Journal of Hazardous Materials 194 (2011) 135–143

Contents lists available at ScienceDirect

Journal of Hazardous Materials

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

Oxidative stress in earthworms short- and long-term exposed to highly Hg-contaminated soils

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ARTICLE INFO

Article history:
Received 4 March 2011
Received in revised form 6 July 2011
Accepted 22 July 2011
Available online 5 August 2011

Keywords:
Mercury
Integrated biomarker responses
Lumbricus terrestris
Oxidative stress
Glutathione redox

ABSTRACT

Exposure to mercury is often assessed by the measurement of molecular and biochemical antioxidant defences against an excessive production of reactive oxygen species. Here we examined some selected biomarkers of oxidative stress in the earthworm *Lumbricus terrestris* short- (2 d) and long-term (44 d) exposed to Hg-contaminated soils (up to 1287 mg/kg dry wt). This level of Hg exposure did not cause earthworm mortality, however it yielded organisms to a situation of oxidative stress which was evidenced by the time-dependent responses of biomarkers. The reduced to oxidized glutathione ratio was a sensitive and early biomarker of Hg exposure, although the glutathione reductase activity back returned their normal physiological concentrations. Metallothioneins and total glutathione seemed to have a significant role in reducing Hg-induced oxidative stress when exposure to Hg prolonged up to 44 d. We combined biomarker responses into an integrate biomarker index which positively correlated with the Hg concentrations measured in the postmitochondrial fraction of the earthworm muscle, and with the available Hg fraction in soil. Current results suggest that glutathione redox cycle can be a complementary tool in the exposure and effect assessment of Hg-polluted soils.

1. Introduction

Mercury is a highly toxic metal of global concern because of its multiple natural and anthropogenic sources, the high volatility of its elemental metallic form and the high atmospheric persistence (~1 yr) of gaseous Hg^0 [1]. Toxicologically, Hg displays a high bioaccumulation factor [2,3], and it acts as a pro-oxidant through the generation of hydrogen peroxide [4]. In general, organisms exposed to Hg show a disturbance of the glutathione homeostasis [5–7]. This thiol tripeptide is an efficient scavenger against reactive oxygen species [8]. The ratio of reduced to oxidized glutathione as well as the enzyme activities involved in this redox balance are commonly used as exposure biomarkers of oxidative stress [9–11].

Biomarkers (sub-individual measurable responses to contaminant exposure) are often used in toxicity testing of environmental contaminants as indirect measurements of bioavailability [12]. Moreover, they are key elements in the understanding of the toxic mechanism underlying observed effects at individual level [13]. Biomarkers are also useful in the distinction of acute toxic effects from long-term effects [14]. In earthworms, biomarkers have been primarily used in organisms experimentally exposed to, or inhabiting in, metal-polluted environments. However, effects of Hg exposure on earthworm biomarkers have been little documented [7,15,16], despite bioaccumulation of this metal by earthworms has been well described. For example, Hg bioaccumulation markedly varied with the ecophysiology of earthworms inhabiting forest soils [17]. Bioaccumulation factors of total Hg and monomethylmercury were higher in *Eisenia fetida* exposed to low Hg-contaminated soils compared to individuals exposed to higher Hg concentrations [3]. Likewise, methylation of inorganic Hg seems to occur in the gastrointestinal tract of *E. fetida* by the action of symbiotic sulfate reducing bacteria [18], which can facilitate the intestinal uptake of Hg. Taken together, these studies suggest that bioaccumulation is a robust tool in the assessment of Hg bioavailability in polluted soils [19]. However, sublethal effects have been scarcely investigated in Hg-exposed earthworms.

Our study is a contribution to the understanding of metal-induced oxidative stress in earthworms exposed to hazardous materials such as Hg-contaminated soils. We used soils collected from the mining district of Almadén (Ciudad Real, Spain); a region considered the largest natural geochemical anomaly of Hg on Earth [20,21]. The aim of this study was to examine whether biomarkers of oxidative stress in earthworms can be a complementary methodology in the assessment of soils historically contaminated by Hg. To test this hypothesis, we measured multiple biochemical and molecular biomarkers directly related to the Hg-induced

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doi:10.1016/j.jhazmat.2011.07.091

oxidative stress in the earthworm *Lumbricus terrestris* acutely (2 d) and chronically (44 d) exposed to soils. Mercury concentration was measured in the soils following a standardized sequential extraction procedure to assess multiple pathways of Hg uptake, and in the postmitochondrial fraction (toxic Hg fraction) of earthworm muscle. An integrated analysis of biomarkers revealed that these sub-individual responses are suitable sublethal endpoints to be included in a risk assessment scheme for monitoring Hg-contaminated soils.

2. Materials and methods

2.1. Chemicals

Reagents used in biomarker assays were obtained from Sigma–Aldrich (Madrid, Spain). The *ortho*-phthalaldehyde stock solution (1 mg/mL) was made in HPLC-grade absolute methanol. Monobromobimane reagent (1 mg/mL) was prepared in HPLC-grade acetonitrile and keep in dark until its use for metallothionein derivatization. Stock solutions of reduced and oxidized glutathione were prepared in 0.1 M Na-phosphate buffer containing 5 mM EDTA (pH 8.0). Stock solution (3 mg/mL) of rabbit liver metallothionein-I (Lot 80K7012, Sigma–Aldrich) was made in 0.1 M Tris–HCl (pH 9.5).

2.2. Earthworms and test soils

Adult earthworms (*L. terrestris*) were obtained from a local commercial supplier in Toledo (Spain) and kept in acclimatized chambers (15 °C and continuous dark) for one month. Before running exposure tests, the animals were left on wet filter paper in Petri dishes (15 mm diameter) for 24 h to reduce soil content into their gut, and the body weight was recorded (3.79 g ± 0.83 fresh weight, mean ± SD of 60 individuals). Non-contaminated soil was collected in Montes de Toledo (Toledo, Spain), and it was the substrate used in the acclimatization period of earthworms as well as the control soil in the exposure trials. Contaminated soils were collected from four sites in the mining district of Almadén: the Almadén mine, the Almadenejos village, the Entredicho mine and the Las Cuevas mine (Fig. 1). Soil samples (<5 mm, particle size) were kept at 4–5 °C until used in bioassays (within one month).

2.3. Experimental setup

Soil toxicity testing was performed in plastic containers (14.5 cm × 14 cm × 12 cm) with perforated lids. The test containers were maintained in a chamber at 15 °C and continuous dark. Each test soil (control and contaminated) was divided into three replicates (800 g dry weight) and four earthworms were released per replicate. Soil moisture was kept at 36% (w/v) throughout the experiment. Two earthworms were removed from each replicate at $t = 2$ and 44 days following soil exposure ($N = 6$ individuals per treatment and sampling time). We selected this sampling frequency according to the most common period of metal exposure in bioaccumulation studies with earthworms [22]. Sampled earthworms were placed in Petri dishes for voiding their gut (24 h at 15 °C and dark) and subsequently they were weighted. Animals were cooled on ice to facilitate dissection process. We took a sample of the body wall muscle (1–2.5 g wet weight) which was washed in distilled water to remove soil particles. Muscle samples were immersed in liquid nitrogen and stored frozen at −80 °C until analysis.

Samples were homogenized (1:10, w/v) in ice-cold 20 mM Tris–HCl buffer (pH 7.6) containing 0.1 mM phenylmethylsulfonyl fluoride, using firstly an Ultra-Turrax T25 (2500 rpm) and subsequently a glass-Teflon Potter-Elvehjem homogenizer. The homogenates were centrifuged at 10,000 × *g* at 4 °C for 30 min, and an aliquot of the supernatant was used for total Hg determination. The supernatant of this centrifugation represents the biologically active fraction of metals (cytosolic and microsomal fractions), whereas the pellet would contain the rich-metal granules, cellular debris, and fragments of tissues [23]. The rest of 10,000 × *g* supernatant was ultracentrifuged (100,000 × *g* at 4 °C for 1 h) and the supernatant or cytosolic fraction (3.78 ± 1.02 mg protein/mL, mean ± SD) was used for antioxidant enzyme assays as well as for determining the concentrations of reduced glutathione, oxidized glutathione and metallothioneins. The pellet or microsomal fraction (1.53 ± 0.33 mg of total protein mL^{-1}, mean ± SD) was resuspended in 0.15% (w/v) KCl and used for the measurement of thiobarbituric acid reactive species as an indicator of lipid peroxidation (oxidative damage).

2.4. Antioxidant enzymes

Glutathione S-transferase (GST; EC 2.5.1.18) activity was measured at 340 nm following the method by Habig et al. [24] in a reaction mixture containing 0.1 M Na-phosphate buffer (pH 6.5), 2 mM CDNB, 5 mM reduced glutathione and the sample (10 μL). A millimolar extinction coefficient of 9.6 mM^{-1} cm^{-1} was used for activity expression. Glutathione reductase (GR; EC 1.6.4.2) activity was determined according to the method described in Ramos-Martínez et al. [25], which measures the oxidation of NADPH at 340 nm in the presence of oxidized glutathione and 0.1 M Na-phosphate buffer (pH 7.0). The measurement of cumene hydroperoxide-dependent glutathione peroxidase (CHP-GPx; EC 1.11.1.9) activity followed the method by Lawrence and Burk [26] by which the oxidation of NAPDH is read at 340 nm in an incubation medium containing 100 mM Na-phosphate (pH 7.5), 1 mM EDTA, 1 mM dithiothreitol, 0.24 mM NADPH, 0.8 mM cumene hydroperoxide and the sample (100 μL). A millimolar extinction coefficient for the NADPH oxidation of −6.22 mM^{-1} cm^{-1} was used for calculation of both GR and CHP-GPx activities. Enzyme activity was expressed in international milliunits (mU) per mg of protein (1 mU was defined as the amount of enzyme that consumes 1 nmol of substrate, or generates 1 nmol of product, per minute). All kinetic assays were carried out at 20–22 °C and blanks (reaction mixture without the sample) were periodically checked for nonenzymatic reaction and enzyme activity was corrected. Total protein content was determined by the Bradford method [27], using bovine serum albumin as the standard.

2.5. Lipid peroxidation

Lipid peroxidation was estimated spectrophotometrically by the formation of malondialdehyde as a lipid peroxidation product according to the method by Ohkawa et al. [28]. Muscle samples (microsomal fraction) was heated (95 °C for 1 h) in a reaction medium containing 8.1% sodium dodecyl sulfate, 20% acetic acid (pH 3.5 with NaOH), and 0.8% aqueous solution of thiobarbituric acid. Afterwards, a mixture n-butanol:pyridine (15:1, v/v) was added to the reaction mixture, shaken vigorously and centrifuged (15,000 × *g* for 20 min at 4 °C) to get a coloured upper organic layer, which was read at 532 nm. Lipid peroxidation was expressed as nmol malondialdehyde/mg protein using a calibration curve made with 1,1,3,3-tetramethoxypropane.

2.6. Glutathione determination

Concentrations of reduced and oxidized glutathione were determined according to the fluorimetric method by Hissin and Russell [29]. Muscle samples were deproteinized by addition of 10% cold trichloroacetic acid and afterward centrifuged (10,000 × *g* for 10 min at 4 °C). Concentration of reduced glutathione was determined by incubation of the sample with *ortho*-phthalaldehyde

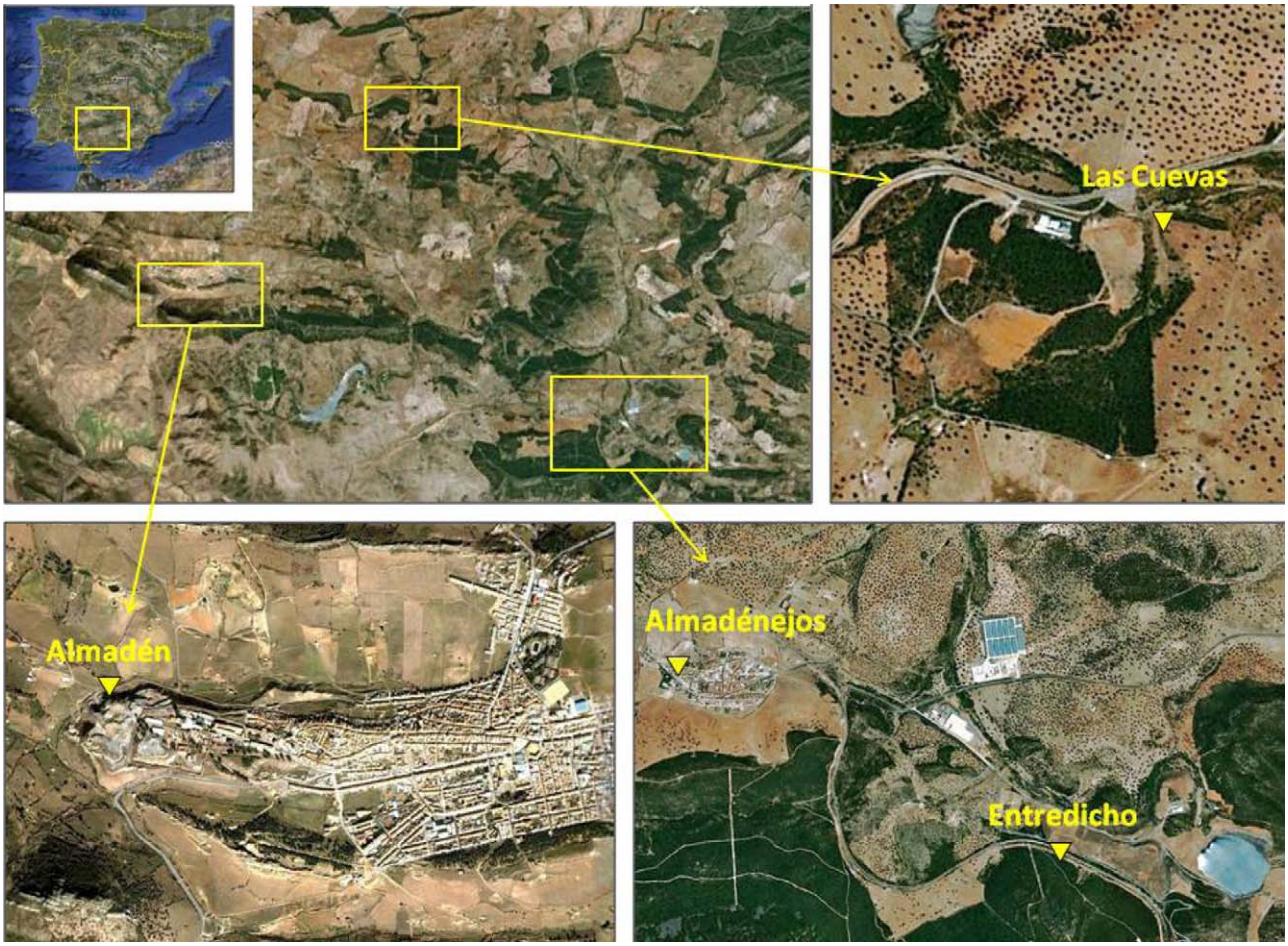

Fig. 1. Location of the sampling sites in the mining district of Almadén (Southern Spain).

(1 mg/mL) in a basic medium (0.1 M Na-phosphate–5 mM EDTA buffer, pH 8.0), whereas determination of the oxidized form of glutathione required a previous step with *N*-ethylmaleimide (40 mM) to prevent glutathione oxidation and 1 N NaOH was used instead of Na-phosphate buffer. The reaction mixture was incubated at 20–22 °C for 15 min and the fluorescence was measured at λ_{EM} = 420 nm and λ_{EX} = 350 nm. Quantitation was performed using external standards of reduced (3.27–327 nmol/mL) and oxidized glutathione (1.6–82 nmol/mL).

2.7. Metallothioneins

Metallothioneins were determined following the chromatographic method by Alhama et al. [30]. Muscle samples (cytosolic fraction) were incubated at 70 °C for 20 min for reduction and denaturation in the presence of 0.1 M Tris–HCl buffer (pH 9.5) containing 2 mM dithiothreitol, 2 mM EDTA and 1% (w/v) SDS (99% purity). Metallothioneins were derivatized with monobromobimane (6 mM, final concentration), and the reaction mixture was incubated at 20–22 °C and dark for 15 min. A 10-μL aliquot was injected in an Agilent 1200 Series HPLC system which included a manual injector (7725i injection valve, 20-μL loop), a vacuum degasser, a quaternary solvent pump and a fluorescence detector. Metallothioneins were separated in a LC-18 column (0.46 cm × 25 cm, 5 μm particle size) under the following solvent program: 70% H_2O containing 0.1% trifluoroacetic acid (solvent A) and 30% acetonitrile containing 0.1% trifluoroacetic (solvent B) for 10 min, then 30% A:70% B for 1 min. After keeping these conditions for 10 min, the initial conditions were re-established within 1 min. Flow rate was 1 mL/min and the fluorescence detector was set up at λ_{EM} = 470 nm and λ_{EX} = 382 nm (gain = 10).

2.8. Mercury determination

Mercury analysis was performed in the muscle 10,000-g supernatant and in soil samples (<63 μm). Concentrations of total Hg in earthworm muscle and soil as well as in the multiple soil fractions obtained from the sequential extraction procedure were determined using an atomic absorption spectrophotometer specifically designed for Hg determination (Advanced Mercury Analyzer – AMA 254 – LECO Company). Both the solid and liquid samples were measured without the need of sample chemical pre-treatments and with a detection limit of 0.01 ng of Hg. When the soil samples showed a high Hg content, above the range limit detection (>600 ng), they were pre-processed by an acidic digestion following EPA 3052 Method (US EPA, 1996). The process was performed in a microwave ETHOS 1 (Milestone SRL). Samples (0.5 g) were digested in 15 mL of an acid mixture composed by concentrated HNO_3:HCl:HF (1:1:1, v/v). The temperature program was set up as follows: 170 ± 5 °C in 15 min, then increased up to 180 °C in 7 min and kept at this temperature for 9.5 min for the completion of specific reactions. After cooling, the vessel contents were allowed to settle and then filtered, diluted to a desired volume with deionized water for Hg determination.

We used the sequential extraction procedure developed by Sánchez et al. [31], in which Hg was extracted in seven fractions: water soluble, exchangeable, carbonates, easily reducible, soluble in 6 M HCl, oxidizable and a final residue. Accuracy of the sequential

extraction procedure was checked by the concomitant analysis of Hg in these multiple extractions with the certified reference material CRM 051. The average recovery percentage was about 90%.

Analytical determinations in each sample were replicated 3 times, and the accuracy of analytical procedures was checked by routine determination of Hg in certified reference materials. These reference materials were BCR-CRM 150 (spiked skim mild powder, 9.4 ± 1.7 μg/kg, mean and standard deviation of the certified Hg concentration), BCR-CRM 151 (spiked skim mild powder, 0.101 ± 0.010 mg/kg), CRM 62 (olive leaves, 0.280 ± 0.020 mg/kg) SRM 2709 (San Joaquin agricultural soil, 1.40 ± 0.08 mg/kg), DOLT-2 (dogfish liver, 2.14 ± 0.28 mg/kg) and CRM 051 (soil from USA contaminated area, 29.90 ± 5.96 mg/kg). The mean (±SD) Hg concentrations determined in our analytical procedures ($N = 10$ independent analysis) were 10.8 ± 0.3 μg/kg (BCR-CRM 150); 0.094 ± 0.002 mg/kg (BCR-CRM 151); 0.292 ± 0.005 mg/kg (CRM 62); 1.38 ± 0.08 mg/kg (SRM 2709); 1.98 ± 0.26 mg/kg (DOLT-2) and 24.1 ± 1.52 mg/kg (CRM 051). At a 95% confidence level, no significant differences were detected between the certified and the experimental values. We considered therefore our analytical method as accurate for total Hg determination.

2.9. Data analysis

Significant differences in pH, electrical conductivity and total organic carbon among the five soils were tested using the non-parametric Kruskal–Wallis ANOVA test, while the impact of earthworm activity on these physicochemical parameters was tested using the Mann–Whitney *U* test. Biomarker data were logarithmically transformed to normalize data and stabilize variances. We found significant differences in the body mass of earthworms exposed to control and the Almadén mine soils over the exposure time. Therefore, biomarker responses were compared using an all-effects generalized linear model with soil type and duration of exposure as the categorical independent variables and the body mass as a continuous independent cofactor. ANOVA tests were followed by a post hoc LSD Fisher test for pairwise comparisons.

We also analyzed the global biomarker response for each soil type and duration of exposure by combining the individual biomarker responses into an index called "integrated biomarker response" (IBR), which is accepted as a measurement of "stress" [32–36]. To calculate the IBR values, data were firstly standardized as described in Beliaeff and Burgeot [32]. The scores (s) or standardized data were used for calculating the IBR values according to the equation: $IBR = [(B1 \times B2)/2] + [(B2 \times B3)/2] + \cdots + [(Bn - 1 \times Bn)/2] + [(Bn \times B1)/2]$, where B1, B2,. . .,B$n$ are the biomarker's scores. The IBR values were finally divided by the number of biomarkers ($N = 7$) involved in the calculation as suggested by Broeg and Lehtonen [37].

3. Results

3.1. Mercury in Almadén soils

Physicochemical data and soil Hg concentrations are summarized in Table 1. Soils showed a wide variation in pH (from 5.5 to 7.7), electrical conductivity (from 60 to 157 μS/cm) and total organic carbon (from 1.9 to 5.6%). We measured these physicochemical parameters once finished the exposure trial, and the earthworm activity did not have a significant impact on these physicochemical parameters (Mann–Whitney *U* test, $p < 0.05$).

Total Hg concentrations were high in soils collected from the mining district compared to control soil (Table 1). The mean total Hg concentration measured at Las Cuevas mine (1287 ± 70 μg/g dry wt) was one order of magnitude higher than those detected

Table 1
Physicochemical properties and Hg concentrations (mean ± standard deviation) in soils from the mining district of Almadén.

Soil	pH	EC (μS/cm)	TOC (%)	Hg[a] concentration (μg/g dry weight)						Total Hg
				Water soluble	Exchangeable	Carbonates	6 M HCl-soluble	Oxidizable	Final residue	
Control soil	7.0 ± 0.1	462 ± 180	9.34 ± 1.56	–[b]	–	–	–	–		0.11 ± 0.013
Almadén mine	7.0 ± 0.1	113 ± 31	1.90 ± 0.08	0.042 ± 0.038 (0.034)	0.18 ± 0.095 (0.15)	2.58 ± 0.24 (2.1)	27.3 ± 7.1 (22)	27.8 ± 1.6 (22)	66.1 ± 9.7 (53)	124 ± 13
Almadenejos village	7.7 ± 0.3	157 ± 17	1.95 ± 0.47	0.039 ± 0.010 (0.034)	0.045 ± 0.055 (0.04)	5.00 ± 2.63 (4.3)	49.5 ± 13.1 (49)	26.0 ± 3.7 (22)	35.7 ± 14.1 (31)	116 ± 22
Entredicho mine	6.1 ± 0.2	101 ± 9	4.42 ± 0.89	0.021 ± 0.023 (0.029)	0.058 ± 0.072 (0.08)	0.045 ± 0.023 (0.06)	15.3 ± 4.7 (21)	10.2 ± 3.5 (14)	48.0 ± 4.0 (65)	73 ± 24
Las Cuevas mine	5.5 ± 0.1	60 ± 12	5.43 ± 0.32	<LOD (0)	0.46 ± 0.12 (0.04)	0.43 ± 0.56 (0.03)	77.8 ± 35.4 (6.0)	311 ± 96 (24)	897 ± 173 (70)	1287 ± 70

[a] Concentrations corresponding to the easily Hg reducible fraction were below the detection limit. Values between brackets denote the percentage of total Hg concentration in the soil sample. EC = electrical conductivity, TOC = total organic carbon.
[b] Not measured.

in soils collected from the rest of sampling sites. To explore what percentage of the total Hg detected in our soils was bioavailable to earthworms, we submitted the soils to a sequential extraction procedure obtaining seven Hg-extractable fractions (Table 1). More than 95% of total Hg was associated to 6 M HCl-soluble, oxidizable and residual fractions, and <0.18% of the total soil Hg was attributed to the chemically available Hg fraction (the sum of water soluble and exchangeable fractions).

3.2. Bioaccumulation of Hg by earthworms

Mercury concentrations did not differ statistically ($F_{(1,25)} = 0.34$, $p = 0.84$) between the controls and the earthworms exposed for 2 d to the Hg-contaminated soils (Fig. 2). However, when exposure extended up to 44 d, concentrations in Hg-exposed earthworms increased significantly compared to that of the corresponding control group (LSD post hoc test, $p < 0.0001$). Mercury concentrations in these earthworms increased as the chemically available and the organic matter-bound Hg fractions increased in soils (Table 1).

3.3. Effects of Hg-contaminated soils on earthworm biomarkers

Despite the high level of Hg contamination in the soils, no earthworms dead and they did not suffer a dramatic decrease of their body mass (Mann–Whitney U test, $p < 0.05$). Mean (±SD) fresh weight ranged between 2.69 ± 0.56 and 4.17 ± 1.02 g at $t = 2$ d, and between 2.48 ± 0.68 and 3.35 ± 0.52 g at $t = 44$ d. The mean body weight of the earthworms exposed to soils from the reference area and the Almadén mine showed, however, a significant decrease after 44 d of soil exposure (Mann–Whitney U test, $p < 0.05$).

Table 2 summarizes the mean (±SD) activities of antioxidant enzymes and glutathione concentrations. No significant differences were detected in the control groups between the sampling periods ($t = 2$ and 44 d) for all biomarkers (LSD post hoc test, $p < 0.05$).

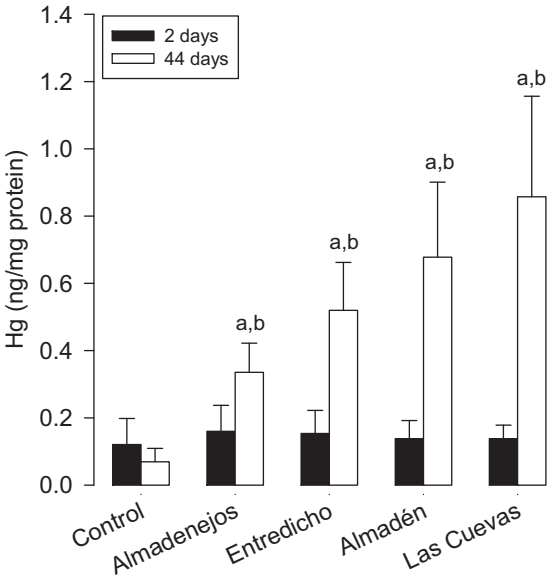

Fig. 2. Mean (±SD) Hg concentrations in earthworm muscle (10,000-g subcellular fraction) following 2 and 44 days of exposure to soils from the mining district of Almadén. Lower case letters denote statistical differences between corresponding controls and treated groups ("a") and within effects ("b") (ANOVA test, LSD post hoc test, $p < 0.05$).

However, soils collected from the four sites in the Almadén mining district had a significant impact on earthworm biomarkers. The ratio of reduced to oxidized glutathione was significantly affected by the soil type, whereas the duration of exposure (and its interaction with soil type) had a significant impact on GR activity, glutathione concentrations (total, reduced and oxidized forms), lipid peroxidation and metallothionein concentrations (Table 2 and Fig. 3). Soils from the Almadenejos village and the Entredicho mine

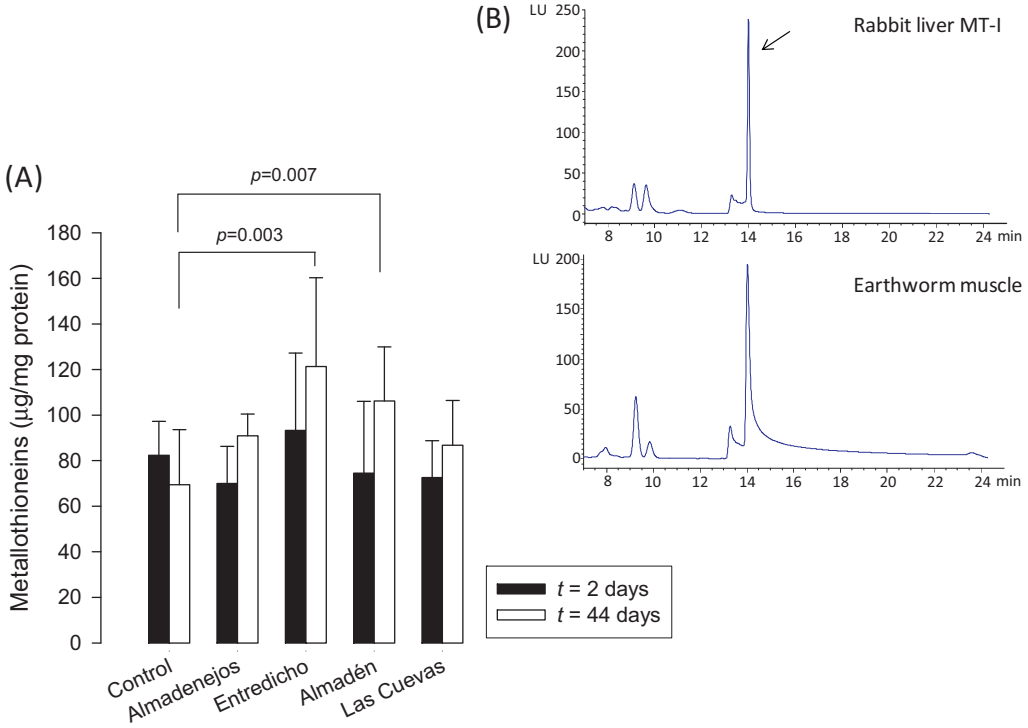

Fig. 3. (A) Metallothionein (MT) concentrations in the muscle of *Lumbricus terrestris* exposed for 2 and 44 days to Hg-contaminated soils. (B) An example of HPLC elutions of rabbit liver metallothionein-I (upper chromatogram) and an earthworm muscle sample (lower chromatogram) derivatized with monobromobimane (chromatographic conditions described in the Section 2.7).

Table 2
Mean (±standard deviation) of biomarker responses in the body wall muscle of *Lumbricus terrestris* following exposure to soils from the mining district of Almadén.

Biomarker	Duration of exposure (days)	Control	Almadén mine	Almadenejos village	Entredicho mine	Las Cuevas mine
GST (mU/mg protein)	2	305 ± 161	263 ± 64	300 ± 91	281 ± 110	257 ± 126
	44	238 ± 127	455 ± 179[a]	217 ± 77	429 ± 181	295 ± 166
GR (mU/mg protein)	2	13.8 ± 4.0	11.0 ± 2.0	10.7 ± 4.8[a,b]	12.5 ± 3.3	9.2 ± 2.8
	44	13.1 ± 6.3	15.9 ± 5.6	16.1 ± 5.8	19.4 ± 5.3	15.7 ± 7.8
CHP-GPx (mU/mg protein)	2	5.7 ± 1.9	2.1 ± 0.5	3.6 ± 0.6	4.0 ± 1.6	4.9 ± 2.6
	44	9.0 ± 2.5	14.4 ± 7.8[b]	6.5 ± 3.7	9.2 ± 2.7	4.6 ± 3.8[a]
Reduced glutathione (nmol/g wet wt)	2	653 ± 113	749 ± 68	430 ± 130[a,b]	436 ± 137[a,b]	674 ± 138
	44	618 ± 126	708 ± 175	776 ± 171	846 ± 88	703 ± 181
Oxidized glutathione (nmol/g wet wt)	2	178 ± 34	170 ± 57	290 ± 94	252 ± 57	173 ± 34
	44	205 ± 114	202 ± 35	187 ± 53	170 ± 29	136 ± 45
Lipid peroxidation (nmol MDA/mg protein)	2	272 ± 117	233 ± 62	322 ± 96	277 ± 74	235 ± 68
	44	257 ± 53	397 ± 58[a,b]	302 ± 91	283 ± 51	333 ± 65[b]

GST = glutathione S-transferase, GR = glutathione reductase, CHP-GPx = cumene hydroperoxide-dependent glutathione peroxidase, MDA = malondialdehyde.
[a] Statistical differences between Hg-contaminated soils and the corresponding controls ($p < 0.05$, post hoc LSD test).
[b] Statistical differences between sampling periods ($p < 0.05$, post hoc LSD test).

caused an increase of total glutathione concentration at $t = 44$ d compared to that observed at $t = 2$ d (Fig. 4A). However, short-term exposure to these two soils led to an oxidation of glutathione which was evident in the reduced/oxidized ratio of this thiol (Fig. 4B), although the oxidized form was back reduced within 44 d of soil exposure.

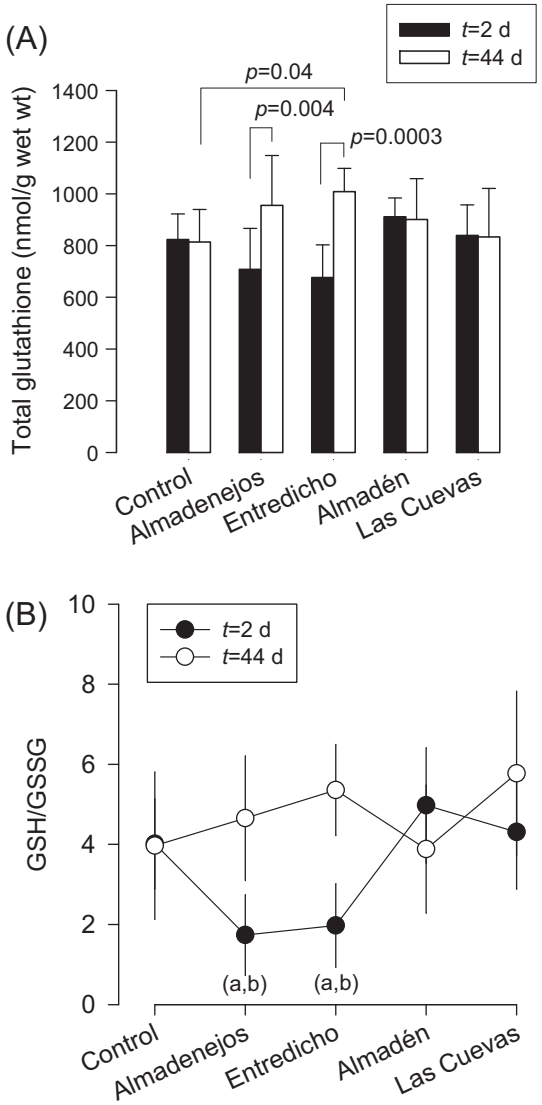

Fig. 4. (A) Total glutathione concentrations and (B) changes in the ratio of reduced to oxidized glutathione (GSH/GSSG) of earthworms exposed for 2 and 44 days to controls and Almadén's soils. Lower case letters as in Fig. 2.

We correlated earthworm Hg concentrations with each biomarker to establish cause-effect relationships, and no significant correlation was found (non-parametric Spearman's correlation test, $p < 0.05$). However, we found a marked response of the IBR index when muscle Hg concentrations dramatically increased following 44 d of exposure (Fig. 5).

4. Discussion

4.1. Mercury in the soil–earthworm system

Total Hg concentrations in our soils were in the same range of magnitude that those reported by others in soils collected from the Almadén's mining district [31,38,39]. Furthermore, these Hg concentrations were among the highest ever reported in soils from Hg mining areas [40–42]. However, the sequential extraction procedure revealed that Hg was not easily bioavailable; an observation already documented by Sánchez et al. [31] and Millán et al. [38]. Furthermore, the oxidizable Hg fraction accounted for a 14–24% of the total Hg in our soils, which had a relative low total organic carbon (1.9–5.4%). Nonetheless, the highest Hg concentrations in this extractable fraction corresponded to the soil with the highest total organic carbon (Las Cuevas mine). This is a usual relationship because of the strong affinity of Hg^{2+} ions by the thiol and carboxyl groups of the soil organic matter [43].

Despite this limited Hg available fraction, we found high Hg concentrations in the earthworm muscle following 44 d of exposure. This was an expected result in view of previous studies that demonstrate these soil organisms accumulate efficiently Hg [3,19,44,45]. In our soils, the bioavailable and the organic matter-bound Hg fractions seemed to account for the accumulation of this metal by earthworms, reflecting therefore the main routes of metal uptake, i.e., dermal and gastrointestinal. However, we found that exposure duration of 2 d was not enough to cause a significant Hg accumulation (Fig. 2). This lack of initial Hg accumulation has been also reported by Burton et al. [3], who found that bioaccumulation of total Hg by *E. fetida* exposed to historically Hg-contaminated soils was very low during the first 5 d of exposure.

4.2. Glutathione redox cycle as an integrated biomarker of Hg exposure

It is widely accepted that glutathione reduces Hg-mediated oxidative stress [4]. The mechanism for such attenuation occurs in a double way. First, Hg^{2+} ions have a high affinity by sulfhydryl groups

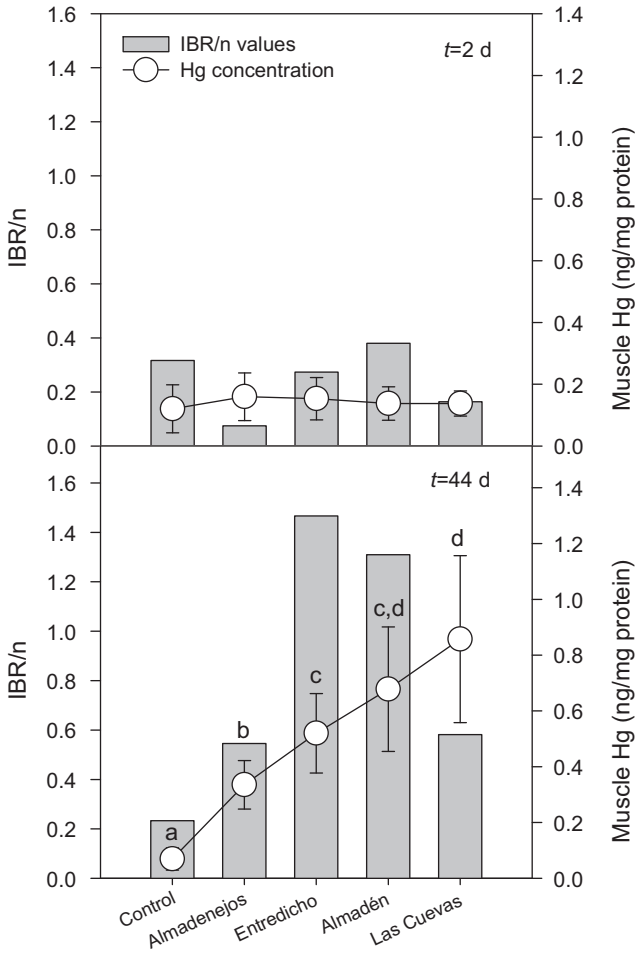

Fig. 5. Integrated biomarker responses values (IBR/*n*) and total Hg concentration (mean ± SD) in the muscle of *Lumbricus terrestris* exposed for 2 (upper chart) and 44 (lower chart) days to Hg-contaminated soils. Different lower case letters denote statistical differences (ANOVA test, LSD post hoc test, $p < 0.05$).

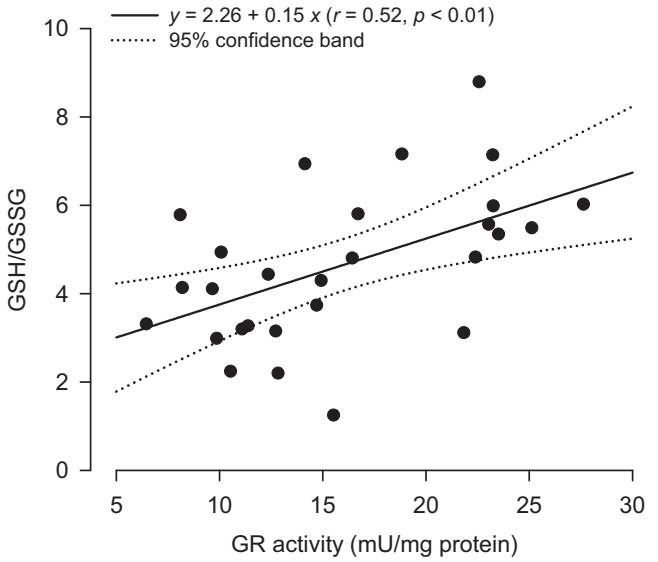

Fig. 6. Relationship between muscle glutathione reductase (GR) activity and the reduced to oxidized glutathione ratio (GSH/GSSG) in *Lumbricus terrestris* exposed for 44 days to soils from the Almadén mining district.

and glutathione acts, therefore, as a mercury scavenger. Second, Hg causes oxidative stress via H_2O_2 production [4,46]. The hydrogen peroxide is reduced to water by action of the GPx activity which uses reduced glutathione as the substrate. As result of this reaction, the glutathione is oxidized to its disulfide form. In these detoxification pathways, it should be expected therefore a decrease of total glutathione concentration or a low reduced to oxidized glutathione ration in Hg-exposed organisms. Our results corroborate these glutathione responses to Hg exposure. We found a rapid and significant decrease of the reduced/oxidized glutathione ratio in the earthworms exposed for 2 d to soils from the Almadenejos village and the Entredicho mine compared to controls. However, total glutathione levels increased significantly in the earthworms exposed to these soils after 44 d. This unexpected increase of total glutathione concentration has been also observed in other organisms such as fish [5,47]. The type of response (decrease or increase) of total glutathione can be attributed to factors such as the chemical form of Hg, the target tissue or organ, species, age, diet or the extent of Hg exposure. In this context, it is suggested that multiple biomarkers of oxidative stress (e.g., enzyme activities involved in the glutathione redox balance) and oxidative damage (e.g., lipid peroxidation) should be used jointly to establish a more consistent cause–effect relationship between contaminant exposure and oxidative stress [9,48]. We measured in the wall muscle of *L. terrestris* the concentrations of reduced and oxidized forms of glutathione as well as the main enzymes involved in its redox status. The transient response of the ratio of reduced to oxidized glutathione can be partially explained by the action of glutathione-dependent enzymes. For example, short-term exposure of *L. terrestris* to soils from the Almadenejos village and the Entredicho mine caused an oxidation of glutathione (Fig. 4B). However, the concentration of the reduced form of glutathione was re-established to normal physiological levels after 44 d by the GR activity. Indeed, a significant (Spearman's correlation test, $p < 0.01$) correlation was found between GR activity and the ratio between the reduced and oxidized glutathione concentrations in the earthworms sampled at $t = 44$ d (Fig. 6). This correlation suggests a significant role of GR activity in the homeostasis of this tripeptide, which has been also observed in other organisms exposed to Hg [4,9,47]. Conversely, the role of CHP-GPx activity in this reduced/oxidized glutathione balance was not clear. The ANOVA test did not reveal a significant impact of soil Hg concentration or duration of exposure on the CHP-GPx activity. In line with other studies (reviewed in van der Oost et al. [10]), our data show that this enzyme activity is a low sensitive biomarker of exposure to pro-oxidants such as Hg.

Glutathione and metallothioneins play a pivotal role in the tolerance of soil organisms to metal toxicity, acting as cellular metal ligands and endogenous antioxidants [49]. For example, Gudbrandsen et al. [7] examined the role of total glutathione levels in the ability of earthworms to tolerate Hg toxicity. These authors found that pre-exposure (1 week) of *E. fetida* to Cl_2Hg (22 mg/kg)-spiked soils resulted in an increase of earthworm survival in subsequent exposure trials with Hg-spiked soils. It was suggested that both total glutathione and metallothioneins (not measured in that study) could be involved in this ability of earthworms to tolerate Hg toxicity [7]. We found a significant increase of total glutathione concentrations in the earthworms long-term exposed to soils from the Almadenejos village and the Entredicho mine. Likewise, these earthworms and those exposed to the Almadén mine soils also displayed an increase of the metallothionein concentrations, corroborating the early findings by Gudbrandsen et al. [7].

In the biological monitoring of environmental contamination, biomarkers are often used as predictors of individual impairment. But this linkage is hard to demonstrate particularly with biomarkers of oxidative stress, which are affected by multiple biological and environmental variables that contribute to the imbalance between oxyradical production and the antioxidant defence. Identification

Table 3
Standardized scores and integrated biomarker responses (IBRs) in *Lumbricus terrestris* exposed for 2 and 44 days to Hg-contaminated soils.

Soil	Exposure duration	GST	GR	CHP-GPx	Total glutathione	GSH/GSSG	Metallothioneins	Lipid peroxidation	IBR/*n*
Control	2	0.95	0.74	0.68	0.63	1.15	0.79	0.68	0.32
	44	0.48	0.61	1.88	0.54	1.12	0.17	0.00	0.23
Almadén mine	2	0.69	0.13	0.42	1.40	1.77	0.59	0.83	0.38
	44	1.87	1.18	1.89	1.31	1.07	1.62	2.38	1.31
Entredicho village	2	0.80	0.49	0.24	0.00	0.16	1.17	1.60	0.27
	44	1.73	1.75	1.75	1.63	1.81	1.93	1.41	1.47
Almadenejos mine	2	0.90	0.21	0.00	0.18	0.00	0.00	0.96	0.07
	44	0.00	1.13	0.97	1.35	1.48	1.25	1.11	0.55
Las Cuevas mine	2	0.61	0.00	0.46	0.79	1.32	0.24	0.68	0.16
	44	0.88	1.03	0.35	0.75	2.10	1.00	1.56	0.58

GST = glutathione S-transferase, GR = glutathione reductase, CHP-GPx = cumene hydroperoxide-dependent glutathione peroxidase, GSH/GSSG = reduced to oxidized glutathione ratio, IBR/*n* = integrated biomarker response values divided by the number of biomarkers (*n*).

of the impact on biomarkers from these confounding environmental and biological factors is a difficult task. Thus, we used multiple biomarkers related to the underlying mechanisms of Hg toxicity, and combined them into an integrated index of health status [32]. We found that the IBR index is a suitable approach for assessing sublethal effects in Hg-exposed earthworms. In fact, the IBR correlated positively with the postmitochondrial Hg fraction suggesting this Hg concentration corresponded to the toxic fraction of the metal [23]. Most of the studies involving the IBR index have used organisms collected from the field in which biomarker responses can be influenced by multiple environmental or biological confounding variables [34,35,50,51]. In the most of these field studies, the IBR was useful to identify polluted sites. Herein, we have used the IBR to link the toxic Hg fraction to observed sublethal toxicity. In general, the highest IBR values corresponded with an increase of the Hg concentration in the muscle postmitochondrial fraction (Fig. 5). Nevertheless, the use of the IBR index presents some limitations (discussed in Broeg and Lehtonen [33]). For example, the algorithm used in the IBR calculation can mask biomarkers having high scores. For this reason, some authors recommend that biomarker scores be individually examined [33]. This is the case of earthworms exposed to the soil from Las Cuevas mine. Although these earthworms had the highest Hg concentrations, their IBR value was lower than those obtained of earthworms exposed to soils from the Almadén and the Entredicho mines. However, biomarker scores summarized in Table 3 shows that exposure to soils from Las Cuevas mine caused a significant response of the ratio of reduced to oxidized glutathione ($s = 2.10$) and the lipid peroxidation ($s = 1.56$).

5. Conclusions

Three main conclusions can be drawn from this microcosm study:

(1) Despite the extremely high total Hg concentrations in the Almadén's soils (up to 1287 ± 70 mg/kg dry wt), they were not acutely and chronically lethal to *L. terrestris*. The low concentrations of available Hg in these soils probably accounted for the lack of lethal toxicity. Nonetheless, earthworms showed evidences of Hg-induced oxidative stress in terms of changes in the glutathione homeostasis, lipid peroxidation and induction of molecular scavengers (metallothioneins).
(2) Exposure duration had a significant impact on the response of total glutathione, concentrations of the reduced and oxidized forms of glutathione, GR activity, metallothionein induction and lipid peroxidation. Short-term exposure (2 d) caused a marked change in the reduced to oxidized glutathione ratio which indicated Hg-induced oxidative stress, although this glutathione balance was re-established within 44 d mainly by the action of GR activity. Increase of metallothionein and total glutathione concentrations had a primary role at long-term exposure (44 d) acting as endogenous scavengers and, probably, reducing oxidative damage.
(3) The IBR index increased as Hg concentrations in the muscle postmitochondrial fraction enhanced, being a good indicator of the toxic Hg fraction (cytosolic and microsomal fractions). The use of this integrative index involving biomarkers related to the mechanism of Hg toxicity is a suitable approach for assessing soil Hg pollution.

Acknowledgments

Financial support was provided by the Ministerio de Ciencia e Innovación (CTM 2006-11828/TECNO). We thank to Dr. José Alhama for a critical reading of the manuscript and two anonymous reviewers for valuable comments that improved the manuscript.

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