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Assessing ecotoxicity and uptake of metals and metalloids in relation to two different earthworm species (*Eiseina hortensis* and *Lumbricus terrestris*)

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A B S T R A C T

Due to diffuse atmospheric fallouts of process particles enriched by metals and metalloids, polluted soils concern large areas at the global scale. Useful tools to assess ecotoxicity induced by these polluted soils are therefore needed. Earthworms are currently used as biotest, however the influence of specie and earthworm behaviour, soil characteristics are poorly highlighted. Our aim was therefore to assess the toxicity of various polluted soils with process particles enriches by metals and metalloids (Pb, Cd, Cu, Zn, As and Sb) collected from a lead recycling facility on two earthworm species belonging to different ecological types and thus likely to have contrasted behavioural responses (*Eiseina hortensis* and *Lumbricus terrestris*).

The combination of behavioural factors measurements (cast production and biomass) and physico-chemical parameters such as metal absorption, bioaccumulation by earthworms and their localization in invertebrate tissues provided a valuable indication of pollutant bioavailability and ecotoxicity. Soil characteristics influenced ecotoxicity and metal uptake by earthworms, as well as their soil bioturbation.

Keywords:

Earthworms
Inorganic trace element
Ecotoxicity
Cast production
Soil properties

1. Introduction

In the last decade, the soil area polluted by atmospheric fine particles enriched with metals and metalloids (noticed PM) has increased (Schreck et al., 2011), especially due to the development of metal recycling activities (Foucault et al., 2013; Uzu et al., 2009). As reported by Cecchi et al. (2008), the different stages in the recycling process in these facilities lead to PM emissions which contaminate the soil and therefore have a potential impact on terrestrial ecosystems (Schreck et al., 2011). Earthworms are the dominant soil organisms in most temperate and tropical grassland soils (Lee, 1985). As reported by Nahmani et al. (2007a) they modify chemical and physical soil parameters, mix leaf litter with the soil, form and stabilize aggregates and generate soil porosity. According to Ruiz et al. (2011), they can also influence the behaviour of inorganic elements

in soils (pollutants and nutrients). Earthworms are therefore important terrestrial model organisms for toxicity testing to assess environmental pollution (Schreck et al., 2008; Capowiez et al., 2010; Schreck et al., 2012). These soil organisms can provide important information about environmental risks and could serve as useful biological indicators of contamination because of the fairly consistent correlation between the concentration of some contaminants in their tissues and in soils (Nannoni et al., 2011). Pauget et al. (2011) concluded that only partial information is obtained from the chemical extractions currently used to assess metal bioavailability: biota measures are therefore needed for risk assessment studies.

In their review about the interactions between earthworms and inorganic trace element (ITE), Nahmani et al. (2007b) considered several parameters such as earthworm species, soil type, metal type and experimental conditions. They suggested several priority areas for further studies: in particular, other earthworm species besides *Eisenia fetida* (currently used in normalized ecotoxicity tests) should be studied in order to apply the large existing database on this

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earthworm to other soil dwelling species. Then, so that studies from different research experiments can be accurately compared, a set of standard protocols for the exposure and depuration periods is needed and precise parameters, such as soil chemical and physical properties, should be defined. It was also recommended that more field or terrestrial model studies using real contaminated soils rather than metal-amended artificial soils should be performed. Recently Schreck et al. (2011) showed that ageing can influence metal transfer and ecotoxicity. Furthermore, although ITE bioaccumulation by earthworms has been widely studied for one ITE in particular (Nahmani et al., 2007b) and with spiked soils (Nahmani et al., 2007a,b), studies on multi-metal pollution under field or close to real conditions remain not numerous (Andre et al., 2010a, 2010b; Nannoni et al., 2011). It is increasingly accepted that for risk assessments, measures of total ITE concentrations must be complemented with data on the available or mobile ITE concentrations in soils. Therefore numerous studies reported the use of chemical extractions such as CaCl_2 extraction to assess metal bioavailability (Uzu et al., 2009; Schreck et al., 2011). Actually, these chemical extractions can break bonds between metals and clay or organic matter, releasing metals and making them bioavailable for earthworms (Owojori et al., 2009). Moreover, few soluble and unfixed to organic matter particles are then available for earthworms via soil ingestion.

According to Morgan and Morgan (1999), earthworm behaviour such as burrowing and feeding affect their exposure to the pollutants. Parameters such as the earthworm species and soil conditions could therefore strongly influence metal ecotoxicity (Tomlin, 1992). In addition, as mentioned by Capowiez et al. (2010), there is currently a lack of ecotoxicity tests adapted to ecologically relevant earthworm species and with endpoints which could be directly related to their ecological role in the soil. A new behavioural biomarker, cast production, was thus recently proposed by Capowiez et al. (2010). This test was first elaborated for *Lumbricus terrestris* (high bioturbation activity) under laboratory conditions, but was then adapted to *Aporrectodea caliginosa* (Dittbrenner et al., 2011). Once deposited on the soil surface, PM can have deleterious effects on soil micro and macrofauna. Earthworm soil activities such as burrowing, casting and mixing of litter and soil (bioturbation) have important consequences for soil processes and properties. Thus earthworms may also modify PM fate, mobility (Sizmur and Hodson, 2009), availability (Ruiz et al., 2011) and speciation. However, those modifications will be less pronounced if PM toxicity affects earthworm behaviour (Vorenhout et al., 2000). It is therefore important to first assess any behavioural changes in earthworms which may be induced by PM.

The aim of the present study was to assess the toxicity of various polluted soils on two earthworm species (*Eisenia hortensis* and *Lumbricus terrestris*). The soils were collected from a lead recycling facility and contaminated with Pb, Cd, Cu, Zn, As and Sb. The two earthworm species belong to two different ecological types and are thus likely to have contrasting behavioural responses. The original and innovative aspect of this paper consists in the utilization of historically polluted soil from a lead recycling facility. In this way, a cocktail of metals and metalloids, as well as the influence of different soil characteristics, could be studied, to carry out complementary ecotoxicity tests. Then, metal bioaccumulation studies and microscopy experiments will be performed to better understand metal localization and tissues storage in earthworms in order to investigate the uptake pathway.

2. Materials and methods

2.1. Soil preparation

The heavily polluted soil (noted as S1) used in this study was collected from a lead recycling facility at a chemical metal treatment company (STCM) which

currently recycles batteries. The facility is located in the urban area of Toulouse, in the southwest of France (43°38'12" N, 01°25'34" E). For several decades, the industrial activities at the site have produced high concentrations of Pb and other ITE such as Cd, Cu, As, Sb, Zn in surface soils (Uzu et al., 2009; Schreck et al., 2011). This historically polluted soil (from the facility courtyard) was sampled from the top 0–25 cm soil layer, air-dried at ambient temperature for a week, disaggregated and, finally, sieved to retain aggregates smaller than 2 mm. Total ITE concentrations were measured according to Dumat et al. (2006) by inductively coupled plasma atomic emission spectrometry (ICP-AES) on an IRIS Intrepid II XXDL apparatus, following triacid (HF, HCl and HNO_3) digestion of the solid samples. A standard reference soil (GRX2) was used to verify the digestion protocol and measurement accuracy. All the results correspond to the mean of two replicates. Values were the following: Pb = 39800, Sb = 2095, Zn = 294, As = 288, Cu = 286 and Cd = 18.36 mg kg^{-1} . This highly contaminated soil was used to establish a pollution concentration gradient to which earthworms were exposed.

The polluted soil was diluted with two uncontaminated control soils: referred to as S2 and S3, according to Uzu et al. (2009). The soil was then left in the open air to stabilize for one month (Schreck et al., 2011). The uncontaminated S2 had similar characteristics to polluted S1 (pH, texture and OM %) whereas S3 was poorly enriched in organic matter and more calcareous. This soil preparation procedure allowed both the influence of ITE concentration and soil type (organic matter concentration and pH, in particular) on earthworm ecotoxicity to be studied. Table 1 provides details on the concentrations and some physico-chemical information of these three soils measured according to normalized procedures.

Table 2 shows the notations used and ITE concentrations in the soils used. When S1 is pure, the pollutant concentration is maximal, noted as C_5 -S1. When the uncontaminated soils S2 and S3 are pure, the concentrations are minimal and noted as C_0 -S2 and C_0 -S3, respectively. The following notations were then used: (C_1 -S2, C_2 -S2, C_3 -S2 and C_4 -S2) or (C_1 -S3, C_2 -S3, C_3 -S3 and C_4 -S3) when mixtures of (S1 + S2) or (S1 + S3), respectively, were studied. For convenience, the different dilutions were referred to for their final total lead concentration: $C_1 = 100$; $C_2 = 825$; $C_3 = 1650$; and $C_4 = 4000 \text{ mg kg}^{-1}$. The emission of particles from the recycling plant is the source of the S1 soil pollution. These PM sources were characterized and their solubility measured by Uzu et al. (2009). All the process particles contained the same major lead phases (Pb, PbS, PbO, PbSO_4 and PbO, PbSO_4) but differed on the nature and amount of minor phases. The size distribution of the particles was previously determined by laser granulometric analyses using a Malvern Mastersizer S: the PM mainly contains fine particles (91% are less than 10 μm). Their total element contents were determined by ICP-OES Intrepid II XXDL after heat digestion (Uzu et al., 2009): lead was the main metal (33.4%) and the other metal contents were Cd, Sb, As, Cu and Zn at 2.7, 1.8, 0.09, 0.09 and 0.7% respectively. The estimation of their metal solubility was performed using 0.01 M CaCl_2 extraction procedure.

2.2. Earthworms

Two earthworm species (*Eisenia hortensis* and *Lumbricus terrestris*) were chosen in the experimental set-up. *Eisenia hortensis* is an epigeic species closely related to *Eisenia fetida*, which is currently used for normalized ecotoxicity tests (Fuller-Espie, 2010). *Lumbricus terrestris* is an anecic species that dominates earthworm biomass in various temperate ecosystems and strongly affects organic matter transformation and soil development (Maleri et al., 2008). Adult earthworms (*L. terrestris* and *E. hortensis*) were purchased from a local supplier (Decathlon®, France). Before exposure experiment, earthworms were acclimatized during one week in the experiment conditions (temperature, obscurity, soil moisture...).

2.3. Experimental set-up

Small plastic Petri dishes (diameter = 8 cm and height = 3 cm) were filled with 100 g of wet soils sieved to 2 mm. Earthworms were added in the boxes: one earthworm by petri dish for *L. terrestris* and *E. hortensis*. Six experimental series were carried out simultaneously for each soil and each earthworm species, one series for each metal concentration. Each series consisted in seven replicates with either *L. terrestris* or *E. hortensis* and three boxes were used as controls without

Table 1
Detailed characteristics of each of three soil types used in this study.

Soil	Soil characteristics
S1	pH = 6 Organic matter: 5.2% 12% clay, 47% silt and 41% sand
S2	pH = 5.7 Organic matter: 4.5% 13% clay, 48% silt and 38% sand
S3	pH = 7.6 Organic matter: 1% 22% clay, 26% silt and 52% sand

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Table 2

Metals and metalloid concentrations in the studied soils (mg kg⁻¹).

Concentrations	Pb	Cd	Cu	Zn	As	Sb
C ₀	19.2	0.27	21.6	75.7	0.05	0.05
C ₁	100	0.31	22.3	76.2	0.7	5.3
C ₂	825	0.64	27.1	80.2	6	43.4
C ₃	1650	1	32.6	84.8	11.9	86.8
C ₄	4000	2.1	48.2	97.6	28.9	210.5

earthworms. Earthworms were exposed for 14 days. The boxes were stored in a dark chamber at a constant temperature of 12 °C. At the beginning of the experiment, soil moisture was measured and adjusted to 19%, corresponding to 65% and 75% of water holding capacity respectively for S2 and S3, then checked regularly by weighing the Petri dishes and adjusted to the desired value by adding distilled water and mixing the soil (without destroying the casts).

2.4. Ecotoxicity parameters

To evaluate the impact of contaminated soils on earthworms, three endpoints were monitored: mortality, biomass changes and cast production (CP). Earthworm weight was determined at the beginning of the experiment, after 7 days and at the end of the exposure period. For this, earthworms were rinsed in distilled water and gently dried on filter paper (without gut voiding). Biomass changes were expressed as a percentage of the initial biomass.

According to Capowiez et al. (2010), CP is an ecologically meaningful and promising biomarker for ecotoxicity tests, but some aspects should be carefully considered for this marker to be widely used and accepted. First of all, preliminary tests need to be carried out to adapt (i) mesh size and test duration to the earthworm species under investigation and (ii) soil water content to soil texture. The key parameters are the soil moisture content and the initial soil particle size distribution. CP was therefore measured according to the protocol recently described by Capowiez et al. (2010) and modified by Dittbrenner et al. (2011). Casts were separated using a set of four sieves (diameter = 15 cm and mesh sizes = 5.6, 4, 3.15, 2.5 mm). The principle is that earthworm activity will modify soil granulometry leading to an increase in the amount of soil retained in some sieves (casting) and a decrease in others (soil consumption). All soil from each Petri dish, including the soil that adhered to the walls of the dishes which was removed with a knife, was sieved taking care not to break up the casts. The set of sieves was manually shaken for 10 s. The soil retained in each sieve was weighed. The effect of earthworm bioturbation was then examined by determining the changes in the particle size distribution (PSD), i.e. weight of fresh soil in each sieve minus the corresponding weight of soil for the control soil (without earthworm). CP was measured immediately after the 14th day of exposure. It was expressed as cast fresh weight per earthworm fresh body mass per day.

2.5. Metal bioaccumulation

ITE concentrations in earthworm bodies were determined after acidic digestion. Invertebrates were digested with a mixture of 1 mL HNO₃ 70% and 0.5 mL of fluorhydric acid 51% in Teflon bombs placed on heating plates at 90 °C for 2 days. After evaporation, 1 mL H₂O₂ 30% and 1 mL of HNO₃ 70% were added and the Teflon bombs replaced on heating plates at 90 °C for 2 days. The samples were then evaporated at 50 °C. Ultra-high purity water was used for dilutions. Solutions were stored at 4 °C in polyethylene tubes before analysis. ITE concentrations were measured by inductively coupled plasma-optical emission spectrometry ICP-OES (IRIS Intrepid II XXDL) or inductively coupled plasma-mass spectrometry ICP-MS (X Series II, Thermo Electron). Ten blank samples were submitted to the same treatment (mineralization and assay) for method control. They were performed to verify the non-contamination during acidic digestion. Each sample was analysed in triplicate. The detection limits for Pb, Cd, Sn, Sb, As, Cu and Zn were 0.3, 0.2, 0.2, 0.2, 0.2, 1.3 and 2.2 µg L⁻¹ respectively, whereas, the limits of quantification were about 0.4, 0.3, 0.3, 0.4, 0.3, 2 and 3 µg L⁻¹ respectively for the same inorganic trace elements. Measurement accuracy was verified using reference material: lobster tissue (TORT-2) was used as a reference material for earthworm mineralization whereas standard soil reference material (from National Institutes of Standards and Technology: NIST) was used for soil acidic digestion. The concentrations found were within 95–102% of the certified values for all measured elements. Internal ITE concentrations were indicated as (IC). Following Nannoni et al. (2011), Bio Accumulation Factors (BAF) were calculated as the ratio of the element concentration in the earthworm (in mg kg⁻¹ of dry weight) to the total soil element content (in mg kg⁻¹ of dry weight).

2.6. Micro-X-ray fluorescence (µXRF) experiments

In order to investigate the distribution of accumulated Pb within earthworm tissues, X-ray fluorescence micro-spectroscopy (µ-XRF) analysis was performed on

transversal histological sections of *L. terrestris* earthworms in the intestinal region. For µXRF experiments, earthworms were exposed to the most polluted soil (S1) during 14 days and lead was the principal metal investigated because of its high content in S1. µXRF measurements were performed after a gut-voiding period on filter paper during five days. During the analysis the samples were fix and kept frozen using a Peltier freezing system. Experiments were carried out using a microscope (XGT7000, Horiba Jobin Yvon) equipped with an X-ray guide tube producing a finely focused beam with a 10 µm or 100 µm spot size (Rh X-ray tube, 30 kV, 1 mA equipped with an EDX detector), as described by Lapiéd et al. (2011).

2.7. CaCl₂ extraction

To assess the available fraction of inorganic pollutants in the soil, extraction with neutral salts is the most useful indication (Menziez et al., 2007). A 0.01 M CaCl₂ extraction procedure gives a good indication of lead availability. The CaCl₂ procedure was performed on S2 + S1 and S3 + S1 for all concentrations. 30 mL of 0.01 M CaCl₂ solution was mixed with 3 g of soil (1:10 solid solution ratio) in 50 mL polypropylene centrifugation tubes placed on a shaker table (Heidolph promax 1020) at 50 oscillations min⁻¹ for 2 h, 20 °C. After extraction, the tubes were centrifuged at 10,000 g for 30 min. The supernatant liquid was then filtered (0.22 µm), acidified to 2% with distilled HNO₃ (15 M, suprapur 99.9%) and stored at 4 °C before analysis. Extracted ITE concentrations were determined by inductively coupled plasma atomic emission spectrometry (ICP-AES) on IRIS Intrepid II XXDL apparatus.

2.8. Statistical analyses

Results were expressed as means ± SD (standard deviation). The relationships between CP and earthworm fresh weight were assessed using linear regression. To study the effects of pollutants concentrations on CP or weight loss, we performed a one way ANOVA. All tests were carried out using the R[®] 2.14.1 package software. Statistical differences were accepted when the probability of the result assuming the null hypothesis (*p*) was less than 0.05.

3. Results

3.1. Mortality and biomass changes

Throughout the experimental period, no earthworms died in the uncontaminated control soils (C₀). Thus, this suggests that the experimental conditions were valid in terms of providing suitable media for earthworm survival. Regardless of the concentration of polluted soil applied, earthworm mortality was less than 5% in S2 and no mortality was observed for S3. For both earthworm species, the most significant weight losses were always observed following incubation in polluted soil S1 (Fig. 1) and the mortality recorded in this S1 soil was about 10.5%. After 14 days of exposure to increasing concentrations of ITE, the *L. terrestris* biomass was significantly reduced in S2 at concentrations C₃ and C₄ compared to the control group and increased significantly for C₁ and C₂. In contrast, no significant differences were found for worms incubated in the S3 soil, regardless of the pollutant concentration. Similar results were observed for *E. hortensis* (Fig. 1), but weight losses were only significant for the C₄ concentration in S2 (-6.6%). No significant decreases in weight were observed for any pollutant concentration mixed with S3 control soil, however earthworm weight did significant increase after 14 days at concentrations C₂, C₃ and C₄.

3.2. Cast production

According to Fig. 2, both earthworms' species produced significantly less casts in the polluted soil S1 compared to the control group. For *L. terrestris*, CP was significantly lower compared to the control group at the C₄ concentration in S2, with a decrease of 30.5%. In contrast, no significant differences in CP were observed in S3 soil mixed with polluted soil compared to controls (Fig. 2). For *E. hortensis*, a significant decrease was observed for the C₃ and C₄ concentrations in S2, with a decrease in CP of approximately 65.5% and 99.5%, respectively, compared to controls. As observed for *L. terrestris*, no significant differences were observed for S3 conditions (Fig. 2).

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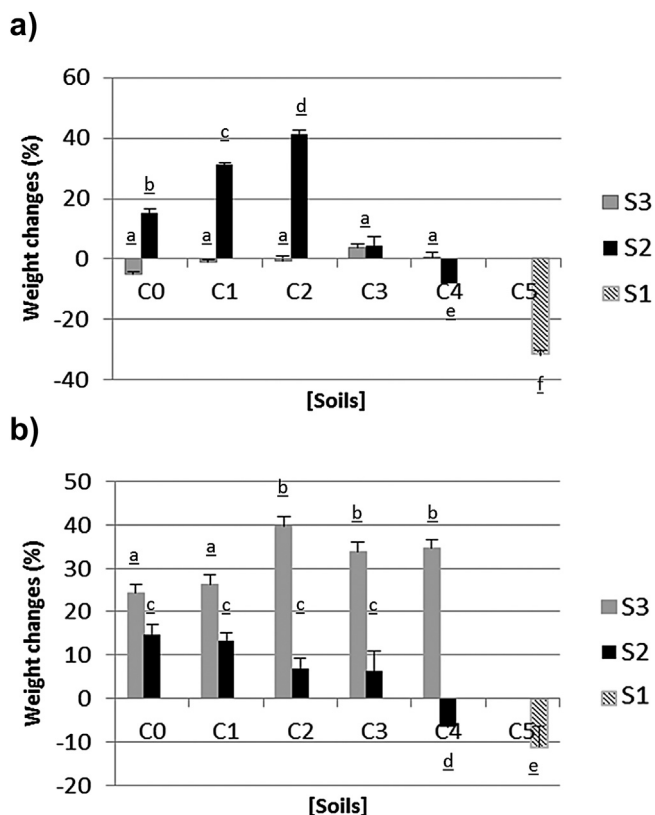


Fig. 1. Biomass changes as a percentage of the initial mass at the end of the 14 day experiment for *Lumbricus terrestris* (a) and *Eisenia hortensis* (b) incubated in different concentrations of polluted soil (a,b,c,d,e,f weight changes significantly different ($p \leq 0,05$) for one way ANOVA.).

3.3. Metal bioaccumulation

The mean concentrations of Pb, Cd, Cu, As, Sb, and Zn (expressed as mg kg^{-1} dry weight) in *L. terrestris* and *E. hortensis* specimens are reported in Table 3 for S1 and in Fig. 3 for S2 and S3. Differences were observed between the element contents in tissues and between the two earthworm species for all the concentrations. Except for Zn, the ITE concentration in *L. terrestris* and *E. hortensis* exposed to C₂, C₃, C₄ was higher than in the control group, in both soil types. Internal concentrations of pollutant (except for Zn and Cd) in *L. terrestris* were significantly higher in earthworms incubated in S2 than in S3 (Fig. 3). IC of Pb and Sb in *E. hortensis* were higher in earthworms incubated in S2 than in S3 (Fig. 3). Table 4 shows all the bioaccumulation factors (BAF) for *L. terrestris* and *E. hortensis*. For both species, BAF values were ranked as follows: Cd > Zn >> As > Cu > Pb Sb. Only Cd and Zn had BAF values above 1, indicating that the two elements were not only taken up but also bio-accumulated in the earthworms. For the other ITE, BAF values were below 1.

3.4. Lead distribution within earthworm tissues: μXRF

The distribution of accumulated Pb within transversal histological sections of earthworms in the intestinal region was performed by $\mu\text{-XRF}$ and microscopy observations were given in (Fig. 4). Results showed that even after depuration, Pb remains localized in the gut wall and above all in the longitudinal muscles of exposed earthworms (Fig. 4). The mean XRF spectra generated from the hyperspectral maps and the presence of the fluorescence lines

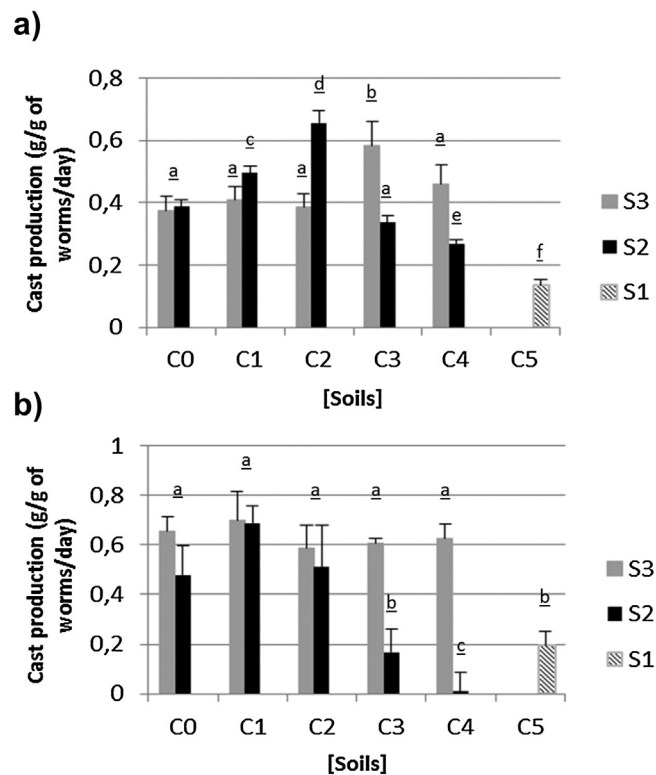


Fig. 2. Casts produced over the course of the experiment (g g^{-1} of earthworm day^{-1}) by *Lumbricus terrestris* (a) and *Eisenia hortensis* (b) incubated in different concentrations of polluted soil (a,b,c,d,e cast production significantly different ($p \leq 0,05$) for one way ANOVA.).

at 10.5 KeV (Pb L α emission line) and 12.6 KeV (Pb L β emission line) confirmed the presence of Pb (Fig. 4).

3.5. CaCl₂ extraction

Table 5 shows the results of CaCl₂ metal extraction from S1, S2 and S3 (expressed as a percentage of the soil concentration) and from the PM emission (expressed as the percentage of total metal content.). The fraction of extractable ITE was always higher from S2 than S3 for all the considered ITE. Table 5 reported the values only for C₄ concentration because ITE extractable fraction was not detectable by ICP-OES for C₁, C₂ and C₃ concentrations.

4. Discussion

4.1. Viability and ecotoxicity

This experiment focused on the effects of a cocktail of ITE (lead being the major pollutant), on earthworm health and behaviour. Lethality effects were only observed in the soil containing the highest ITE concentrations ($40,000 \text{ mg Pb kg}^{-1}$ and other ITE, see Table 1). These results confirm that the control experimental conditions were suitable for earthworm survival and that PM polluted soil is not toxic enough to have lethal effects on earthworms until

Table 3
Internal concentrations of metals in *Lumbricus terrestris* and *Eisenia fetida*.

		Pb	Cd	Cu	Zn	As	Sb
S1	<i>Lumbricus terrestris</i>	8659	26.2	85.3	503.2	24	695
	<i>Eisenia hortensis</i>	932	7.2	23.9	130.6	8.2	45.9

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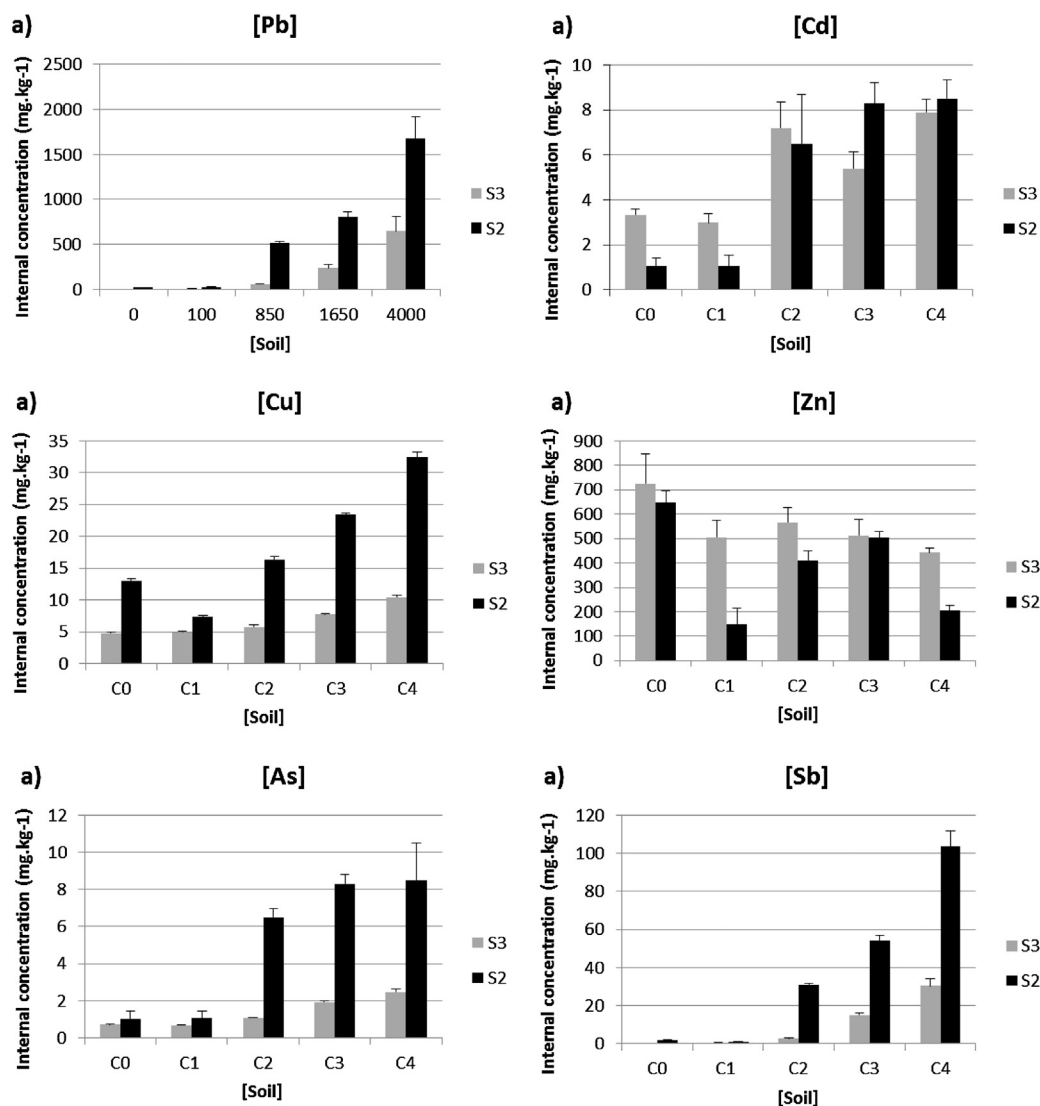


Fig. 3. Total internal content of each element in *Lumbricus terrestris* (a) and *Eisenia hortensis* (b) incubated in S2 and S3 and different concentrations of polluted soil.

4000 mg Pb kg⁻¹. Davies et al. (2003) measured a LC₅₀ of 5311 mg Pb kg⁻¹ for earthworms exposed for 14 days to nitrate lead spiked soil. This lower lethal concentration could be explained by the different experimental conditions. The stability and lack of lethality for biological organisms observed here were previously reported by Schreck et al. (2011) working with the same long-term polluted soil.

However, several sublethal effects were observed in this Petri dish experiment. Changes to physiological and behavioural endpoints such as biomass and CP were observed at the highest concentrations. These effects suggest that atmospheric pollution can have a significant impact on earthworm physiology (locomotion, nutrition...). As shown in Table 6, ITE can impact earthworm physiology and ecotoxicity depends on earthworm species and soil characteristics. Indeed, Reinecke et al. (2001) studied the cocoon viability of three earthworm species following exposure to substrates contaminated with lead nitrate, cadmium sulphate and zinc sulphate for 36 months. They concluded that different earthworm species can have significantly different sensitivity to ITE. As reported by Ernst et al. (2008), soil properties can modify metal bioavailability and their subsequent impacts on earthworm physiology and behaviour. In the present study we compared a clayed,

organic matter rich, acidic soil (S2) with a higher pH sandy soil (S3), and showed that S3 had less impact on earthworm health. Furthermore, extraction with CaCl₂ (Table 4) to simulate solid-solution transfer for pollutants, showed that metals were more mobile in the more acidic soil S2 which suggests that soil pH plays an important role. According to Bradham et al. (2006), soil pH can increase the fraction of soluble metals. Vandecasteele et al. (2004) reported that, compared to the surrounding environment, earthworm biomass was four times lower in contaminated dredged sediment-derived heavy clay soils, but comparable when earthworms were incubated in sandy loam soils. The same results were observed in the present experiment.

4.2. Metal bioaccumulation

Excepted for Zn, a significant (r^2) positive linear correlation was observed between ITE concentration in earthworms and total contents in soil, for both earthworm species (Table 7). Logically, the highest ITE concentrations were found in earthworms incubated in S1 (historically polluted soil). These findings are consistent with previous studies (Morgan and Morgan, 1993, 1998). Unlike the other ITE, there was no linear relationship between the internal Zn

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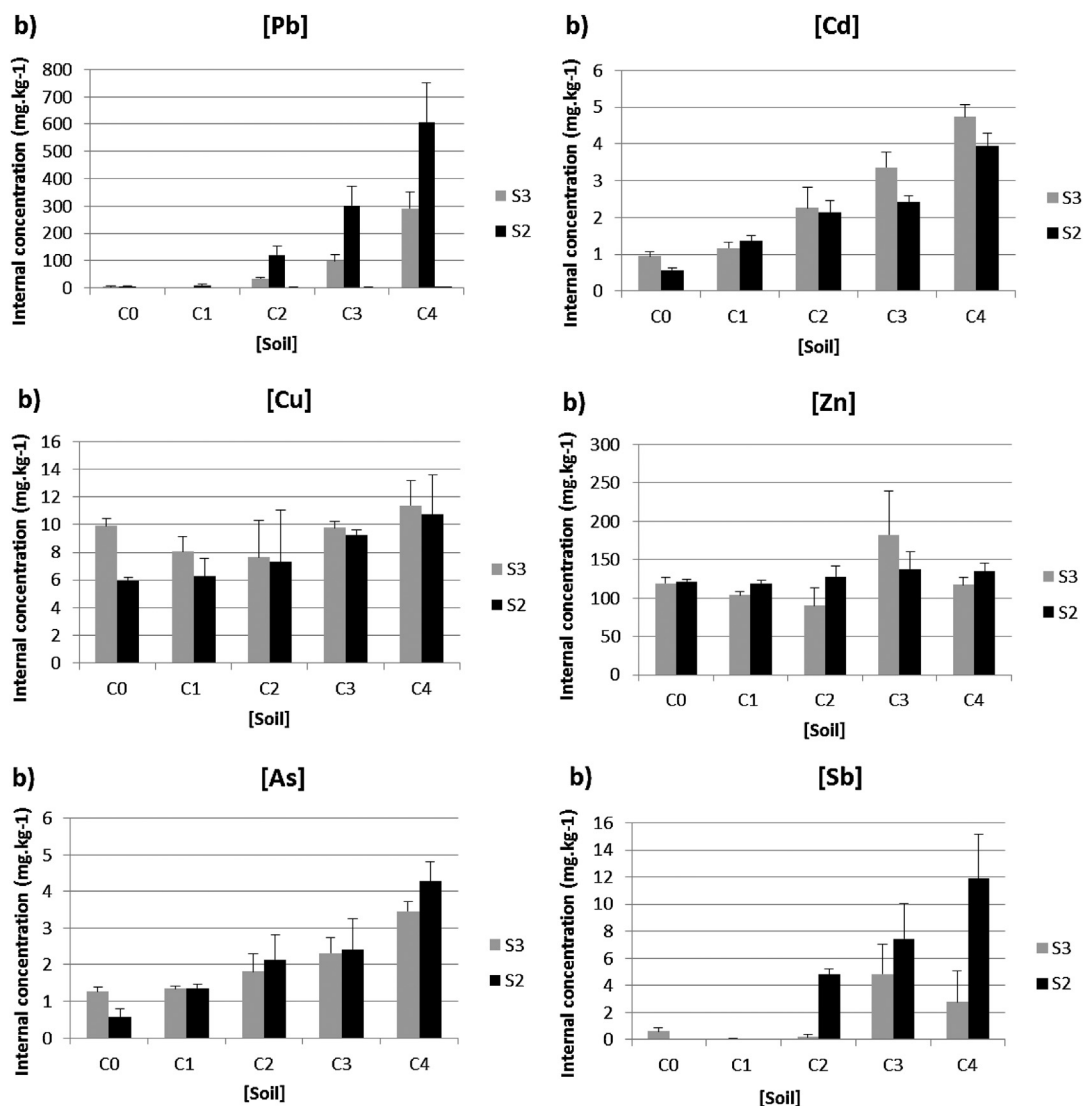


Fig. 3. (continued).

concentration in earthworms and its total soil content. Indeed Zn is an essential element and its internal level is regulated directly by earthworms (Nannoni et al., 2011). It has been suggested that in earthworms Zn plays essential roles (respiratory control and tissue growth, development and regeneration processes) suggesting that it is specifically taken up by earthworms (Wròblewski et al., 1979; Morgan, 1984). In addition, the bioavailable fraction estimated by 0.01 M CaCl₂ extraction (Table 4) showed that Zn should be highly bioavailable to earthworms (in comparison with other metals), even if its soil concentration was low. In this present study, the BAF for Zn was above 1, which indicates that Zn was bioaccumulated by the two earthworm species. The efficiency of Zn accumulation probably relates to a necessity for a stored pool of available Zn in anticipation of future physiological demand (Nannoni et al., 2011). At such high internal exposure levels, earthworms must either suffer from Zn toxicity or have adapted to resist toxicity, resulting in a higher capacity to store this element (Hobbelen et al., 2006).

Despite its toxicity, Cd was also highly accumulated by earthworms. This phenomenon could be explained by its high mobility and availability in soil together with its chemical analogy with Zn (Li and Thornton, 2001; Nannoni et al., 2011). In a smelter contaminated area in northern Kosovo, Nannoni et al. (2011)

previously observed that earthworms bioaccumulate both Zn and Cd. Thus, internal concentrations and bioaccumulation factor values both suggest that earthworms could be used as bioindicators of ITE pollution in soils. In addition, less uptake and bioaccumulation of all ITE was observed in *E. hortensis* than in *L. terrestris*. Indeed, Suthar et al. (2008) reported that a species-specific metal accumulation pattern depending on the earthworm ecological type could be observed. They suggested that species-specific feeding behaviour, earthworm niche structure, the ecological category of inhabiting earthworms and even the horizontal distribution of contaminants in soil layers are major determinants for metal accumulation patterns in soil dwelling earthworms. The well-characterized differences in burrowing patterns between endogeic and anecic worms could influence the patterns of metal bioaccumulation, although other factors may also contribute (Suthar et al., 2008). Nannoni et al. (2011) did not observe any significant differences in terms of metal uptake and bioaccumulation between different species within a same ecological group. Lukkari and Haimi (2005) showed that *Eisenia fetida* (epigeic) appeared to be more tolerant to metals than *A. tuberculata* (endogeic) and seemed to regulate the tissue metal concentrations more strictly. Morgan and Morgan (1999) studied the accumulation

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Table 4
 Heavy metal bioaccumulation factors (BAF) for *L. terrestris* and *E. hortensis*.

	Sols	Pb	Cd	Cu	Zn	As	Sb	
<i>Lumbricus terrestris</i>	S2	C ₀	1.2	3.9	0.6	8.55	ND	ND
		C ₁	0.19	3.33	0.33	1.97	1.454	0.19
		C ₂	0.62	10.1	0.6	5.12	1.090	0.71
		C ₃	0.41	8.13	0.27	5.93	0.695	0.78
		C ₄	0.42	4.07	0.67	2.10	0.294	0.49
	S1	C ₅	0.31	1.8	0.42	2.27	0.121	0.46
	S3	C ₀	0.22	12.33	0.22	9.58	ND	ND
		C ₁	0.06	9.46	0.22	6.63	0.93	0.10
		C ₂	0.07	11.16	0.21	7.05	0.18	0.06
		C ₃	0.14	5.28	0.23	6.04	0.16	0.17
<i>Eisenia hortensis</i>	S1	C ₄	0.16	3.77	0.21	4.53	0.08	0.14
		C ₅	0.12	1.06	0.17	1.14	0.04	0.19
	S2	C ₀	0.28	2.11	0.27	1.60	ND	ND
		C ₁	0.07	4.29	0.28	1.56	1.87	ND
		C ₂	0.14	3.30	0.27	1.59	0.35	0.11
		C ₃	0.18	2.37	0.28	1.62	0.20	0.08
		C ₄	0.15	1.89	0.22	1.38	0.14	0.05
	S1	C ₅	0.03	0.40	0.07	0.44	0.05	0.04
	S3	C ₀	0.26	3.49	0.45	1.57	ND	ND
		C ₁	0.003	3.70	0.36	1.36	1.85	ND
	C ₂	0.04	3.50	0.28	1.12	0.30	0.004	
	C ₃	0.06	3.29	0.30	2.15	0.19	0.05	
	C ₄	0.07	2.26	0.23	1.2	0.12	0.01	
S1	C ₅	0.02	0.38	0.09	0.44	0.007	0.004	

of ITE by two ecologically contrasting earthworm species and found that Cd, Cu and Pb concentrations were significantly higher in *A. caliginosa* (endogeic) than in *L. rubellus* (epigeic). However, concerning Zn accumulation, a difference between the two earthworm species was only observed at the lowest soil concentrations, suggesting differences in metal behaviour inside the earthworms, mostly related to elimination mechanisms (Morgan and Morgan, 1999). Internal concentrations and bioaccumulation factors were also different depending on the soil characteristics. According to our results, soil properties influence ITE bioavailability in relation to mobility (solid–solution transfer) and consequently the mechanisms of transfer (ingestion or transdermal uptake). van Gestel et al. (2011) reported that Mo uptake by earthworms is affected by soil pH, with the highest uptake levels occurring at a pH = 4.

Table 5
 The lead fraction extractable using the 0.01 M CaCl₂ procedure in PM and S1 and at the C₄ concentration in S3 and S2.

Soil	Metal extractable by CaCl ₂ (% of total metal content)			
	Zn	Cu	Cd	Pb
S1	1.91 ± 0.6	0.5 ± 0.1	1.1 ± 0.5	0.3 ± 0.1
S2	5.2 ± 0.4	1.9 ± 0.2	4.5 ± 0.1	0.5 ± 0.05
S3	4.1 ± 0.2	4.9 ± 0.4	4 ± 0.1	0.3 ± 0.05
PM	3.1 ± 0.8	ND	9.8 ± 0.4	0.01

*Note: ITE extractable fraction was not detectable by ICP-OES for C₁, C₂ and C₃ concentrations.

Diez-Ortiz et al. (2010) also found that BAF total values for metal uptake in *E. andrei* correlated with the soil organic matter content. In the present experiment, ITE were generally more bioavailable in S2 which was acidic and enriched in organic matter and uptake of Pb, Cu, Sb and As by earthworms (especially for *L. terrestris*) was also higher when they were incubated in this soil type. As reported by Nannoni et al. (2011), organic matter is the main food source for earthworms and consequently represents an important source of ITE, which are largely bound to this component and later absorbed in the gut. Cecchi et al. (2008) observed a strong association of lead with the soil organic matter from a historically polluted soil impacted by process particles from lead recycling batteries. Thus, S2, enriched in organic matter, was a more important source of toxic ITE than S3 for the two species in the study. In contrast, the same proportion of Cd and Zn, which are more mobile in soils, bioaccumulated regardless of the soil type. These two ITE are more present in soluble and extractable fractions, allowing an easy uptake in sandy soil.

4.3. Uptake mechanisms and ecotoxicity test sensitivity

Even at very low metal concentration in solution (maximum 0.5% of lead was extracted by CaCl₂ for more acidic soil S2), the absorption of lead by earthworms was observed. Our hypothesis is therefore a strong absorption of metals from solid particles throughout the gut and only a small absorption achieved throughout teguments. Depending on ITE type, the mechanism by

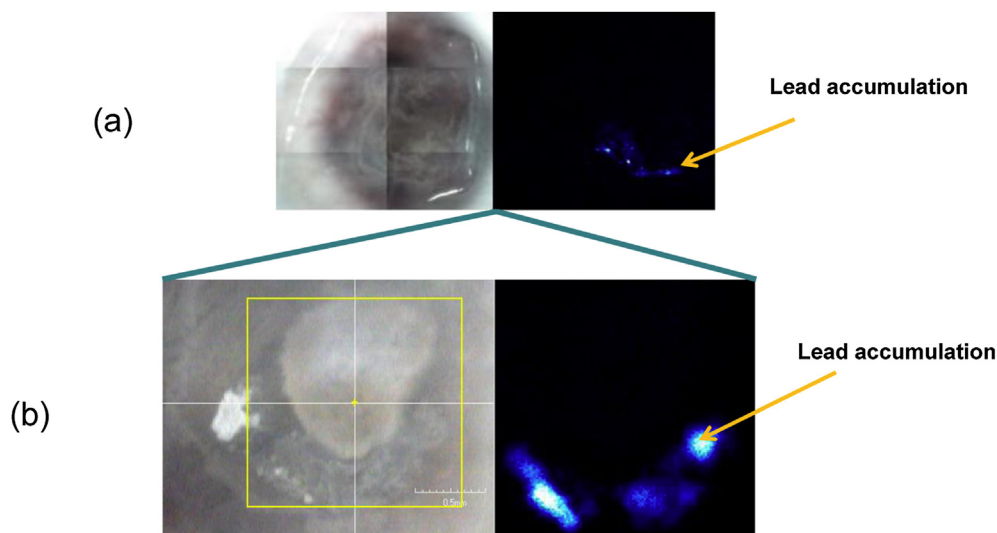


Fig. 4. Micro-XRF images of a transversal section (10× magnification) of the earthworm *L. terrestris* (intestinal region, about 10 segments behind the clitellum) after exposure to S1. (a) Cross-section of *L. terrestris* (after 14 days of exposure to S1) observed by μXRF. Lead localization (1 pixel = 14 μm, 512 px, 1000 s/px). (b) Zoom on the cross section. Lead localization (1 pixel = 12 μm, 128 px, 1000 s/px) in the gut wall and the longitudinal muscles of earthworm.

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Table 6
 Review of ITE impact on earthworms.

References	ITE	Soil total content	Time of exposure	Earthworm species	Soil preparation	Observation
Zheng and Li, 2009	Pb	0–7500 mg kg ⁻¹	14 days	<i>Pheretima guillelmi</i>	Soil spiked with lead nitrate solution	- LC50 = 4300 mgPb kg ⁻¹ soil - Biomass and locomotion decreased at 2800 mgPb kg ⁻¹ soil
Nahmani et al., 2007a,b	Cd, Cu, Fe, Pb, Zn, Ag, Al, Ba, Sr	30–42,100 mgPb kg ⁻¹ 120–53,400 mgZn kg ⁻¹ 0.5–347 mgCd kg ⁻¹	7,14,21,28 and 42 days	<i>Eisenia fetida</i>	Historically polluted soils	- Effect on earthworm survival, body weight, cocoon production and hatching - Those effects are influenced by soils parameters (texture, pH, [Ag], [Cd]...)
Lukkari and Haimi, 2005	Cu, Zn	2–246 mgCu kg ⁻¹ 13–453 mgZn kg ⁻¹	48 h–14 days	<i>Aporrectodea tuberculata</i>	Soil spiked with copper and zinc solution and field soils	- Artificial soils: (Cu/Zn) LC50 = 134/234 mg kg ⁻¹ soil - Field soils: (Cu/Zn) LC50 = 333/582 mg kg ⁻¹ soil - Avoidance test: 80% at 79/138 (Cu/Zn) mg kg ⁻¹ soil
Wu et al., 2012	Pb, Cd	0.1–10 mgCd kg ⁻¹ 5–500 mgPb kg ⁻¹	7 days	<i>Eisenia fetida</i>	Soil spiked with lead and cadmium solution	- Cd + Pb: inhibit cellulase activity - For low [Cd], addition of Pb = ↗ DNA damages - For high [Cd], addition of Pb = ↘ DNA damages
Li et al., 2008	Cd, Zn	0.1 μM–10 μMCd 0–1000 nn	2 days	<i>Eisenia fetida</i>	Cattle manure spiked with simulated soil solution	- LC 50 = 145 μM Zn - LC 50 = 316 μM Zn with pre exposure of Cd (1 μM) - Cd uptake is regulated by Ca
Jones et al., 2009	Pb	300–2249 mgPb/kg	4–90 days	<i>Eisenia fetida</i>	Soil spiked with lead nitrate solution	- LC50 = 2490 mgPb kg ⁻¹ soil - Effect on reproduction (cocoon count and juvenile count) at 400 mgPb kg ⁻¹ soil
Owojori et al., 2008	Zn	0–750 mgZn kg ⁻¹	28 days	<i>Eisenia fetida</i>	Soil spiked with ZnCl ₂ and NaCl solution	- Mortality observed at 750 mgZn kg ⁻¹ soil - Cocoon viability decreased at 500 mgZn kg ⁻¹ soil - ↗ toxic effects with salinity

which earthworms are exposed can vary: direct soil ingestion versus transfer through the teguments (Vijver et al., 2003). The study of lead localization in earthworm tissues by μXRF have bring precious information about lead distribution in exposed earthworms and could bring hypothesis on the uptake way of Pb. Actually, as showed by the Fig. 4, Pb remains localized in the gut wall and the longitudinal muscles of exposed earthworms. These kind of observations were recently reported by Kılıç (2011) working with autometallography in the same earthworm species: their observations revealed that heavy metals were found to be accumulated mainly in circular and longitudinal muscles of gut. By the way, ITE absorption by earthworms often depends on the proportions of reducible and oxidable fractions in the soil, suggesting also that the intestine is likely the most important uptake route (Nannoni et al., 2011). If bioavailable ITE concentrations are high in the solid soil phase and low in the soil solution, the absolute uptake of metals could be higher via the gut exposure route than via the dermal exposure route (Hobbelen et al., 2006) (Fig. 5). It is possible that ITE which bind strongly to organic matter in earthworm food, are taken up in the gut, as was found in this study for S2. However, as previously mentioned, the water soluble fraction plays an important role especially for the most mobile elements such as Cd and Zn, suggesting that dermal uptake is considerable, especially in acidic soils such as the experimental S2. To conclude, Cœurduassier et al. (2002) suggested that digestive and cutaneous contamination routes for Cd are responsible for sublethal toxic effects on snail growth in laboratory bioassays. However, cutaneous transfer of Cd was thought to be a secondary intoxication pathway, compared to digestive absorption, in the accumulation and induction of toxic effects in natural conditions.

Finally, antagonistic and synergistic phenomena between ITE can also occur. Wu et al. (2012) recently reported that Cd and Pb

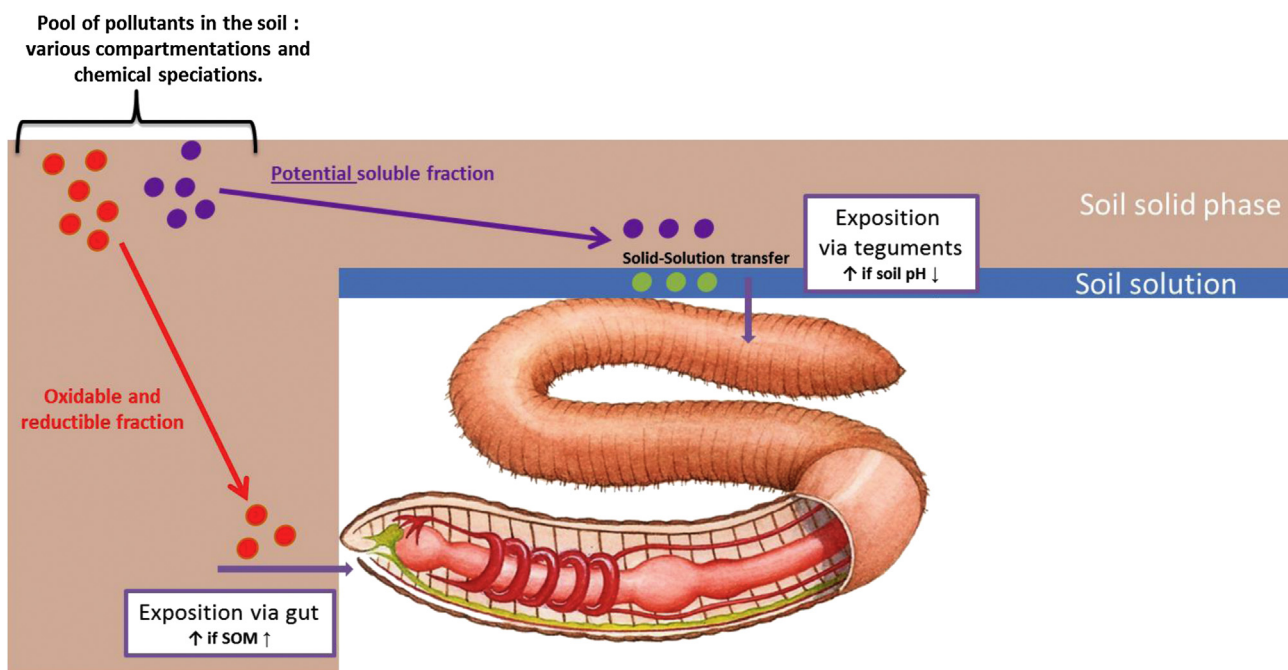
have combined and complex toxicological effects on earthworms. Cd bioavailability is often modified by its competitive sorption with Pb in soil fractions. Li et al. (2008) found that the presence of Pb significantly decreased the percentage of Cd in the cytosolic fraction but increased it in the cell membrane fraction of *E. fetida*, which could influence Cd bioaccumulation and toxicity. In the case of acute toxicity due to a cocktail of metals on an estuarine meiobenthic harpacticoid copepod, the mixture had a significantly greater than additive effect on survival, with the mixture being more toxic than that expected by simple additivity of the toxic effects of each metal (Hagopian et al., 2001). In order to assess ITE ecotoxicity, it is necessary to perform several complementary tests with various sensitivity levels. Indeed, the use of internal ITE concentrations in earthworms appears to be an effective and sensitive indicator of soil contamination, even in the case of pollution due to atmospheric fallout deposition near a lead recycling factory. This extractable fraction could become a good predictor of ITE bioavailability and thus ecotoxicity in soils, taking into account soil properties, ITE type and mobility. Lukkari and Haimi (2005) reported that a battery of tests on earthworms (avoidance, acute toxicity and reproduction tests) can be an efficient tool to assess the ecological risks of contaminated soils. However, careful choice of

Table 7
 Linear correlation coefficients between the internal heavy metal concentration in earthworms and the total element content in the soil.

Soil	Species	Pb R ²	Cd R ²	Cu R ²	Zn R ²	As R ²	Sb R ²
S2	<i>Lumbricus terrestris</i>	0.9987	0.9522	0.9866	0.252	0.9589	0.9996
	<i>Eisenia hortensis</i>	0.8454	0.85	0.939	0.0809	0.975	0.9967
S3	<i>Lumbricus terrestris</i>	0.9986	0.9336	0.9992	0.5137	0.9962	0.9993
	<i>Eisenia hortensis</i>	0.8752	0.7237	0.9827	0.0114	0.9943	0.7371

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Fig. 5. Heavy metal exposure pathways in earthworms related to soil characteristics.

the species and even populations is largely recommended to gain the most insight into soil contamination and its toxicity for invertebrates.

5. Conclusions and perspectives

This study focused on the ecotoxicity of two soils polluted with a cocktail of metals and metalloids on two earthworm species belonging to two different ecological types (*Eisenia hortensis* and *Lumbricus terrestris*). Earthworms were exposed under laboratory conditions but with a realistic source of pollution using soil collected from a lead recycling facility. The combination of behavioural factor measurements (cast production) and earthworm ITE absorption, their bioaccumulation and localization in invertebrate tissues provided a valuable indication of pollutant bioavailability and ecotoxicity. The soil properties significantly affected the impact of soil ecotoxicity and ITE uptake by earthworms, as well as their bioturbation activities in the soil.

Studies examining uptake pathways and toxicity mechanisms are still in progress on various metals and the effect of ITE on metabolic enzymes or gene expression which could be highly sensitive markers, should be investigated further. The role of earthworms in the fate of ITE in the environment (phytoavailability and human bioaccessibility) associated with their burrowing activities should also be investigated in further studies.

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