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

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19 **Abstract**

20 Abandoned mining and quarry areas are sources of potentially toxic elements (PTEs), through lixiviates
21 or transfer processes of bioavailable fractions from mining wastes and tailings. In this study, earthworms
22 (*Eisenia fetida* Savigny, 1826) were exposed for 28 days to two mining soils from a lead/zinc mine and
23 two quarry soils from an old serpentine quarry. Despite their pseudo total metal contents, a previous
24 characterization of these soils pointed out for a low chemical availability of PTEs. Therefore, a
25 multibiomarker approach was used and the response of *E. fetida* to soils was assessed through the analysis
26 of neurotoxic, oxidative stress, energy metabolism and DNA damage biomarkers (acetylcholinesterase,
27 catalase, glutathione-s-transferase, lactate dehydrogenase, lipid peroxidation and DNA strand breaks).
28 Metal bioaccumulation was also assessed to evaluate bioavailability and organism's exposure. Results
29 showed that high contents of PTEs were recorded in the whole body of earthworms exposed to lead/zinc
30 mine. However, the bioaccumulation factors for worms exposed to soils from both sampling sites were <
31 1 due to the high PTEs contents in soils. Earthworms exposed to both types of soils displayed neurotoxic
32 and energy metabolism effects. However, significant levels of oxidative stress and DNA damage were
33 recorded only for earthworms exposed to lead/zinc mine soils. This study demonstrated that despite the
34 low availability of PTEs showed by previous sequential chemical extractions, the results obtained from
35 the direct toxicity assessment performed in this study, highlight the importance of a multibiomarker
36 approach using soil organisms to provide a better evaluation of soils pollution.

37

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

39

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

43 1. Introduction

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

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

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

73 After exposure, the bioaccumulation of PTEs was determined through the quantification of earthworm's
74 body burdens. PTE's toxicity was determined through the evaluation of changes in the activity of
75 several biomarkers of neurotoxicity [acetylcholinesterase (AChE)], oxidative stress [catalase (CAT)],
76 biotransformation and oxidative stress [glutathione-S-transferases (GST)], energy metabolism [lactate
77 dehydrogenase (LDH)], and lipid peroxidation [thiobarbituric acid reactive substances-TBARS (LPO)].
78 The analysis of DNA damages also was measured by the alkaline comet assay. These biomarkers are
79 sensitive, time- and cost-effective and have been used in a wide range of scenarios, species and toxicity
80 assessment approaches (e.g. Cataldo et al. 2011; Colacevich et al. 2011; Lourenço et al. 2011; Bessa et al.
81 2016; Boughattas et al. 2016; Correia et al. 2017; Rodríguez-Seijo et al. 2018).

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

87

88 **2. Materials and methods**

89 **2.1. Study sites and soil properties**

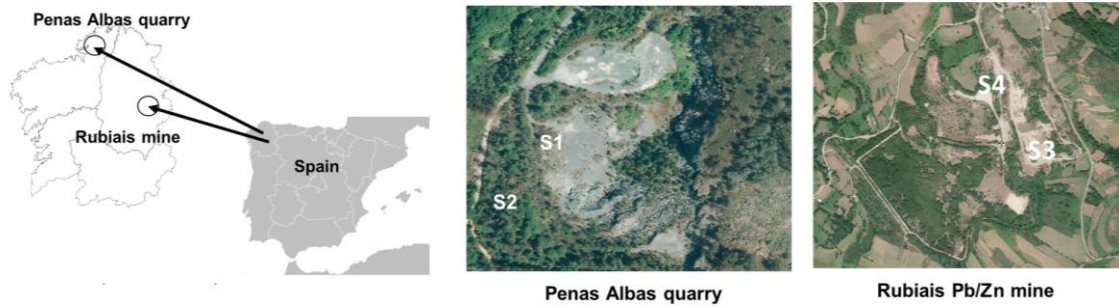
90 This study was carried out with four contaminated soil samples from Galicia (NW Spain): i) two soils
91 from an old serpentine quarry (Penas Albas, Moeche, NW Spain) (Soils S1 and S2), and ii) two soils from
92 an abandoned lead/zinc mine (Rubiais mine, NW Spain) (Soils S3 and S4) (Fig. 1). The selected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

99

100 **2.2. Determination of pseudo total and available PTEs content in soil samples**

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

103 conditions: 9 bar, 190°C and 45 min) (Lago-Vila et al. 2017). A single CaCl₂ extraction was performed to
104 determine the available content of PTEs in the studied soils (0.01 M CaCl₂ acidified with HCl 0.1 M, 1:10
105 w/v soil to extractant ratio, 2 h shaking) (Houba et al. 2000) (Table 1). In both cases, the concentration of
106 PTEs in the extracts was determined by ICP-OES (Perkin Elmer Optima 4300 DV) at CACTI-
107 Universidade de Vigo (Vigo, Spain).



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

110 2.3. Earthworms analysis

111 2.3.1. Experimental procedure

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

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

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

126

127 *2.3.2. Potentially Toxic Elements exposure and accumulation by earthworms*

128 Earthworms were thawed at room temperature and dried at 60°C until constant weight. Hence, a pool of
129 two worms from each replicate (4 replicates per soil tested) was digested with a mixture of 1 ml H₂O₂
130 (30%) and 3 ml (HNO₃) (70%) (Ultra-pure reagents) (Rodríguez-Seijo et al. 2017). The solution was
131 filtered and diluted to 50 ml with Milli-Q water. Sample blanks were obtained following the same
132 procedure but without the biological sample. All samples were analysed using an ICP-OES. The Cd, Co,
133 Cr, Ni, Pb and Zn concentrations were expressed as mg kg⁻¹ dry weight.

134 The bioaccumulation factor (BAF) of studied PTEs was calculated according to $BAF = C_b/C_s$, where
135 C_b is the concentration of a given element in earthworms, while C_s is the concentration of the element in
136 the soil sample (OECD, 2010).

137

138 *2.3.3. Neurotoxicity and oxidative stress biomarkers*

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

147 Three earthworms from each replicate, randomly selected and previously depurated (24 h), were
148 pooled and homogenised in an ice-cold phosphate buffer (50 mM, pH = 7.0 with 0.1% Triton X-100)
149 using a tissue homogeniser (T 10 basic ULTRA-TURRAX®). The homogenates were then centrifuged
150 (3000rpm for 15 min, at 4 °C). Finally, the obtained supernatant was separated and used for biochemical
151 analyses. The same procedure was followed for all the experimental replicates. All biomarkers were
152 measured in triplicate for each replicate using a spectrophotometer equipped with a microplate reader
153 (Thermo Scientific™ Multiskan™ GO UV/Vis microplate spectrophotometer). For all assays, the protein
154 concentration of the samples was determined by Bradford's method (Bradford, 1976), adapted to the
155 microplate reader and measured spectrophotometrically at 595 nm in triplicate (Thermo Scientific™
156 Multiskan™ Go UV/Vis microplate spectrophotometer). Results were expressed as nmol min⁻¹ mg⁻¹

157 protein (AChE, CAT and GST), $\mu\text{mol min}^{-1} \text{mg}^{-1}$ protein (LDH) and nmol MDA equivalents by mg^{-1} of
158 protein (LPO).

159

160 *2.3.4. Coelomocytes extrusion and DNA damage quantification*

161 DNA damage quantification was performed in earthworm coelomocytes (five worms from each
162 replicate), obtained following the methodology described by Lourenço et al. (2012) and Correia et al.
163 (2017). Finally, DNA damage was visually scored, through the observation of one-hundred nucleoids,
164 randomly selected, and graded into five classes (García and Mandina 2005; Correia et al. 2017): from 0 to
165 4, being 0 a nucleoid without damage and 4 a nucleoid with almost all the DNA in the tail (most damaged
166 cells). The results were reported as arbitrary units, calculated by multiplying the number of observed
167 comets (0–100) by the comet classification (0–4).

168

169 **2.4. Statistical analyses**

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

178

179 **3. Results and discussion**

180 **3.1. Soil properties and PTEs content in selected soil samples**

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

187 (19.59 cmol₍₊₎ kg⁻¹). The studied soils presented a base saturation between 99.8 and 100%, due to parent
188 rock material, and in the case of quarry soils, they had an imbalance of Ca/Mg (Rodríguez-Seijo et al.
189 2014; Lago-Vila et al. 2015).

190 The studied soils also had high levels of pseudo total PTE concentrations above the generic reference
191 limits for ecosystem protection and industrial uses, established for the Galician region (DOG 2009). In
192 each area, samples have differences between them because they originate from different grades of activity
193 (Table 1). As expected, the levels of available PTEs were lower than the pseudo total contents (< 20% for
194 mine soils and 12 to 17% for quarry soils). The exception was recorded for Pb (up to 46% for S3) and Co
195 (23% for S1), respectively) (Table 1). The PTEs with higher levels were Ni, Pb and Zn (Table 1).

196

197 **3.2. Exposure and accumulation of PTEs by earthworms**

198 At the end of the 28 days of exposure, there was no mortality and weight loss as not statistically
199 significant differences in exposed organisms were recorded when compared to the control (OECD soil)
200 (ANOVA: F: 1.808; *df*: 4, 14; *p* = 0.1834). Significantly, higher levels of PTEs were found in exposed
201 organisms when compared to the control (Table 2). This was probably due to both direct dermal contact
202 with PTEs in interstitial water and the ingestion of soil particles. Soil ingestion may increase PTEs
203 bioavailability, due to pH variability in the different compartments of the gastrointestinal tract of
204 earthworms, potentially increasing mobilization (Peijnenburg and Jager 2003; Becquer et al. 2005;
205 Hobbelen et al. 2006; Sizmur et al. 2009; Lourenço et al. 2011; Boughattas et al. 2016). According to
206 Song et al. (2002), the threshold concentrations in earthworm's tissues that can lead to increased mortality
207 in *E. fetida* are 300, 1300, 1700 and 300 mg kg⁻¹ dw for Cu, Zn, Pb, and Cd, respectively. The
208 concentrations found in this study were lower than those required to induce death, except for Zn levels
209 (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	<i>Bdl</i>	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	<i>Bdl</i>
USDA classification	Sandy Loam	Sand	Sandy Loam	Sand	Sand
Pseudo total content of studied PTEs (mg kg ⁻¹)					
Co	-	<i>109 ± 1</i>	<i>147 ± 1.2</i>	<i>92 ± 3.8</i>	<i>141 ± 1.7</i>
Cr	-	<i>1672 ± 110</i>	<i>2604 ± 38</i>	78 ± 2.1	82 ± 3.5
Ni	-	<i>2039 ± 107</i>	<i>1861 ± 62</i>	36 ± 2.9	29 ± 1.2
Cd	-	<i>Bdl</i>	<i>Bdl</i>	<i>14 ± 0.6</i>	<i>43 ± 0.7</i>
Pb	-	<i>Bdl</i>	<i>Bdl</i>	<i>2137 ± 370</i>	<i>6761 ± 1352</i>
Zn	-	33 ± 3	63 ± 5	<i>12000 ± 559</i>	<i>32000 ± 3570</i>
CaCl ₂ available contents of studied PTEs (mg kg ⁻¹)					
Co	-	26 ± 0.8	25.2 ± 0.19	<i>Bdl</i>	<i>Bdl</i>
Cr	-	4.02 ± 0.05	7.65 ± 0.17	<i>Bdl</i>	<i>Bdl</i>
Ni	-	274 ± 6.7	153 ± 3.2	<i>Bdl</i>	<i>Bdl</i>
Cd	-	<i>Bdl</i>	<i>Bdl</i>	0.35 ± 0.01	0.80 ± 0.01
Pb	-	<i>Bdl</i>	<i>Bdl</i>	987 ± 42	1022 ± 38
Zn	-	<i>Bdl</i>	<i>Bdl</i>	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).

217 **Table 2.** Concentration of Cd, Co, Cr, Ni, Pb and Zn (mg kg⁻¹ dry weight) in *E. fetida* exposed to control
 218 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	<i>Bdl</i>	3.40 ± 0.95 <i>a</i>	3.10 ± 0.74 <i>a</i>	-	-
Cr	<i>Bdl</i>	18.76 ± 4.4 <i>a</i>	15 ± 3.7 <i>a</i>	-	-
Ni	<i>Bdl</i>	43.71 ± 10.6 <i>a</i>	35.7 ± 8.4 <i>a</i>	-	-
Cd	<i>Bdl</i>	-	-	1.63 ± 0.34 <i>a</i>	1.7 ± 0.6 <i>a</i>
Pb	<i>Bdl</i>	-	-	23.5 ± 6.3 <i>b</i>	398.4 ± 108.4 <i>a</i>
Zn	<i>Bdl</i>	-	-	250 ± 66.1 <i>b</i>	1405 ± 368 <i>a</i>

219 Values are expressed as mean ± standard deviation; Bdl: below detection level. “-” not measured.

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

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

230 In fact, and as mentioned above, Arenas-Lago et al. (2014; 2016) showed through sequential
 231 extractions that only a small proportion of soil PTEs was associated with exchangeable and organic
 232 matter fractions for both sampling areas; therefore it was not the organic matter that had the main role in
 233 reducing the chemical availability of PTEs in these soils. However, earthworms can ingest soil particles
 234 and contaminants sorbed to poorly labile fractions (bound to oxide and organic fractions), and their
 235 passage through the gut can change the availability from sorbed to exchangeable PTEs (Becquer et al.
 236 2005; Sizmur et al. 2009; Nannoni et al. 2011; Sizmur et al. 2011a,b) also observed that the ingestion of
 237 PTEs bounded to soil components (mineral or oxidable fractions) was an uptake route more significant
 238 than the dermal uptake of dissolved ions from the soil solution. This could explain why higher levels of
 239 PTEs were found in earthworms exposed to soils with a low levels of bioavailable PTEs. Despite the
 240 exposure, in all cases, the BAFs levels were all below 1, pointing for no bioaccumulation of these
 241 elements in the organisms (Table 3).

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

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

244 Values are expressed as mean ± standard deviation. “-” not measured.

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

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

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

257

258 3.3. The neurotoxic and oxidative stress responses of earthworms exposed to PTEs rich soils

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

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

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

268 1996; Gaitonde et al. 2006; Lionetto et al. 2012; Dongxing et al. 2016). However, stimulatory effects of
269 metals mixtures (Cr, Cu, Ni, Pb, Zn) on AChE activity were also reported (Zheng et al. 2013). In the
270 present study, a significant inhibition of AChE activity compared to the control group (OECD soil) was
271 observed for earthworms exposed to samples from the quarry area (ANOVA: $F=65.60$; $d.f.=2, 26$; $p <$
272 0.0001 ; Fig. 2a) and lead/zinc mine area (ANOVA: $F= 29.6$; $d.f.=2, 26$; $p < 0.0001$; Fig. 2a). Inhibitions
273 of AChE in exposed earthworms were ranged from 47% (S3) to 68% (S2), and inhibitions of AChE
274 activity above 20% has been proposed as indicative of exposure to anticholinesterase agents (Ludke et al.
275 1975; Menéndez-Helman et al. 2015; Fajardo and Ocampo 2018). Different PTEs such as Cd, Co, Cr, Pb
276 and Zn have been proposed as inhibitors of AChE (Elumalai et al. 2002; Frasco et al. 2005; Gaitonde et
277 al. 2006; Dongxing et al. 2016; Hayat et al. 2017; Mkhinini et al. 2019), while Ni has shown
278 contradictory results (Frasco et al. 2005; Hayat et al. 2017). Our results indicate that the exposure to both
279 types of soils displayed neurotoxic effects on earthworms.

280 Catalase is an important component of the antioxidant defence system, an antioxidant enzyme that
281 regulates the amount of H_2O_2 , protecting cells from their toxic effects (Ghribi et al. 2019). In our assay,
282 CAT activity was significantly inhibited on S2 from the quarry area (ANOVA: $F=5.719$; $d.f.=2, 15$; $p =$
283 0.0143 ; Fig. 2b), but their activity was significantly increased in the S3 sample from the lead/zinc mine,
284 (ANOVA: $F=9.789$; $d.f.=2, 12$; $p =0.003$; Fig. 2b).

285 In the earthworms exposed to quarry soils, other enzymes may have been activated to avoid an
286 oxidative stress response (Zhang et al. 2009; 2013, Santana et al. 2018; Yin et al. 2018; Ghribi et al.
287 2019). In fact, despite the inhibition of CAT activity, no significant increase in lipid peroxidation was
288 recorded in these samples, as a reduction on the MDA concentration was observed (ANOVA: $F=54.52$;
289 $d.f.=2, 28$; $p < 0.0001$; Fig. 2e).

290 Earthworms exposed to the lead/zinc mine samples showed a different enzymatic activity profile with
291 an increase in CAT activity, but only significant for S3 (Fig. 2b). These results suggest that earthworms'
292 exposure to high levels of Cd, Pb and Zn may induce an oxidative stress response and, consequently,
293 catalase activation (Ghribi et al. 2019). Such response was able to prevent lipid peroxidation on exposed
294 organisms, as lipid peroxidation levels were significantly decreased for exposed organisms (ANOVA:
295 $F=6.744$; $d.f.=2, 34$; $p = 0.0034$; Fig. 2e). Although other enzymes of the anti-oxidant system may have
296 been involved in oxidative stress response, this was not the case of GST, because no significant
297 differences, between worms exposed to control and contaminated soils, were detected in our study (Fig.

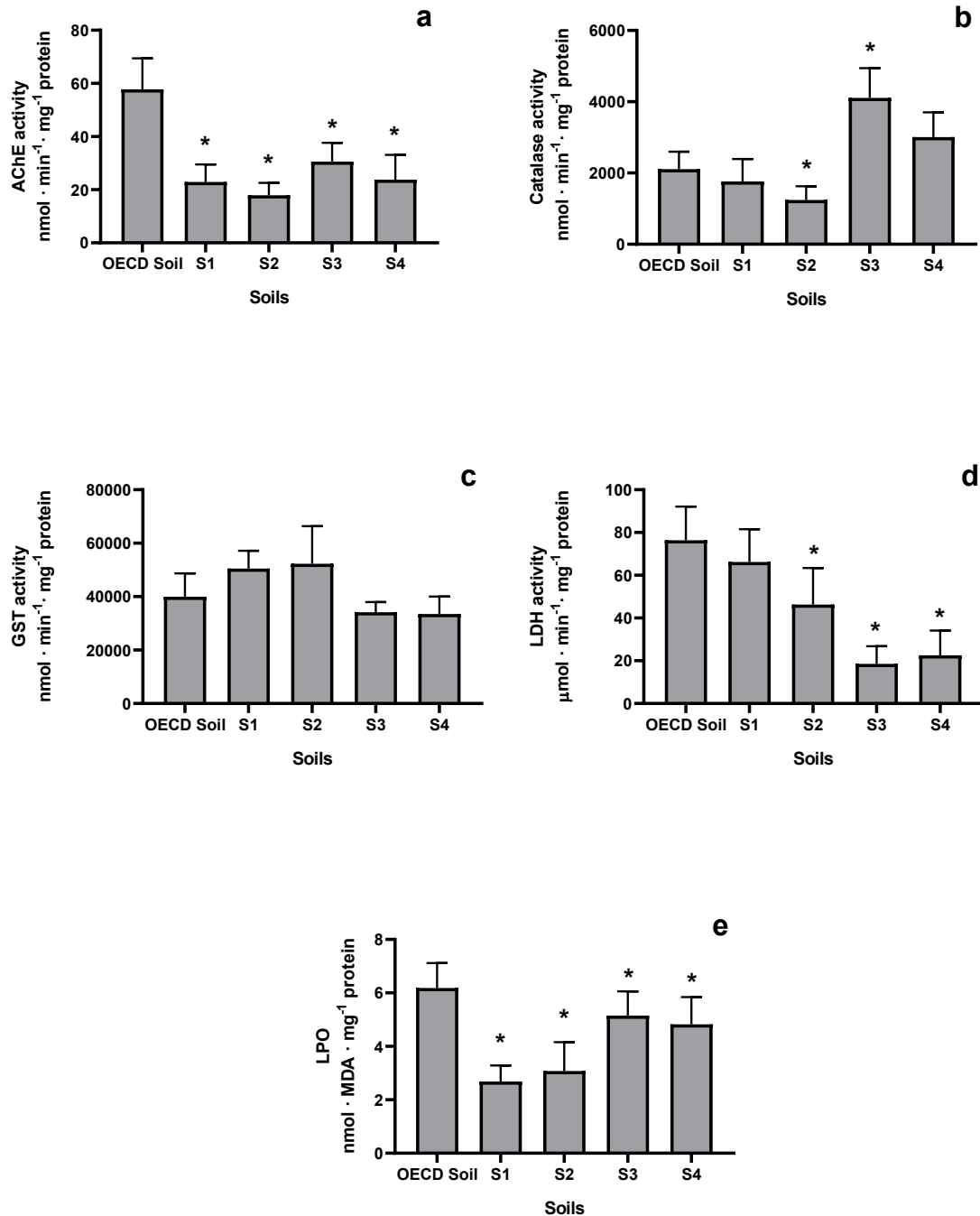
298 2c). According to Grelle and Descamps (1998) or Dhainaut and Scarps (2001), GST activity in *E. fetida*,
299 is not affected by PTEs, but that is not supported by other studies (e.g., Cataldo et al. 2011; Wang and Xie
300 2014; Ojo et al. 2016). Therefore, there is still no consensus in the usefulness 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).

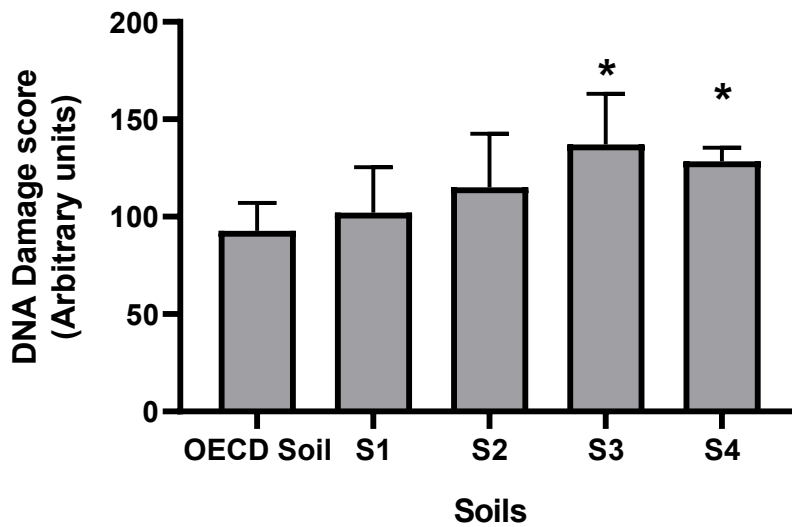
313

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).



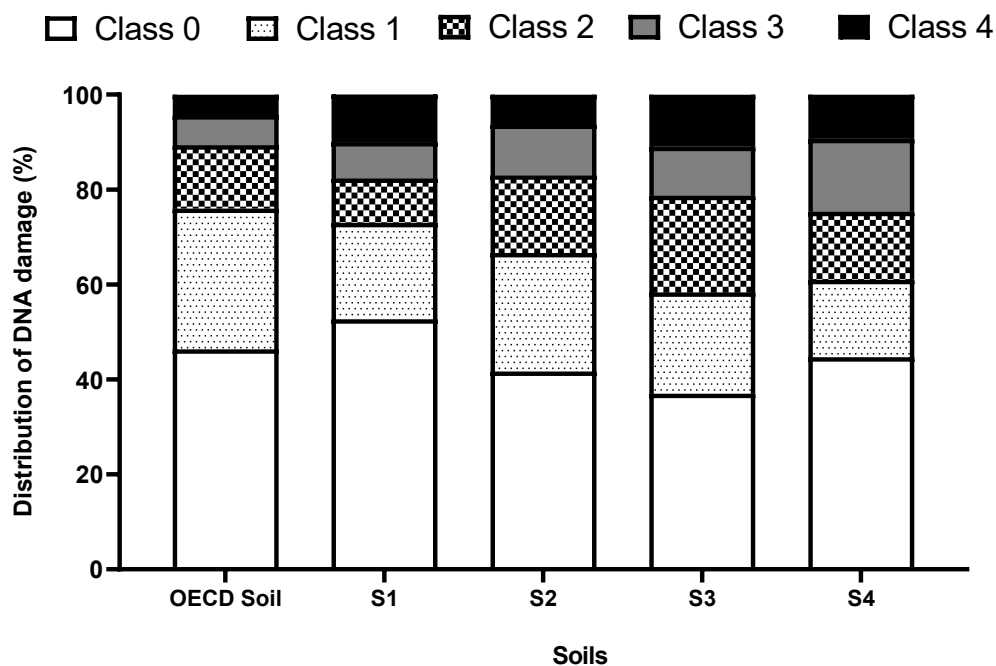
321
 322 **Fig. 2.** Mean activity of acetylcholinesterase (a), catalase (b), glutathione S-transferase (c), lactate
 323 dehydrogenase (d) and lipid peroxidation (e), in *E. fetida* following 28 days exposure to control (OECD
 324 soil), quarry (S1 and S2) and mine soils (S3 and S4). The error bars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



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

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

337



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

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

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

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

367

368 **4. Conclusions**

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

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

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

388

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402

403 **Conflict of Interest Statement**

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

406

407 **Research involving human participants and/or animals**

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

410

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