Arch Environ Contam Toxicol (2015) 68:181–192
DOI 10.1007/s00244-014-0060-4

Effects of Soil Organic Matter Content on Cadmium Toxicity in *Eisenia Fetida*: Implications for the Use of Biomarkers and Standard Toxicity Tests

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Received: 11 March 2014 / Accepted: 2 June 2014 / Published online: 12 July 2014

Abstract Bioavailability is affected by soil physicochemical characteristics such as pH and organic matter (OM) content. In addition, OM constitutes the energy source of *Eisenia fetida*, a well established model species for soil toxicity assessment. The present work aimed at assessing the effects of changes in OM content on the toxicity of Cd in *E. fetida* through the measurement of neutral red uptake (NRU) and mortality, growth, and reproduction (Organisation for Economic Co-operation and Development [OECD] Nos. 207 and 222). Complementarily, metallothionein (MT) and catalase transcription levels were measured. To decrease variability inherent to natural soils, artificial soils (Organization for Economic Cooperation and Development 1984) with different OM content (6, 10, and 14 %) and spiked with Cd solutions at increasing concentrations were used. Low OM in soil decreased soil ingestion and Cd bioaccumulation but also increased Cd toxicity causing lower NRU of coelomocytes, 100 % mortality, and stronger reproduction impairment, probably due to the lack of energy to maintain protection mechanisms (production of MT). Cd bioaccumulation did not reflect toxicity, and OM played a pivotal role in Cd toxicity. Thus, OM content should be taken into account when using *E. fetida* in in vivo exposures for soil health assessment.

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Chemical bioavailability describes quantitatively the transport of chemicals from soil to soil living organisms in a finite time period (Katayama et al. 2010). It is a parameter that is affected by the physicochemical properties of the soil. pH and organic matter (OM) content are in this sense directly related with the uptake, toxicity, degradability, and accumulation of pollutants (Kennette 1997; Nahmani et al. 2007). Many pollutants that reach the soil are electrically charged or ionisable; as a result, their bioavailability depends on the presence of charged molecules in the OM (Sizmur & Hodson 2009). These charged molecules confer buffering capacity and mediate adsorption and desorption reactions, so some soils display capacity to decrease contaminant bioavailability (Porta et al. 2003).

Metals, the most common pollutants in soils, coming from industrial, mining, and agricultural activities, are among such ionisable or electrically charged chemicals. Cadmium (Cd), with a release of >7,000 tons/y, is one of the metal pollutants whose concentration in soil has increased due to anthropogenic activity (Nriagu & Pacyna 1988, World Health Organization 1992). Cd does not have any known role for living organisms and, in addition, is one of the most toxic metals (Robards and Worsfold 1991). Thus, it is commonly used as a model metal in toxicological studies aimed at determining the suitability of toxicity biomarkers and their application in soil health assessment (Fugère et al. 1996; Olchawa et al. 2006; Spurgeon et al. 1994).

Eisenia fetida earthworms have been widely used as model experimental organisms in soil toxicity assessment (Spurgeon et al. 2003) and are included in the guidelines for chemical toxicity testing proposed by several agencies and organizations (International Organization for Standardisation International Organization for Standardization 1993; Organization for Economic Cooperation and Development 1984; United States Environmental Protection Agency

[USEPA] 1996). Thus, several standard toxicity tests employing *E. fetida,* such as the acute toxicity test (ATT; OECD No. 207) and the reproduction test (RT; OECD No. 222), have been applied for soil toxicity assessment of real polluted soils (Asensio et al. 2013; Ávila et al. 2009; Rodríguez-Ruiz 2010; Spurgeon et al. 2003, Spurgeon et al. 2004, Spurgeon et al. 2005). The biological end points analysed in both two tests (survival, growth, and reproduction) provide ecologically relevant information on the effects caused by polluted soils on earthworms. However, as mentioned previously, the toxicity exerted by pollutants depends on their bioavailability and hence on the physicochemical properties of the soil (Corp & Morgan 1991; Janssen et al. 1997; Spurgeon et al. 2006). Thus, the results of standard toxicity tests are useful for the screening of the toxicity of chemicals but may be misleading if toxicities of real soils with different physicochemical properties are compared or if dilutions of the soils are used in the test battery (pH and OM content change with dilution). Biomarkers are prompt responses to pollutants at lower level of biological organisation that provide an invaluable screening tool to estimate the toxicity exerted by chemicals on biota before the effects at individual and population levels can be detected (Asensio et al. 2013; Huggett et al. 1992; Schlenk 1999). In *E. fetida*, destabilisation of the lysosomal membrane in coelomocytes is a widely used biomarker that is measured through either the neutral red retention time (NRRT) or neutral red uptake (NRU) assays (Asensio et al. 2013; Svendsen et al. 1996, 2004; van Gestel et al. 2009). Coelomocytes are essential for the homeostasis of earthworms and for their immune function because these cells are the effectory immunocytes in charge of the defense against pathogens (Bilej et al. 2010). Thus, alterations in the stability of their lysosomal membrane may be linked with physiological or pathological responses to pollutant exposure (Weeks & Svendsen 1996). Changes in the transcription of specific target genes—such as those encoding metallothioneins [MTs (metal-sequestering proteins)] or catalase [CAT (antioxidant enzyme)] can be directly related to metal exposure or its effects and are widely measured in earthworms and other invertebrates (Asensio 2009; Brulle et al. 2006, 2008; Demuynck et al. 2006; Irizar et al. 2014; Stürzenbaum et al. 1998).

Thus, the present work was aimed at determining how the OM content in soil affects Cd toxicity in *E. fetida* through the measurement of neutral red uptake, mortality, growth, and reproduction. Changes in the transcription levels of the *mt* and *cat* genes, which encode MTs and CAT, respectively, were quantified as exposure biomarkers. For this purpose, artificial soils composed of peat, clay, and sand according to OECD guidelines (Organization for Economic Cooperation and Development 1984) and with different OM contents (6, 10, and 14 %) were spiked with Cd at concentrations of 0, 5, 12.5, 25, 62.5, 125, and 625 mg Cd/kg soil dry weight (dw).

Table 1 Proportions of sphagnum peat, sand, and kaolin clay mixed to obtain modified OECD soils

Soil label	Sphagnum peat (%)	Sand (%)	Kaolin clay (%)
OECD 6	6	73	21
OECD 10	10	70	20
OECD 14	14	67	19

Materials and Methods

Artificial Soil Preparation and Contamination

Soils were prepared to obtain three different organic matter concentrations according to OECD guide 207 (Organization for Economic Cooperation and Development 1984). OECD 10 soil contained 10 % sphagnum peat, 20 % kaolin clay, and 70 % sand with a final pH 6 ± 0.5 (determined as specified in International Organization for Standardization 11274 1998). The OM content was modified adding 14 % (OECD 14) and 6 % (OECD 6) sphagnum peat and adjusting sand and kaolin clay proportionally (Table 1). The humidity was adjusted by adding deionised water to obtain 40 % of the maximum water-holding capacity [WHC (determined as specified in International Organization for Standardization 11274 1998)]. OECD 6, 10, and 14 soils were spiked with Cd (as $CdCl_2$ diluted in deionised water according to the calculation of the 40 % WHC) at different concentrations (0, 5, 25, 125, and 625 mg Cd/kg soil dry-wt). Soils were stabilized for 45 days, sealed, and maintained at 4 °C until needed.

Earthworms and Exposures

E. fetida earthworms used for the experiments were healthy adults, clitellated, and of similar size (350–500 mg fresh weight) obtained from the stock population provided by a commercial dealer (Hezieko SA, Aizarnazabal, Spain) and maintained in the laboratory under controlled conditions of temperature (18 ± 1 °C), humidity, and food (horse manure) supply.

Earthworms were acclimated for 24 h in control soils [OECD 10 (Table 1)] and afterward were placed (ten individuals/750 g soil) in three glass containers (2.25 kg soil each) containing soils [OECD 6, 10, or 14 (Table 1)] at 19 °C and under controlled humidity and 24-hour light regime for 3 days.

NRU Assay

After a 3-day exposure, the posterior part of the gut of five animals per treatment was cleaned by softly massaging the body. The extraction of coelomocytes was performed by applying a 9 V electric shock for 10 cycles of 3 s after

submerging the five animals in the extrusion solution (0.2 % ethylene diamine tetraacetic acid [EDTA] in Hanks balanced salt solution [HBSS]; Sigma-Aldrich H9394, pH 7.2 to 7.4). Worms were removed, and extruded coelomocytes were cleaned through two cycles of centrifugation (212 g, 4 °C, 10 min). The remaining pellet was resuspended in HBSS (Asensio et al. 2007). The quantification of coelomocyte number was performed by automatic cell-counting equipment (Z2TM Coulter Counter; Beckman Coulter, USA). Coelomocytes ($n = 2 \times 10^5$) were then seeded in microplate wells and incubated for 1 h in a cell incubator at 18 °C.

Coelomocytes were incubated for a 1-hour period with the freshly prepared neutral red solution (0.05 % neutral red in HBSS) in a cell incubator at 18 °C with CO_2-free atmosphere. Then cells were washed twice with HBSS followed by a 15 min exposure to extraction solution (1 % acetic acid, 50 % ethanol) and shaken for 5 min. Absorbance was read in a microplate spectrophotometer (Multiskan Spectrum, Thermo Scientific, Thermo Fischer Scientific, Inc., USA) at 540 nm.

Quantitative Polymerase Chain Reaction of mt *and* cat *Genes*

Five animals exposed to 0, 5, and 25 mg Cd/kg soil dw (LC50/10 for *E. fetida* in OECD artificial soil) (Asensio et al. 2007) were retrieved and anesthetized with alcohol, and the hindgut (Irizar et al. 2014) was dissected and immersed in 500 µl de RNAlater (Sigma-Aldrich, USA), frozen in liquid nitrogen, and stored at −80 °C until their posterior analysis.

The total RNA extraction was performed using the TRIzol reagent (Invitrogen, USA) according to the manufacturer's instructions with some modifications. Samples were homogenized after RNAlater removal in a HYBAID RiboLyser FP120-HY-230 for 45 s and at maximum speed. RNA concentration and purity were tested through spectrophotometry (BioPhotometer, Eppendorf, Germany). cDNA synthesis was performed with the SuperScript First Synthesis System Kit for Real Time-Polymerase Chain Reaction (RT-PCR; Invitrogen) taking 100 ng cDNA in a 2,720 Thermal Cycler (Applied Biosystems, USA).

The gene sequences for primer design were obtained in the National Center for Biotechnology Information database [http://www.ncbi.nlm.nih.gov (Table 2)], and specific primers were designed with the aid of the Primer Express 3.0 software for RT-PCR (Applied Biosystems). Primer sequences for β-actin were obtained from Brulle et al. (2006). The optimal amplification conditions for the primers were standardized by testing the annealing temperature and the concentrations of the primers and cDNA. For that, 35 cycles were run in a 2,720 Thermal Cycler (Applied Biosystems) using the conditions illustrated in Irizar et al. (2014). β-actin and 18S rRNA were amplified as reference genes to calculate the relative transcription levels of *mt* and *cat* genes. Q-PCR reactions were performed using the FastStar Universal SYBR Green Master (Roche, Germany) in a thermal cycler (7300 Real Time PCR System; Applied Biosystems, Inc., USA). Three replicates were quantified for each cDNA, and only samples with SDs among replicates <0.3 CTs were accepted.

OECD ATT and RT

Tests were performed according to OECD guideline 207 (Organization for Economic Cooperation and Development 1984) for mortality and growth assessment and OECD guideline 222 (Organization for Economic Cooperation and Development 2004) for the reproduction test. Briefly, for mortality and growth assessment (ATT), 10/treatment were exposed for 14 days to the three experimental soils (OECD 6, 10, and 14, Table 1), which were artificially spiked with Cd (0, 5, 25, 125, and 625 mg Cd/kg soil dw) as explained before. A comparison between nominal and real concentrations is listed in Table 3. After 14 days, surviving earthworms were weighted and counted. Weight loss was calculated as the percentage of weight lost after exposure to soils for 14 days. Median lethal concentration (LC50) was calculated after 14 days of exposure.

For the RT, 10 clitellated earthworms/500 g soil (OECD 6, 10, and 14, Table 1) artificially spiked with Cd (0, 5, 25, 125 and 625 mg Cd/kg soil dw) were exposed for 28 days at 19 °C under controlled humidity and 8:16 × hour light-to-dark regime. Two replicates per treatment were performed. Earthworms were fed weekly adding 5 g dw moist horse manure per container. Afterward, living adult worms were removed from the containers and weighted. Soils were maintained for additional 28 days and the number of juveniles hatched from the cocoons as well as the cocoon numbers were counted. Results are given as cocoon number and juvenile number; the median effect concentration (EC50) was calculated for each parameter.

Cd Tissue Concentration in Earthworms and Soils (ATT and RT)

Cd concentration in tissues of earthworms was measured after their exposure in soils with different OM content and Cd concentrations following ATT and RT for 14 days and 28 days, respectively. Earthworms were depurated on filter paper for 24 h and dried in an oven at 120 °C for 48 h until stable dry weight was reached. Pools of five animals were then weighted and crushed in a mortar to facilitate digestion in concentrated nitric acid. After evaporation of the acid, samples were resuspended in 0.05 M nitric acid, and Cd was analysed in the SGIker General Research Services (UPV/EHU) by atomic absorption spectroscopy (AAS; Perkin Elmer 2,280 spectrophotometer). Cd concentrations

Table 2 Sequences of specific primers used for the amplification of selected genes

Gene	GenBank accession no.	Primer sequences	Amplicon size (nt)
MT	AJ236886	Fw 5́-AAATGCTCGGCTGGTTCGT-3́	103
		Rv 5́-CTGATGACAGAGTTCCGTATTTCAA-3́	
CAT	DQ286713	Fw 5́-GCCGACGGAGAAGCTGTGTA-3́	125
		Rv 5́-TAAAGGTCACGGGTCGCATAG-3́	
β-actin	DQ286722	Fw 5́- TCTCCACCTTCCAGCAGATG-3́	209
		Rv 5́- CGAAAAATGTCCTCCGCAAG-3́	
18S rRNA	AB558505	Fw 5́-CCGGCGACGTATCTTTCAA-3́	145
		Rv 5́-CTGCCTTCCTTGGATGTGGTA-3	

Fw forward, *Rv* reverse

Table 3 Nominal Cd concentration (mg/kg) added to the tested OECD soils and real Cd concentration measured in soils (mg/kg)[a]

Nominal Cd (mg/kg)	Real Cd concentration in soil (mg/kg)		
	OECD 6	OECD 10	OECD 14
Control	0.1 ± 1	0.1 ± 0	0.2 ± 1
5	3.6 ± 1	5.9 ± 2	3.8 ± 1
25	17 ± 1	22 ± 6	19.2 ± 4
125	98.5 ± 9	110 ± 7	108.1 ± 10
625	ND	ND	301.3 ± 9

ND not detected

[a] Real Cd concentration values are shown in averages ± RSD

are expressed as micrograms of metal per gram of tissue dry weight. Merck standard solutions were diluted in 0.05 M nitric acid for AAS calibration (Soto et al. 2000).

For quantitative determinations of Cd in soils, three replicates were sieved, digested in a microwave, and analysed in the SGIker General Research Services (UPV/EHU) by inductively coupled plasma-mass spectrometry. Briefly, acid digestion (0.5 g dried soil in 15 mL of HCl 20, HNO_3 20, and H_2O 60 %) was performed in Teflon vessels in a microwave oven (USEPA Method No. 3,051). After cooling, the extracts were filtered through syringe Polytetrafluoroethylene (PTFE) filters (25 mm, 5 µm; Waters, Milford, USA) and diluted to 50 ml in MilliQ water. Samples were analysed in an isotope pattern vector mass spectrometry (IPV-MS) detector (7700x; Agilent, USA) using a MicroMist microuptake glass concentric nebulizer (Glass Expansion, Australia). The equipment included a collision cell (helium gas, ORS^3 system; Agilent) to discriminate spectral interferences with high performance.

Statistical Analysis

Statistical analysis was performed with the aid of SPSS statistical package (SPSS, Microsoft, version 19). Data of the NRU assay and *mt* and *cat* transcription levels were analysed with Mann–Whitney *U* test ($p < 0.05$). Data of Cd tissue concentrations were analysed with Student *t* test ($p < 0.05$). LC50 and EC50 values regarding cocoon and juvenile productions were estimated by means of the log-probit method provided by the SPSS statistical package. Regressions were analysed with the *t* score ($p < 0.05$).

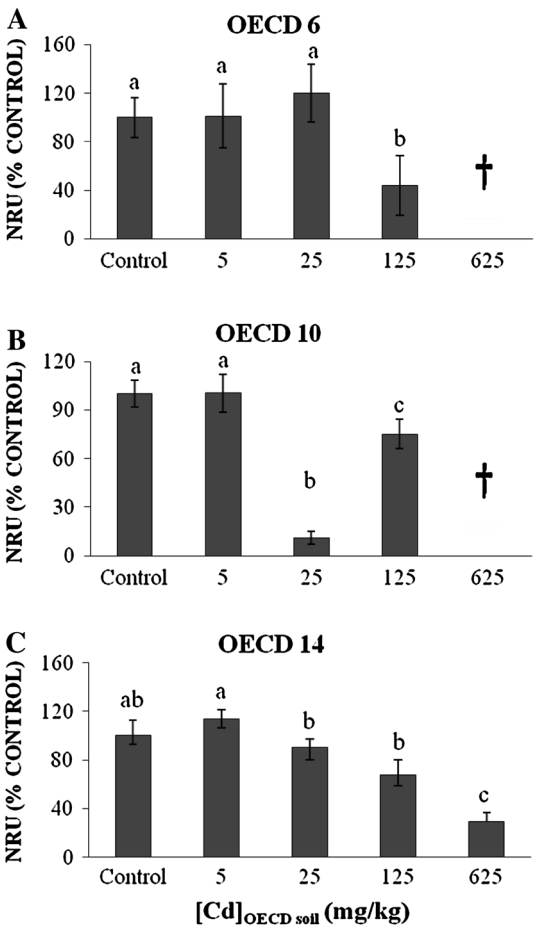

Fig. 1 NRU (% control) in coelomocytes of *E. fetida* exposed for 3 days to 5, 25, 125, and 625 mg/kg Cd in **a** OECD 6, **b** OECD 10, and **c** OECD 14 soils. Values are shown as average ± SD. *Significantly different from controls (Mann–Whitney *U*; $p < 0.05$). †Earthworms died before the end of the experiment

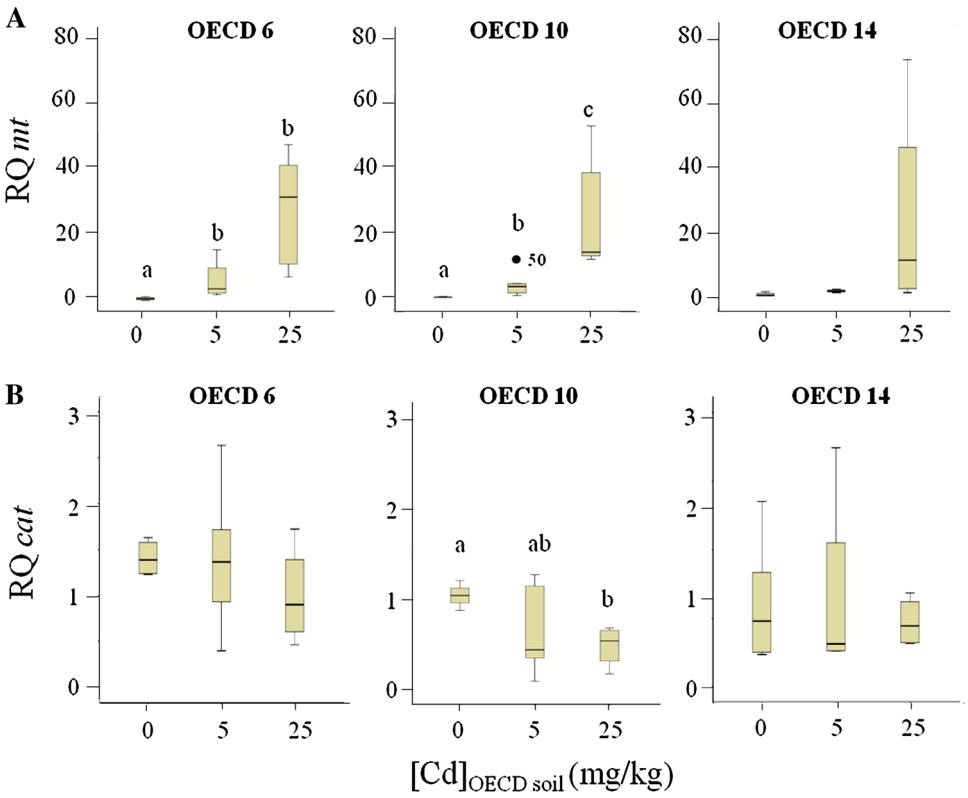

Fig. 2 Box *plots* indicating relative quantification (RQ) of the transcription of *mt* gene (**a**) and *cat* gene (**b**) normalised with transcription levels of 18S rRNA in *E. fetida* exposed for 3 days to OECD 6, OECD 10 and OECD 14 soils contaminated with Cd (0, 5, and 25 mg Cd/kg). Box *plots* represent the data within the 25th and 75th percentiles with the median indicated by a *line*. *Different letters* indicate significant differences between Cd exposure groups (Mann–Whitney *U*; $p < 0.05$)

Results

NRU Assay

Results of the NRU assay performed with coelomocytes retrieved from earthworms exposed to Cd in different OECD soils for 3 days are illustrated in Fig. 1a significant decrease in NRU was observed after exposure to 125 mg Cd/kg soil in OECD 6 soil (Fig. 1a). The NRU assay was not performed with coelomocytes of earthworms exposed to 625 mg Cd/kg soil because 100 % mortality occurred before 3-day exposure (Fig. 1a). Exposure to OECD 10 soil with 25 mg Cd/kg soil caused a significant decrease in NRU compared with the control group. In contrast, there was a significant increase in NRU after exposure to 62.5 mg Cd/kg soil, and a second decrease at 125 mg Cd/kg soil was observed (Fig. 1b). Exposure to 625 mg Cd/kg soil also produced 100 % mortality before 3-day exposure (Fig. 1b). In OECD 14 soil, NRU decreased significantly only in soils spiked with 625 mg Cd/kg (Fig. 1c).

mt *and* cat *Gene Expression*

Regarding the analysis of gene transcription levels, only *18S rRNA* could be used as reference gene due to the unspecific bands generated during PCR amplification of *β-actin*. *mt* gene transcription was significantly upregulated on exposure to 5 and 25 mg Cd/kg soil in OECD 6 and OECD 10 soils for 3ays following a dose-dependent trend (Fig. 2a). In contrast, this trend was not observed in OECD 14 soil, although the highest Cd concentration caused an increase (albeit not significant) in *mt* gene transcription levels (Fig. 2a). The levels of *cat* transcripts were decreased on exposure to 25 mg Cd/kg soil in OECD 10 (Fig. 2b), whilst no significant change was recorded in OECD 6 and OECD 14 soils after exposure to Cd, although a certain decreasing trend was envisaged in 25 mg Cd/kg soil in OECD 6 (Fig. 2b).

OECD ATT and RT

Animals lost weight after a 14-day exposure (without food supply) under all experimental conditions, even in control soils (Table 4). The smallest decrease in weight was observed in earthworms maintained in OECD 14 soil for all of the Cd concentrations analysed. The smallest difference between exposure groups and controls also corresponded to OECD 14 (11.44 vs. 33.52 % in OECD 6 and 40.56 % in OECD 10). One hundred percent mortality occurred in OECD 6 and 10 soils contaminated with the highest Cd concentration (625 mg Cd/kg soil). The calculated LC50 values for Cd were 354 mg Cd/kg soil for OECD 6 and 10. In contrast, no mortality was observed in OECD 14 soils (LC50 > 625 mg Cd/kg soil).

Table 4 Growth (% initial weight) [14 days and 28 days and LC50 values (mg Cd/kg soil)] (14 days for *E. fetida* earthworms exposed to soils containing different OM concentrations (OECD 6, 10 and 14) and spiked with Cd[a]

Nominal (mg/kg)	Growth (% initial weight) 14 days			Growth (% initial weight) 28 days		
	OECD 6	OECD 10	OECD 14	OECD 6	OECD 10	OECD 14
0 (control)	−14.52	−16.4	−12.71	−8.74	−7.46	−13.67
5	−21.17	−17.24	−12.89	−2.86	−6.56	5.53
25	−17.61	−15.58	−15.16	−1.40	0.65	8.82
125	−18.9	−22.26	−16.6	15.02	10.94	6.11
625	†	†	−14.06	†	†	11.51
LC50 (mg Cd/kg)	354	354	>625			

[a] LC50 values were calculated with nominal Cd concentration values (mg Cd/kg soil dw)

† All earthworms died before the end of the experiment

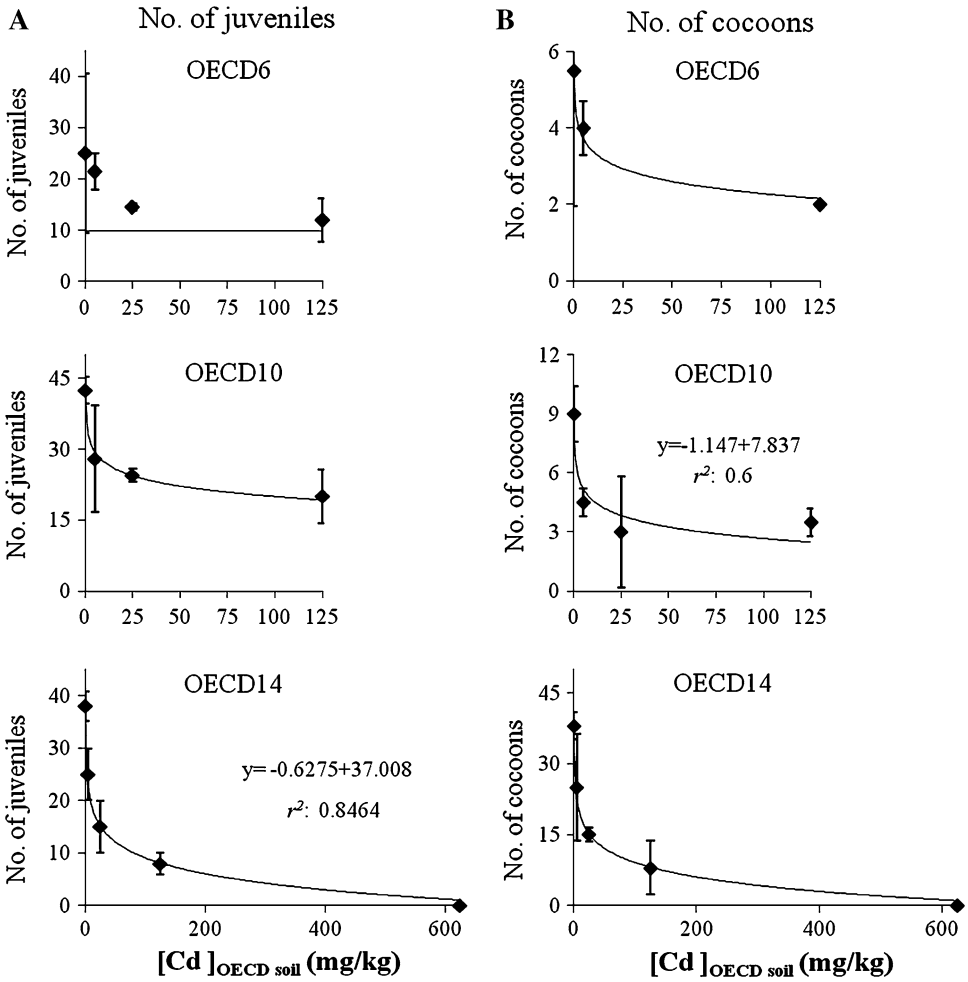

Fig. 3 Number of juveniles (**a**) and cocoons (**b**) produced in OECD 6, 10, and 14 soils polluted with 0, 5, 25, and 125 mg Cd/kg in OECD 6 and 10 soils and with an additional 625 mg Cd/kg dose in OECD 14 soil. The logarithmic regression function is only represented in the cases when it is statistically significantly (t score, $p < 0.05$) dependent on Cd concentration

The number of produced juveniles followed a decreasing Cd dose-dependent trend independently of the OM content of soils (Fig. 3). Cd toxicity was highest in OECD 6 and 10 soils with similar EC50 values ($EC50_{OECD6} = 15.13$ mg Cd/kg soil, $EC50_{OECD10} = 10.45$ mg Cd/kg soil, and $EC50_{OECD14} = 51$ mg Cd/kg soil). Earthworms maintained in the control OECD 6 soil did not produce the minimum number of juveniles required ($n = 30$) for an adequate control series according to OECD RT 222 (Organization for Economic Cooperation and Development 1984).

The highest number of cocoons was recorded in the OECD 14 soils (Fig. 3). Cd caused a dose-dependent

Table 5 Cd tissue concentrations (μg/g dw) in *E. fetida* after 14 and 28 days of exposure to OECD soils with different OM content contaminated with different concentrations of Cd

Cd in soil (mg/kg)	Cd in tissue (μg/g dw)					
	14 days			28 days		
	OECD 6	OECD 10	OECD 14	OECD 6	OECD 10	OECD 14
0	UDL	UDL	UDL	UDL	UDL	UDL
5	13.8	22.8	45.3	19.9 ± 3.8[a]	31.1 ± 1.8[a*]	17.7 ± 7.3[a]
25	49	68.3	50.8	93 ± 3.4[b]	84.7 ± 60.5[a]	74.9 ± 42.4[a]
125	54.5	110.5	ND	153.8 ± 101.8[ab]	295.7 ± 55.6[b]	301.8 ± 17.8[b]
625	†	†	108.7	†	†	343.5 ± 78.9[b]

Different superscript letters indicate statistical differences ($p < 0.05$) among values of the same soil type [OECD 6, 10, and 14 (same columns)]

ND no available data, *UDL* under detection limit

† All earthworms died before the end of the experiment

* Statistical differences [Student *t* test ($p < 0.05$)] among values of soils with the same nominal Cd concentration (same line)

Values are shown as average ± SD

decrease in cocoon number, but the logarithmic regression function was only significant in OECD 10 soils. The EC50 values indicated the highest toxicity of Cd in OECD 6 soils, followed by OECD 10 and 14.

Bioaccumulation of Cd

Cd concentration in tissues increased with increasing metal concentration in the three OECD soils (Table 5). The highest metal concentrations were measured in earthworms exposed to 125 mg Cd/kg in OECD 10 and to 625 mg Cd/kg in OECD 14 soil in both sampled times with similar values of Cd in tissue between the two Cd doses.

In the RT (28-day exposure), Cd tissue levels increased linearly at increasing Cd concentrations in soil, even at concentrations >25 mg Cd/kg soil in OECD 10 soil (Table 5). As a result, Cd tissue levels were higher after 28- than after 14-days ATT in all of the soils studied. The maximum Cd concentration that earthworms accumulated in OECD 10 soil before dying was as high as the values measured after exposure to 625 mg/kg Cd in OECD 14 soil after 14- and 28-day exposure (Table 5). Moreover, the Cd accumulation pattern in animals exposed to the metal in OECD 10 and OECD 14 soils was similar after 14- and 28-day exposure. Animals exposed to 625 mg Cd/kg soil did not accumulate more Cd than those exposed to 125 mg Cd/kg soil in any case because they reached a plateau approximately 300 μg Cd/g tissue dw.

Discussion

The effect of OM content on toxicity tests in soil organisms has been poorly studied, although its importance governing metal bioavailability in soils and earthworm activity is well known (Nieder & Benbi 2008; Römbke et al. 2005). Presently variants of the standard OECD testing soil with different OM content but a constant ratio of sand/kaolin to clay have been tested. Although clay content varied between soils and this component could adsorb metals, it seems that it does not exert a significant influence on Cd bioavailability (Osté et al. 2001). Overall, the sensitivity of the different biological end points studied (bioaccumulation, NRU in coelomocytes, *mt* and *cat* transcription levels, mortality, weight loss, and juvenile and cocoon production) varied with the OM content in the soil, which is in agreement with previous observations by Scott-Fordsmand et al. (1998).

Earthworms are known to accumulate Cd in their tissues (Brewer & Barrett 1995; Lapinski & Rosciszewska 2008; van Gestel et al. 1993). Specifically, Cd tissue concentration in *E. fetida* increases in a dose dependent manner at exposure concentrations ≤1,000 mg Cd/kg soil dw (Asensio 2009; Lock & Janssen 2001). Accordingly, a dose-dependent accumulation of Cd was found in the present study with Cd concentrations in soil <625 mg Cd/kg soil dw. Cd tissue concentration in earthworms maintained in OECD 6 soil was lower than in the other two soils. This unpredicted result could be understood in terms of diminished feeding activity as recorded in previous studies (Drobne & Hopkin 1995; Laskowski & Hopkin 1996) and decreased growth in a soil with low OM content because in theory Cd should be more bioavailable when OM content in soil is low (Nieder & Benbi 2008; Römbke et al. 2005). Indeed, earthworms in OECD 6 soil showed the highest rate of mortality and weight loss and the lowest reproductive performance.

Although the toxicological bioavailability of nonregulated pollutants such as Cd is represented by the internal critical concentration of the chemical [named "critical

body residues" (CBR)] (Lanno et al. 2004; Lock & Janssen 2001; Ma et al. 2002; Rodríguez-Ruiz 2010; van Wensem et al. 1994), toxicity is caused after the chemical is transported to the site(s) of toxic action in the organism. Thus, it is possible to have accumulation without toxicity (Lanno et al. 2004), and different toxicity values corresponding to similar Cd tissue concentrations are not unexpected.

Earthworms have several mechanisms to avoid the toxic effects of Cd, such as the production of MTs. Cd is known to upregulate *mt* gene expression (Asensio et al. 2007; Bundy et al. 2008; Galay-Burgos et al. 2005; Homa et al. 2010; Spurgeon et al. 2005) and, accordingly, in the present study *mt* transcription levels were significantly upregulated after 3-day Cd exposure both in OECD 6 and 10 soils. In the case of OECD 14 soil, a less marked upregulation of the *mt* gene expression than in OECD 6 and 10 soils was observed together with weaker toxic effects. Interestingly, the presence of high OM contents in soil can also enhance earthworm activity, which would promote pollutant bioavailability by mobilising and metabolising chemicals from the soil (Cheng & Wong 2002; Devliegher & Verstraete 1996; Ma et al. 2002). Thus, the high Cd tissue concentration found in OECD 14 soil earthworms could be the result of the entry of Cd-bound to SOM (associated with organic molecules) by way of the digestive tract. In such case, Cd tissue concentrations would be high, but MT induction and biological effects would be less marked than if dissolved Cd enters by way of the skin pores (*e.g.*, as expected in OECD 6 soil). In addition, OM itself would contribute to improve the nutritional status of the earthworms by providing more energy supply to cope with metal detoxification and to contribute to growth and reproduction. This was evidenced by the larger weight loss of earthworms in soils with lower OM concentration.

cat transcription levels were decreased after exposure to Cd in OECD 10 soil, and to a lesser extent in OECD 6 soil, but not in OECD 14 soil. In agreement, *cat* gene has been reported to be downregulated after Cd exposure in standard OECD (equivalent to OECD 10) (Brulle et al. 2007; Chen et al. 2011; Yang et al. 2012). Indeed, CAT activity is known to be inhibited in earthworms at short-exposure times as a result of the high quantity of superoxide anions produced in response to metal exposure, whereas at later stages *cat* gene expression can be upregulated as a part of the adaptive response against metal exposure (Wu et al. 2011; Yang et al. 2012; Zhang et al. 2009, 2013; Zheng et al. 2013).

According to the mortality data, the 14-day LC50 for Cd registered in the present study (354 mg Cd/kg soil) was similar to that obtained by Fitzpatrick et al. (1996) in OECD soils (374 mg/kg dw) and is in agreement with the range of 300 to 2,000 mg Cd/kg soil dw reported in OECD 10 soil (Asensio 2009; Neuhauser et al. 1985; Rodríguez-Ruiz 2010; Spurgeon et al. 1994; Spurgeon & Hopkin 1995). This heterogeneity among values could be due to differences in soil physicochemical properties (Lock & Janssen 2001), which can be found even between OECD soils prepared in different laboratories (Bielskà et al. 2012). Certainly, although earthworms presented high Cd tissue concentrations, no mortality was recorded in OECD 14. Thus, OECD 14 soil exerted a protective role for earthworms exposed to Cd (LC50 $>$ 625 mg Cd/kg soil dw). Likewise, growth was affected by Cd exposure in OECD 6 and 10 soils but not in OECD 14 soil. Nevertheless, the effect on growth was not dependent on the Cd concentration in the soils; therefore, critical toxic values could not be calculated. In addition, in the present study the control earthworms also lost weight, thus indicating that the experimental conditions had been suboptimal. Indeed, the negative effects of OECD soils in toxicity assays have been repeatedly reported, and modifications concerning OM content have been proposed (Spurgeon et al. 1994; van Gestel et al. 1989).

Adults exposed in OECD 14 soils for 28 days tended to gain weight compared with earthworms exposed to the same Cd concentration (5 and 25 mg Cd/kg dw) in soils with less OM content, which is in agreement with previous studies (Rodríguez-Ruiz 2010). However, data on growth could be directly related with the decreased humidity of soils. OM confers greater WHC to soils, and thus the water required to reach the optimal humidity for the preparation of OECD soils is directly proportional to the OM content. However, during the RT, soils were moistened equally leading to a lower moisture level in OECD 14 and a higher level in OECD 6 soils. Because soil humidity is directly related with earthworm wet weight (Lee 1985; Rundgren 1975), it is possible that the measured weight values were more dependent on soil hydration than OM or Cd concentrations.

Reproduction was 5 to 7 times more sensitive to Cd exposure than mortality, which is in agreement with previous reports on metal toxicity to earthworms (Asensio 2009; Rodríguez-Ruiz 2010; Scott-Fordsmand et al. 1998; Spurgeon et al. 1994; van Gestel et al. 1991, 1993). The 56-day EC50 for cocoon production after Cd exposure was reported to be in a wide range between 22.0 and 91.4 mg Cd/kg soil dw in OECD soil (Lock & Janssen 2001; Spurgeon et al. 1994; van Gestel et al. 1993), which is slightly higher than the results presently obtained with OECD 10 soil. However, effects on reproduction have been recorded after exposure to concentrations as low as 18 mg Cd/kg (van Gestel et al. 1992). Thus, greater OM contents in soil seem to be protective for *E. fetida* reproduction, whereas Cd toxicity increases extremely when OM content is decreased to 6 %. Similarly, Ávila et al. (2009) observed that OM modulated the reproductive toxicity of Cu.

NRRT and NRU assays have been successfully employed as sensitive and early warning biomarkers in different earthworm species exposed to metals (Asensio et al. 2007; Asensio 2009; Gupta 2000; Maleri et al. 2008 Scott-Fordsmand et al. 1998; Weeks & Svendsen 1996). In addition to its high sensitivity and rapid responsiveness against a wide range of pollutants, its simplicity and low cost makes the NRU assay a suitable tool for ecotoxicity assessment (Asensio et al. 2013). Nevertheless, NRRT and NRU assays do not necessarily correlate with ATT and RT because interindividual variability can be high and because membrane stability can be affected by conditions other than the presence of toxic metals (Asensio 2009; Rodríguez-Ruiz 2010; Scott-Fordsmand et al. 1998). Presently, the results confirm that NRU assay is suitable to assess Cd toxicity in *E. fetida* after 3-day exposure in artificially polluted OECD soils; nevertheless, the response pattern is intricate due to the interactions between Cd, OM content, and earthworm condition.

First, a bimodal dose–response curve of NRU response was observed in the OECD 10 soil, which draws an initial decrease of NRU followed by an increase as previously described (Asensio 2009; Irizar 2013). The initial NRU decay would reflect a general stress associated with sublethal toxicity, whereas the subsequent increase and decrease in NRU would be the result of coelomocyte mortality (Asensio 2009). Environmental stressors provoke alterations in the eleocyte-to-amoebocyte (the two major types of coelomocytes) ratio (Plytycz et al. 2009, 2010a, b, 2011a, b). Thus, in vitro exposure of coelomocytes to a variety of metals seems to cause a decrease in eleocyte numbers respective to the amoebocyte numbers, which could result in an increase in NRU (e.g., presumably by phagocytosis of dead eleocytes by amoebocytes) and a further decrease in NRU as the numbers of cells decrease. This second peak in NRU has been shown to precede individual death in *E. fetida* (Asensio 2009). Presently, similar results were obtained. The remarkable weight loss (125 mg Cd/kg soil dw) and 100 % mortality recorded on exposure to 625 mg Cd/kg soil dw in OECD 10 soil for 14 days were preceded by increased NRU (second peak of the bimodal NRU response).

Second, this bimodal response was not observed in OECD 6 and 14 soils. In case of OECD 14 soils, the lower bioavailable Cd concentration (Devliegher & Verstraete 1996) and greater food content would decrease metal toxicity. However, the Cd tissue concentration in earthworms in OECD 14 soil was as high as that in OECD 10 soil both after 14 days (without food supply) and 28 days of exposure (with food supply). As previously discussed, similar toxicity values would be expected in earthworms treated with OECD 10 and 14 soils that exhibited equivalent CBRs for Cd. Thus, it might be assumed that Cd bound to OM is less toxic for coelomocytes than the fraction of Cd dissolved in porewater that would enter the dorsal pores (Saxe et al. 2001; Vijver et al. 2003) and impact directly on coelomocytes, with dermal uptake being more relevant in OECD 10 than in OECD 14 soils. However, this argument also would imply that both bioaccumulation and toxicity would be the highest in OECD 6 soil, and this is not the case because Cd tissue concentrations are the lowest and the NRU response is apparently intermediate between those found in the other two soils. It might be possible that the second peak of the bimodal NRU response was anticipated by Cd concentrations in OECD 6 soil <25 mg Cd/kg soil dw (NRU is slightly greater than in the control group) such that the NRU decrease recorded at 125 mg Cd/kg soil dw would correspond to the second part of the aforementioned bimodal response curve. Indeed, weight loss and mortality recorded in the ATT and the RT showed a high toxicity of Cd in OECD 6 soil. However, differences between the NRU values recoded in OECD 6 soil in the range of 0 to 25 mg Cd/kg soil dw are seemingly trivial. Feeding activity and growth, and its dependence on OM and their interactions with Cd bioavailability, may be crucial to interpret our results. Low Cd tissue levels and high weight loss were recorded in OECD 6 (i.e., at 125 mg Cd/kg soil dw in Tables 4, 5) as was limited reproduction success (even below the minimum OECD standard for the controls in the RT), which might be related to poor nutritional status in a soil with too low an OM content that does not seem to be optimal for *E. fetida*.

In summary, it can be concluded that OM content in soil affects both Cd bioaccumulation and toxicity in *E. fetida*. However, the response pattern is intricate because it results from the interaction between a lower capacity for metal retention in soils with low OM content and the consequences of the nutritional restrictions created when OM content in soil is low. Whereas Cd tissue concentration is greater in soils with high OM content (OECD 10 and 14) than in soils with low OM content, the effects of Cd exposure on NRU are highest in OECD 10 soil and the effects on mortality, growth and reproduction less marked in soils with high OM content (OECD 14 soil). Moreover, it seems that low OM content in soil has an effect on reproduction even in absence of pollutants. Therefore, it can be concluded that using OECD standard soil in toxicity testing provides unrealistic estimates of Cd bioavailability and toxicity for environmental risk assessment in soil unless the OM is considered as a variable in the design of the toxicity test battery. The uncertainty might be diminished by applying a suite of bioassays and biomarkers that would enable us to understand the mechanisms of bioaccumulation and toxicity. Further research regarding in vitro exposure of coelomocytes would help avoid the confounding effects inherent to the natural variability in the physicochemical properties of the soil (e.g., OM content).

Acknowledgments This research was supported by the Basque Government (ETORTEK IE10-273) and Grant to the Consolidated Research Group (GIC07/26-IT-393-07; IT-810-13) as well as the University of the Basque Country (Research and Formation Unit in Ecosystem Health Protection [UFI 11/37]). A. Irizar was the recipient of a predoctoral fellowship from the Basque Government.

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