAT, GST, LDH, LPO, DNA damage and PTEs content), one-way analysis of variance (ANOVA) was carried out, after checking the homoscedasticity of variances and the normality with Levene's and Shapiro-Wilk tests, respectively. A significance level of $p < 0.05$ was chosen to reject the null hypothesis (no differences between each group of exposed earthworms). When significant differences were recorded, Dunnett's test was applied to determine which soil sample induced significant responses in earthworms when compared to the CTL (OECD soil).

3. Results and discussion

3.1. Soil properties and PTEs content in selected soil samples

The studied soils are classified as sand or sandy loam soils according to USDA classification, with slightly alkaline or alkaline pH values (Table 1). The organic matter (OM) and the Total Kjeldahl Nitrogen contents were very low in the lead/zinc mine soils (S3 and S4). Also, soil samples from the quarry area had low levels of nitrogen, however, the OM contents were within the range described for Galician regional soils and Spanish topsoils (Calvo de Anta et al. 2015; Rodríguez-Martín et al. 2015). All samples had low levels of ECEC (effective cation exchange capacity (< 7 cmol $_{(+)}$ kg^{-1}), except S2

(19.59 cmol $_{(+)}$ kg^{-1}). The studied soils presented a base saturation between 99.8 and 100%, due to parent rock material, and in the case of quarry soils, they had an imbalance of Ca/Mg (Rodríguez-Seijo et al. 2014; Lago-Vila et al. 2015).

The studied soils also had high levels of pseudo total PTE concentrations above the generic reference limits for ecosystem protection and industrial uses, established for the Galician region (DOG 2009). In each area, samples have differences between them because they originate from different grades of activity (Table 1). As expected, the levels of available PTEs were lower than the pseudo total contents (< 20% for mine soils and 12 to 17% for quarry soils). The exception was recorded for Pb (up to 46% for S3) and Co (23% for S1), respectively) (Table 1). The PTEs with higher levels were Ni, Pb and Zn (Table 1).

3.2. Exposure and accumulation of PTEs by earthworms

At the end of the 28 days of exposure, there was no mortality and weight loss as not statistically significant differences in exposed organisms were recorded when compared to the control (OECD soil) (ANOVA: F: 1.808; *df:* 4, 14; p = 0.1834). Significantly, higher levels of PTEs were found in exposed organisms when compared to the control (Table 2). This was probably due to both direct dermal contact with PTEs in interstitial water and the ingestion of soil particles. Soil ingestion may increase PTEs bioavailability, due to pH variability in the different compartments of the gastrointestinal tract of earthworms, potentially increasing mobilization (Peijnenburg and Jager 2003; Becquer et al. 2005; Hobbelen et al. 2006; Sizmur et al. 2009; Lourenço et al. 2011; Boughattas et al. 2016). According to Song et al. (2002), the threshold concentrations in earthworm's tissues that can lead to increased mortality in *E. fetida* are 300, 1300, 1700 and 300 mg kg^{-1} dw for Cu, Zn, Pb, and Cd, respectively. The concentrations found in this study were lower than those required to induce death, except for Zn levels (average value of 1405 mg kg^{-1} dw for S4) (Table 2).

210 **Table 1.** Physical and chemical properties and PTEs concentrations for each studied soil (adapted from

211 Rodríguez-Seijo et al. 2014 and Lago-Vila et al. 2015).

Parameter	**Sampling sites**				
		S1	S2	S3	S4
Soil use	Control soil	Quarry tailings. Serpentine quarry		Mine tailings. Lead/Zinc mine	
pH $_{H2O}$	6.25 ± 0.3	7.94 ± 0.04	7.80 ± 0.05	7.13 ± 0.11	7.91 ± 0.15
Organic matter (%)	4.53 ± 0.25	3.68 ± 0.11	5.70 ± 0.13	0.43 ± 0.03	0.14 ± 0.02
TKN (g kg^{-1})	2.24 ± 0.2	*Bdl*	0.42 ± 0.03	0.33 ± 0.02	0.30 ± 0.02
ECEC ($cmol_+$ kg^{-1})	8.25 ± 0.97	5.19 ± 0.17	19.75 ± 0.37	6.56 ± 0.19	4.91 ± 0.31
Water holding capacity (%)	28.16 ± 3.1	22.9 ± 2.1	21.9 ± 2.5	54.33 ± 5.4	22.71 ± 2.9
Soil porosity (%)	-	26.13 ± 0.02	46.91 ± 0.2	43.39 ± 0.03	29.68 ± 0.1
Bulk density (g cm^{-3})	-	1.84 ± 0.01	1.35 ± 0.01	1.44 ± 0.01	1.8 ± 0
	Particle size distribution				
Sand (%)	75.05 ± 0.72	89.55 ± 0.14	59.73 ± 0.04	90.32 ± 0.07	88.48 ± 0.49
Silt (%)	17.39 ± 0.72	6.62 ± 0.14	26.06 ± 0.04	7.90 ± 0.07	11.52 ± 0.49
Clay (%)	8.43 ± 0.53	4.05 ± 0.45	14.10 ± 0.1	1.78 ± 0.01	*Bdl*
USDA classification	Sandy Loam	Sand	Sandy Loam	Sand	Sand
	Pseudo total content of studied PTEs (mg kg^{-1})				
Co	-	*109 ±1*	*147 ±1.2*	*92 ±3.8*	*141±1.7*
Cr	-	***1672 ± 110***	***2604 ± 38***	78 ± 2.1	*82* ± 3.5
Ni	-	***2039 ± 107***	***1861 ± 62***	36 ± 2.9	29 ± 1.2
Cd	-	*Bdl*	*Bdl*	*14 ±0.6*	***43 ± 0.7***
Pb	-	*Bdl*	*Bdl*	***2137 ± 370***	***6761 ± 1352***
Zn	-	33 ± 3	63 ± 5	***12000 ± 559***	**32000 ± 3570**
	$CaCl_2$ available contents of studied PTEs (mg kg^{-1})				
Co	-	26 ± 0.8	25.2 ± 0.19	*Bdl*	*Bdl*
Cr	-	4.02 ± 0.05	7.65 ± 0.17	*Bdl*	*Bdl*
Ni	-	274 ± 6.7	153 ± 3.2	*Bdl*	*Bdl*
Cd	-	*Bdl*	*Bdl*	0.35 ± 0.01	0.80 ± 0.01
Pb	-	*Bdl*	*Bdl*	987 ± 42	1022± 38
Zn	-	*Bdl*	*Bdl*	2688 ± 33	4390 ± 26

212 Average values ± standard deviation (n ≥ 3). *Bdl* Below detection level; *TKN* Total Kjeldahl

213 Nitrogen; *ECEC* Effective cation exchange capacity. Values in italics and bold letter highlight values above the

214 guidelines for soils delivered by the Galician regional government considering ecosystems protection and

215 industrial uses, respectively, for Cd (1 and 20 mg kg^{-1}), Co (40 and 150 mg kg^{-1}), Cr (80 and 300 mg kg^{-1}), Ni

216 (75 and 200 mg kg^{-1}), Pb (80 and 500 mg kg^{-1}) and Zn (200 and 1000 mg kg^{-1}) (DOG 2009).

Table 2. Concentration of Cd, Co, Cr, Ni, Pb and Zn (mg kg^{-1} dry weight) in *E. fetida* exposed to control soil (OECD), quarry area (S1 and S2) and lead/zinc mine (S3 and S4) sampling sites.

Element	Sampling Sites				
	OECD	S1	S2	S3	S4
Co	*Bdl*	3.40 ± 0.95 *a*	3.10 ± 0.74 *a*	-	-
Cr	*Bdl*	18.76 ± 4.4 *a*	15 ± 3.7 *a*	-	-
Ni	*Bdl*	43.71 ± 10.6 *a*	35.7 ± 8.4 *a*	-	-
Cd	*Bdl*	-	-	1.63 ± 0.34 *a*	1.7 ± 0.6 *a*
Pb	*Bdl*	-	-	23.5 ± 6.3 *b*	398.4 ± 108.4 *a*
Zn	*Bdl*	-	-	250 ± 66.1 *b*	1405 ± 368 *a*

Values are expressed as mean ± standard deviation; Bdl: below detection level. "-" not measured. For each row, different letters in different samples means significant differences from the worms exposed to the control soil (one-way ANOVA test, LSD post hoc test, $p < 0.05$).

Soil physicochemical properties have a great influence in metal's bioavailability and therefore, PTEs uptake and accumulation by soil organisms. For example, earthworms exposed to S2 had similar PTEs content in their bodies, when compared to those exposed to S1 (Table 2), although S2 has a higher content of PTEs than S1. This could be explained by the higher ECEC levels and by the higher clay, organic matter (Table 1) and Fe/Mn oxides content of S2 compared to S1 (15.65 vs 2.24 g kg^{-1}, respectively), that contributed for reducing the bioavailability of PTEs (Owojori et al. 2010; Arenas-Lago et al. 2016). The solubility of PTEs is also influenced by soil pH however, this factor was not of major relevance in these soils as pH values were similar between soils and slight to moderately basic.

In fact, and as mentioned above, Arenas-Lago et al. (2014; 2016) showed through sequential extractions that only a small proportion of soil PTEs was associated with exchangeable and organic matter fractions for both sampling areas; therefore it was not the organic matter that had the main role in reducing the chemical availability of PTEs in these soils. However, earthworms can ingest soil particles and contaminants sorbed to poorly labile fractions (bound to oxide and organic fractions), and their passage through the gut can change the availability from sorbed to exchangeable PTEs (Becquer et al. 2005; Sizmur et al. 2009; Nannoni et al. 2011; Sizmur et al. 2011a,b) also observed that the ingestion of PTEs bounded to soil components (mineral or oxidable fractions) was an uptake route more significant than the dermal uptake of dissolved ions from the soil solution. This could explain why higher levels of PTEs were found in earthworms exposed to soils with a low levels of bioavailable PTEs. Despite the exposure, in all cases, the BAFs levels were all below 1, pointing for no bioaccumulation of these elements in the organisms (Table 3).

Table 3. Bioaccumulation Factor (BAF) values for earthworms exposed to control (OECD), quarry area (S1 and S2) and lead/zinc mine (S3 and S4) samples.

Element	Sampling Sites				
	OECD	S1	S2	S3	S4
Co	-	0.04 ± 0.01*a*	0.02 ± 0 *b*	-	-
Cr	-	0.01 ± 0 *a*	0.01 ± 0 *a*	-	-
Ni	-	0.02 ± 0 *a*	0.02 ± 0.01 *a*	-	-
Cd	-	-	-	0.11 ± 0.02 *a*	0.04 ± 0.01 *b*
Pb	-	-	-	0.01 ± 0 *b*	0.06 ± 0.01 *a*
Zn	-	-	-	0.02 ± 0.01 *b*	0.04 ± 0.01 *a*

Values are expressed as mean ± standard deviation. "-" not measured.

For each row, different letters in different samples means significant differences from the worms exposed to the control soil between (one-way ANOVA test, LSD post hoc test, $p < 0.05$).

Similar results were also shown by several authors, as BAFs values decline with increasing PTEs concentrations in soils (e.g., Nahmani et al. 2007, Peijnenburg and Vijver 2009, Colacevich et al. 2011, Li et al. 2018), as it was observed for BAF levels of Zn, despite the high levels of Zn found in the body of organisms exposed to quarry soils. Alike results were also reported for earthworms exposed to metalliferous soils with levels of contamination similar to those found in our studies (e.g., Morgan and Morgan 1998, Andre et al. 2009, Colacevich et al. 2011). Earthworms also did not bioaccumulate some of the studied PTEs, probably because of their ability to regulate and excrete them efficiently.

In general, BAF values were consistent with those provided by the sequential extraction carried out by Arenas-Lago et al. (2014, 2016) consistently pointing for the low chemical availability and low bioavailability of PTEs in the studied soils.

3.3. The neurotoxic and oxidative stress responses of earthworms exposed to PTEs rich soils

Increased uptake of PTEs does not always mean increased toxicity for earthworms. Uptake and adverse effects of PTEs can be modified by earthworms' physiological factors involved into regulation of metal levels in their tissues or in their ability to eliminate the excess of PTEs, such as, an increment of chloragosomes for metal sequestration or metal biotransformation into less toxic species (Dai et al. 2004, Nahmani et al. 2007, Stankovic et al. 2014, Wang and Cui 2016, Li et al. 2018).

The impact of PTE concentrations on neuromuscular interactions and in the oxidative stress system of *E. fetida* is shown in Fig. 2 for organisms exposed to samples from the quarry and mining area.

Although the acetylcholinesterase (AChE) has been proposed as a biomarker of exposure to neurotoxic compounds such as organic contaminants, PTEs may also inhibit AChE activity (Labrot et al.

1996; Gaitonde et al. 2006; Lionetto et al. 2012; Dongxing et al. 2016). However, stimulatory effects of metals mixtures (Cr, Cu, Ni, Pb, Zn) on AChE activity were also reported (Zheng et al. 2013). In the present study, a significant inhibition of AChE activity compared to the control group (OECD soil) was observed for earthworms exposed to samples from the quarry area (ANOVA: $F=65.60$; d.f.=2, 26; $p < 0.0001$; Fig. 2a) and lead/zinc mine area (ANOVA: $F= 29.6$; d.f.=2, 26; $p < 0.0001$; Fig. 2a). Inhibitions of AChE in exposed earthworms were ranged from 47% (S3) to 68% (S2), and inhibitions of AChE activity above 20% has been proposed as indicative of exposure to anticholinesterase agents (Ludke et al. 1975; Menéndez-Helman et al. 2015; Fajardo and Ocampo 2018). Different PTEs such as Cd, Co, Cr, Pb and Zn have been proposed as inhibitors of AChE (Elumalai et al. 2002; Frasco et al. 2005; Gaitonde et al. 2006; Dongxing et al. 2016; Hayat et al. 2017; Mkhinini et al. 2019), while Ni has shown contradictory results (Frasco et al. 2005; Hayat et al. 2017). Our results indicate that the exposure to both types of soils displayed neurotoxic effects on earthworms.

Catalase is an important component of the antioxidant defence system, an antioxidant enzyme that regulates the amount of H_2O_2, protecting cells from their toxic effects (Ghribi et al. 2019). In our assay, CAT activity was significantly inhibited on S2 from the quarry area (ANOVA: $F=5.719$; d.f.=2, 15; $p = 0.0143$; Fig. 2b), but their activity was significantly increased in the S3 sample from the lead/zinc mine, (ANOVA: $F=9.789$; d.f.=2, 12; $p =0.003$; Fig. 2b).

In the earthworms exposed to quarry soils, other enzymes may have been activated to avoid an oxidative stress response (Zhang et al. 2009; 2013, Santana et al. 2018; Yin et al. 2018; Ghribi et al. 2019). In fact, despite the inhibition of CAT activity, no significant increase in lipid peroxidation was recorded in these samples, as a reduction on the MDA concentration was observed (ANOVA: $F=54.52$; d.f.=2, 28; $p < 0.0001$; Fig. 2e).

Earthworms exposed to the lead/zinc mine samples showed a different enzymatic activity profile with an increase in CAT activity, but only significant for S3 (Fig. 2b). These results suggest that earthworms' exposure to high levels of Cd, Pb and Zn may induce an oxidative stress response and, consequently, catalase activation (Ghribi et al. 2019). Such response was able to prevent lipid peroxidation on exposed organisms, as lipid peroxidation levels were significantly decreased for exposed organisms (ANOVA: $F=6.744$; d.f.=2, 34; $p = 0.0034$; Fig. 2e). Although other enzymes of the anti-oxidant system may have been involved in oxidative stress response, this was not the case of GST, because no significant differences, between worms exposed to control and contaminated soils, were detected in our study (Fig.

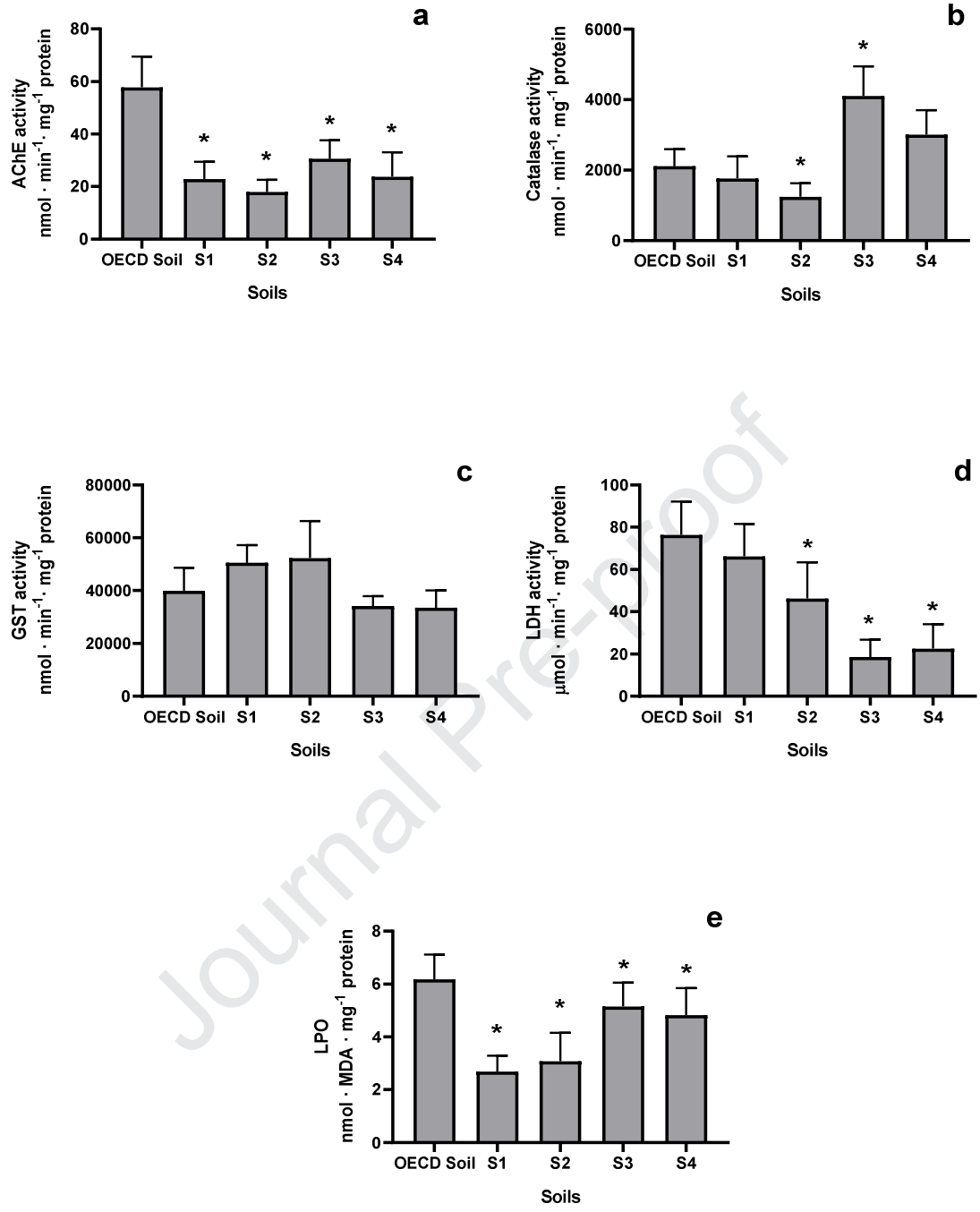
298 2c). According to Grelle and Descamps (1998) or Dhainaut and Scarps (2001), GST activity in *E. fetida*,
299 is not affected by PTEs, but that is not supported by other studies (e.g., Cataldo et al. 2011; Wang and Xie
300 2014; Ojo et al. 2016). Therefore, there is still no consensus in the usefulness of GST as a biomarker of
301 PTEs exposure. Similar results have been previously described by other authors for multi-metallic
302 contamination on marine and terrestrial invertebrates (Rodríguez-Ariza et al. 1992, Labrot et al. 1996,
303 Ramos-Gómez et al. 2008, Babić et al. 2016). In any case, our results, suggest that these variations could
304 be indicative of compensatory antioxidant defence or adaptative mechanisms for long-term exposures and
305 high PTE concentrations, as indicated by Labrot et al. (1996) and Babić et al. (2016).

306 The energy metabolism was affected in earthworms exposed to the most contaminated sample from
307 the quarry area (S2) (ANOVA: F= 5.465; d.f.=2, 15; p = 0.0165) (Fig. 2d), while the LDH activity was
308 also reduced in earthworms exposed to both samples from the lead/zinc mine (ANOVA: F=42.31; d.f.=2,
309 17; $p < 0.0001$) (Fig. 2d). These results suggest an increment in the anaerobic metabolism under PTEs
310 stress (Diamantino et al. 2001, Bessa et al. 2016), that was more evident for samples from the lead/zinc
311 mine (Cd, Pb, and Zn as primary contaminants) than for the quarry area (Co, Cr and Ni as primary
312 contaminants).

313

314 **3.4. DNA damage**

315 The effect of PTEs exposure on DNA was evaluated through comet assay for earthworms exposed to both
316 study areas (Fig. 3). An increase in DNA damage was observed for earthworms exposed to both areas,
317 although with some differences. While no significant differences were observed between worms exposed
318 to samples from the quarry area and control soils (ANOVA, F: 0.747; d.f.: 2,6; p = 0.5129) (Fig. 3),
319 significant differences were detected between earthworms exposed to lead/zinc mine samples and those
320 exposed to the control (ANOVA, F: 5.277; d.f.: 2,6; p = 0.0476) (Fig. 3).



321
322 **Fig. 2**. Mean activity of acetylcholinesterase (a), catalase (b), glutathione S-transferase (c), lactate
323 dehydrogenase (d) and lipid peroxidation (e), in *E. fetida* following 28 days exposure to control (OECD
324 soil), quarry (S1 and S2) and mine soils (S3 and S4). The error bars represent the standard deviation.
325 Asterisks indicate a significant differences to the control ($p < 0.05$).

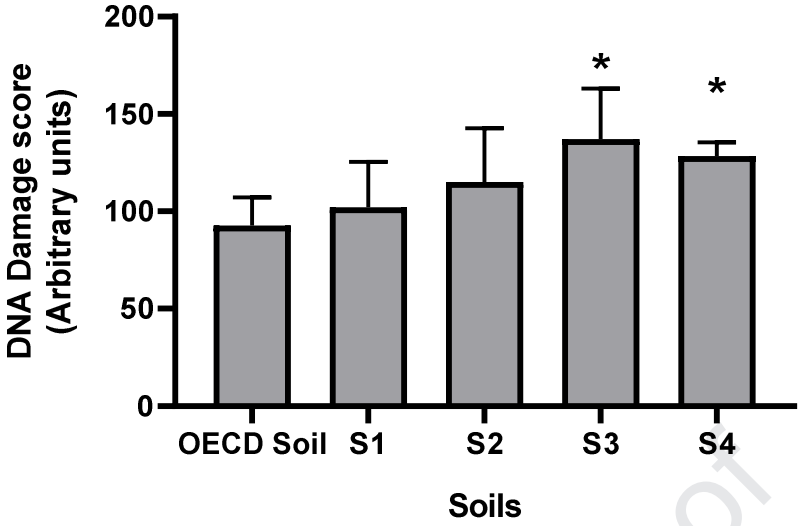
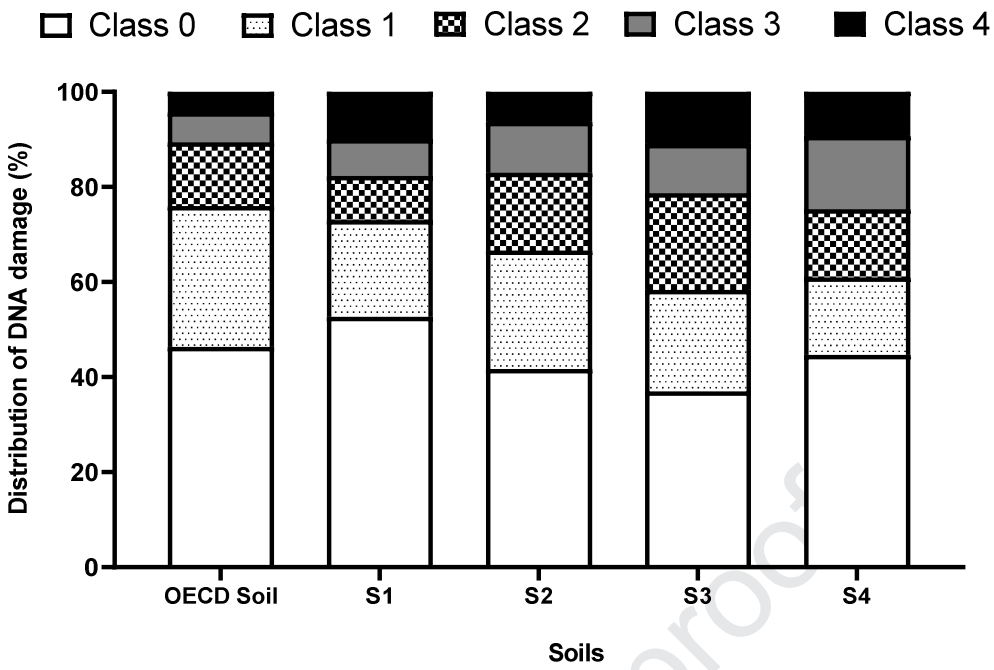


Fig. 3. Mean DNA damage score values (arbitrary units), in *E. fetida* following 28 days exposure to control (OECD soil), quarry (S1 and S2) and mine soils (S3 and S4).. The error bars represent the standard deviation. Asterisks above the bar indicate a significant difference between the samples and the control ($p < 0.05$).

Earthworms exposed to samples from the lead/zinc mine (Cd, Pb and Zn as contaminants) showed a higher frequency of cells with more considerable DNA damage (classes 2, 3 and 4) than organisms exposed to samples from the quarry area (Co, Cr and Ni) (Fig. 4). The differences observed between the soils analysed, may be explained by the differences of soil properties and available contents between both areas, as the bioavailable fraction of PTEs was higher for the lead/zinc mine area (up to 20%, although Pb showed up to 43% of Pb for soil S3) than for the quarry area (around a 12-17%, although Co showed up to 23% for S1).



338 **Fig. 4.** Percentage of coelomocytes with various levels of DNA damage in *Eisenia fetida* after exposure
339 to control (OECD soil), quarry (S1 and S2) and mine soils (S3 and S4)..

340 The higher concentration of PTEs observed in earthworms exposed to S3 and S4 may have increased
341 oxidative stress, which in turn may have increased DNA damage (Reinecke and Reinecke 2004; Taze et
342 al. 2016; Wu et al. 2016), however other factors may also be involved. According to Bigorgne et al.
343 (2010) in a study performed with OECD soils spiked with Cr and Ni, soil properties can also have
344 significant impact for the occurrence of genotoxic effects in *E. fetida*. The interaction between soil
345 components and PTEs, depending on their nature and speciation, may change metal availability and their
346 genotoxicity, as some metal species interact with DNA more efficiently than others, conferring them a
347 higher genotoxic potential (Reinecke and Reinecke 2004; Manerikar et al. 2008; Bonnard et al. 2010). In
348 this case, for lead/zinc mine (Fig. 4), DNA damage results may be related to Cd toxicity, rather than Pb or
349 Zn, as indicated by Li et al. (2009), Muangphra and Gooneratne (2011) or Wu et al. (2012). These authors
350 reported, for soils contaminated by Cd and Pb, that DNA damage was more severe under Cd exposure
351 than Pb for earthworms (*E. fetida* and *Pheretima peguana)*, under monometallic and combined exposure
352 of these metals. Wu et al. (2012) indicated that the combination of Cd and Pb can give antagonist results,
353 due to the competition of both elements by the same receptors at the biomembrane. Voua Otomo et al.
354 (2014) described a similar situation with *E. andrei* exposed to an artificial soil spiked with Cd, Zn and

Cd/Zn, where Cd was more genotoxic than Zn, and that antagonist interactions were also indicated for the metal mixture.

Regarding the response observed for oxidative stress biomarkers and the DNA damage observed, it was expected that the inhibition of CAT and LDH activities recorded in earthworms exposed to S2 from the quarry area (Fig. 2b, 2d), would increase DNA vulnerability to oxidative damage. However, that was not observed in this study. Also, the increment in CAT activity observed for S3 from lead/zinc mine was expected to contribute to the protection of DNA against oxidative stress in earthworms exposed to this mine soil. However, significant DNA damage was detected in the organisms exposed to S3 soil. These results suggest that alterations in CAT activity did not play a significant role in the protection of DNA.

The lack of extensive DNA damage, in the organisms exposed to quarry soils (Fig. 4), despite the known genotoxicity of Cr and Ni (Bigorgne et al. 2010), may be explained by their low availability and also the presence of metals species that may not interact with DNA so efficiently.

4. Conclusions

In the previous works, sequential chemical extractions for both areas showed that the proportion of soil PTEs levels associated with exchangeable and organic matter fractions were very low, pointing for the low bioavailability of PTEs. However, in this study a mild salt extraction showed a clear difference between the quarry and mine soils, as the latter showed a higher chemical availability of Cd, Pb and Zn in parallel with higher contents of these metals in the body of earthworms. This was also coincident with the observed neurotoxic and oxidative stress effects, as well as with the detection of significant DNA damage in earthworms exposed to mine soils. The opposite was recorded for the quarry soils, as the low chemical availability was coincident with no oxidative stress and no DNA damages. Only neurotoxic effects were recorded in earthworms exposed to the quarry soils. BAF values never indicated a significant PTEs bioaccumulation, given the high concentration of these elements in the soils. However, this study, demonstrates that depending on the method, chemical availability may give a wrong perception of the risks posed by contaminated soils. Therefore, studies for risk assessment of abandoned mining areas should be performed using an integrated approach that includes chemical and biological analyses, to obtain a realistic perspective on the toxicity posed to exposed organisms.

Complex contaminated environments such as abandoned quarry and mining areas, with a mixture of contaminants, chemical transformations, inherent environmental factors, and the potential for contaminant

interactions, can cause a myriad of effects on exposed organisms. Multibiomarker assessments should be carried out to improve the knowledge and reduce uncertainties on complex environments such as those that involve metal mixtures.

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Conflict of Interest Statement

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Research involving human participants and/or animals

This article does not contain any studies with human participants or vertebrate animals performed by any of the authors.

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Highlights:

- Sequential chemical extractions may underestimate the hazard of soils contaminated with PTEs.

- A multibiomarker approach provides a better evaluation of PTEs bioavailability in complex soils.

- BAF values may provide misleading conclusions about soils hazardous to earthworms.

Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: