Comparative Biochemistry and Physiology, Part C 255 (2022) 109276

Contents lists available at ScienceDirect

Comparative Biochemistry and Physiology, Part C

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

Research article

Impacts of sewage sludges deposition on agricultural soils: Effects upon model soil organisms

Erik Urionabarrenetxea, Nerea Garcia-Velasco, Beñat Zaldibar, Manu Soto *

Cell Biology in Environmental Toxicology (CBET) Research Group, Dept. Zoology and Animal Cell Biology, Faculty of Science and Technology and Research Centre for Experimental Marine Biology and Biotechnology PIE-UPV/EHU, University of the Basque Country UPV/EHU, E-48080 Bilbao, Basque Country, Spain.

A R T I C L E I N F O

Edited by Martin Grosell

Keywords:
Waste landfills
Polluted soils
Earthworms
Lettuce
Standard tests
Biomarkers

A B S T R A C T

During years sewage sludges have been worldwide poured in agricultural soils to enhance vegetal production. The “Landfill 17” located in Gernika-Lumo town (43°19′28.9”N 2°40′30.9”W) received for decades sewage sludges from the local Waste Water Treatment Plant (WWTP) with agricultural purposes. To this WWTP, several pollutants as heavy metals (Cd, Cr, Ni, Pb), PAHs (benzo(*a*)pyrene among many others) and pesticides (i.e. dieldrin) could have arrived from local industry and be widespread all over the landfill. Soil invertebrates like earthworms and plants are of special interest due to their close contact with the polluted matrix and their potential effects by the presence of pollutants. In this context, the aim of the present work was to determine the health status of landfill soils by evaluating the effects on model soil organisms exerted by long-lasted pollutants after on site deposition of WWTP active sludges. With such a purpose, different standard toxicity tests and cellular level endpoints were performed on lettuce and earthworms. Indeed, germination (EPA 850.4100) and root elongation (EPA 850.4230) tests were carried out in *Lactuca sativa*, while OECD acute toxicity test (OECD-204), reproduction test (OECD-222) and Calcein-AM viability test with coelomocytes were applied in *Eisenia fetida* worms. For the exposure, soils collected in the landfield containing low, medium and high concentrations of pollutants were selected, and as reference LUFA 2.3 natural standard soil was chosen. While no differences were shown in the assays with L. *sativa*, significant differences between sludge exposed groups and control group were recorded with *E. fetida*, with lower coelomocyte number and viability and higher tissue metal accumulation after 28 days of exposure to polluted soils. These results confirmed the impact of contaminants to soil biota even after long periods of time.

1. Introduction

The scientific and technological advances during the last century allowed to quadruple world population (Gómez-Sagasti et al., 2019). This demographic boost, along with a shortage of usable land and the establishment of a consumer culture, brought with the increase of landfills and spilling or filling points. In the Basque Country Government inventory (Decree No.165/2008, relative to soils supporting potentially polluting activities or facilities), 1277 landfills are inventoried, including spilling points. That inventory includes areas as the Landfill 17, where sewage sludges from Gernika-Lumo Waste Water Treatment Plant (WWTP) have been poured for decades as fertilizers for agricultural purposes. However, along with the organic matter (OM), different industrial, commercial or domestic pollutants that could not be eliminated by the WWTP ended up in soil. In the aforementioned area (WWTP northern lands) sewage sludges were spilled over 250 m setting the Landfill 17 with an area of 3.38 Ha.

Some soil organism based guidelines use vegetal processes to address the toxicity of pollutants. OECD (2003) and USEPA (1996) recommend seed germination and root elongation bioassays carried out with lettuce (*Lactuca sativa L.*), especially due to their usefulness to evaluate pollutants toxicity in a simple, fast and sensitive manner (Bagur-González et al., 2010; Adamo et al., 2014). Lettuces are widely used in soil health evaluations due to their ability to extract pollutants by roots and accumulate them in roots and shoots (Adamo et al., 2014; Mtisi and Gwenzi, 2019).

Earthworms are efficient metal accumulators and respond to pollution in a sensitive and measurable manner. For that reason, the use of these sentinel organisms is widely used in soil pollution studies. Among the most used earthworms *E. fetida/E. andrei* species must be

* Corresponding author.
E-mail address: manu.soto@ehu.eus (M. Soto).

https://doi.org/10.1016/j.cbpc.2022.109276
Received 15 October 2021; Received in revised form 23 December 2021; Accepted 19 January 2022
Available online 31 January 2022

highlighted. These earthworms have been broadly used on soil standard toxicity tests (OECD, 1984; ISO 2008) or field studies (Sinha et al., 2008; Urionabarrenetxea et al., 2021) to evaluate the potential risk exerted by pollutants or determine the soil health status of a given area. Acute toxicity tests, allow to determinate different lethal and effect parameters (LCx,ECx), helping on risk evaluation or regulation of chemical compounds (Spurgeon et al., 1994). The OECD artificial soil tests (OECD, 1984) are based on the two principal uptake paths for testing substances: the dietary (soil ingestion) and the dermal (contact of the epidermis with soil) (Laycock et al., 2016; Verdú et al., 2018) to study then changes upon live parameters like earthworms mortality and weight loss. For these tests, OECD or LUFA standard substrates are employed, allowing obtaining same results in different region laboratories and facilitating comparisons and increasing the reliability of established toxicity measurements (Lopes Alves and Nogueira Cardoso, 2016). Indeed, LUFA soils are natural substrates used especially for studies according to GLP (Good Laboratory Practice) investigating pot experiments in the laboratory and in the field (Garcia-Velasco et al., 2017; Urionabarrenetxea et al., 2020).

In chronic ecotoxicological assays, reproduction is a very important factor due to its role on population dynamics (Kooijman and Metz, 1984; Spurgeon et al., 1994; Hertel-Aas et al., 2011). In fact, changes on growth or reproduction could be useful elements to predict changes at population level (Moriarty, 1983). Among these tests, OECD-222 (2016) was designed to evaluate the sublethal effect of different chemicals (single or combined) upon reproduction parameters, such as counting the offspring (cocoons and juveniles) of worms grown in polluted soil.

Apart from the standardized tests, in the recent years a large list of biomarkers have been developed to study sublethal effects exerted by pollutants, and so, evaluate soil health status. Biomarkers provide useful information about the impact exerted by pollutants upon organisms at different levels of biological complexity (Marigómez et al., 1996; Cajaraville et al., 2000). In the case of earthworms, biomarker approach has targeted their immune cells or coelomocytes. Actually, the cellular responses measured in coelomocytes demonstrated the capacity to predict impairments caused by polluted soils at organism level (Curieses et al., 2017). Among in vitro approaches carried out with earthworm coelomocytes, the Calcein AM test is a rapid and accurate microplate assay to assess the impact of chemical substances upon cells viability (Claiborne, 1985). During the last decades, toxicity of single elements (Cd, Cr, Ni, Pb, Hg, BaP, TPH, etc.), complex mixtures (pesticides, metal complexes or organic compounds) or natural soils have been studied in *E. fetida* organisms, using for that the integration of chemical analysis, standardized tests and biomarkers (Irizar et al., 2014a, 2014b, 2015a; Li et al., 2019; Deng et al., 2021).

When assessing soil toxicity, aging is a key factor to be considered. Heavy metals are mostly available immediately after poured and availability decreases over time (Wijayawardena et al., 2015) as reactions between metal and soil ions transform highly soluble forms into less soluble forms (Kulikowska et al., 2015). Thus, metal toxicity is expected to decrease over time as adsorption, complexation, chelation, exchange, diffusion or precipitation processes occur (Jalali and Khanlari, 2008).

The aim of this work is to determine the health status of landfill soils by evaluating the effects on model soil organisms exerted by long-lasted presence of pollutants after on site deposition of WWTP active sludges.

2. Materials and methods

2.1. Experimental organisms

2.1.1. Lettuce: Lactuca sativa

Lactuca sativa seeds for germination and root elongation studies were purchased from Ariatza Lorategia commercial supplier (Bizkaia, Spain). Seeds were maintained at constant temperature (4 °C) in darkness conditions on hermetically closed packages with humidity not exceeding 10%. The seeds selected for the tests were free of abnormalities such as lesions, decolouration or swelling.

In addition, stock germinability was tested before the assays in order to ensure a minimum germination of 75%.

2.1.2. Earthworms: Eisenia fetida

For the present work *Eisenia fetida* earthworms were purchased from Lombrinatur commercial supplier (Almeria, Spain). Earthworms were maintained as stock at constant temperature (19 °C) and humidity (60%) conditions, and weekly fed with horse manure. The earthworms used for this experiment were all healthy, clitellated and of similar size (300–600 mg fresh weight).

2.2. Soil conditioning for testing

Agricultural soils amended with sewage sludges were collected from Landfill 17, located in Gernika-Lumo (Basque Country, Spain; 43°322,434 N, −2°675,425 W). Based on previous chemical characterizations (up to 18 sample points for a complete chemical characterization), soils containing high (H), medium (M) and low (L) lead, nickel, cadmium and chromium contents were chosen among the soil sample collection (Table 1). Glass containers (1.8 l) were filled with 500 g of each soil and moistened to 60% of their water holding capacity (WHC, previously measured) to be left stabilizing for 48 h at constant temperature (19 °C) and humidity conditions (60%). At the same time, LUFA 2.3 (Speyer, Germany) natural and standard soil was used as control.

2.3. Leachate preparations

Soil leachates were prepared as indicated by Spanish legislation (BOE, 2005 516/id) following German standard DIN 38414-S4 (Deutsches Institut fur Normung, 1984 480/id). For that, 10 g of soil (dry weight) were mixed with 100 ml distilled water and the mixture was stirred for 24 h at room temperature to be filtered through 0.45 μm. This process was carried out with H, M and L soil samples. Leachates were chemically analysed and stored at 4 °C until its use in germination and elongation tests.

2.4. Lactuca sativa seeds exposure to leachates from polluted soils

2.4.1. Germination test (modified from EPA 850.4100)

Seeds ($n = 15$) were placed in Petri dishes filled with 15 g of silica sand and 3 ml of leachates coming from the different soils, 3 replicates for each experimental group (H, M and L). In control treatments distilled water (DW) was used. In addition, and in order to validate the test, a positive control was carried out with seeds exposed to legal threshold concentrations for public park use ($n = 3$; 25 mg kg^{-1}ppm Cd, 400 mg kg^{-1}ppm Cr, 500 mg kg^{-1}ppm Ni and 450 mg kg^{-1}ppm Pb, IHOBE, 2015). Samples were kept in darkness, constant temperature (22 °C) and humidity conditions (70%) until measurements at times 48 h and 7 d.

2.4.2. Elongation test (modified from EPA 850.4230)

Pre-germinated seeds ($n = 25$) were transferred (t0) to Petri dishes

Table 1
Organic matter (OM, in %), pH, cadmium (Cd), chromium (Cr), nickel (Ni) and lead (Pb) contents (mg kg^{-1}) in Landfill 17 soils with low (L) medium (M) and high (H) heavy metal concentrations. Concentrations and contents measured in dry weight (D.W.).

	Control	L	M	H
Organic matter (LOI) %	±1	6.2	11	19
pH	6.81	7.42	6.28	7.30
Cadmium (Cd)	<LOD	0.33	8.7	26
Chromium (Cr)	<LOD	41	190	400
Nickel (Ni)	<LOD	32	56	120
Lead (Pb)	<LOD	27	96	170

containing filter paper moistened with 3 ml of soil leachate or DW in the case of control treatments. Germinates were allowed to grow for another 48 h (tf) in same conditions (22 °C, 70% humidity and 16/8 light-dark period) to then measure root length (tf-t0).

2.5. *Eisenia fetida* exposure to polluted soils

2.5.1. Short term exposure (modified from OECD- 207, 1984)

Earthworms of similar size were weighted in groups of 10 and introduced in containers with 750 g soil from the landfill (H, M and L), with 3 replicates per treatment. All the experimental groups were maintained at 19 °C and constant light conditions without feeding in order to guarantee permanent earthworm-soil contact. After 3 days, weight loss and mortality were measured, earthworms were collected for chemical analysis and Calcein-AM viability test was carried out with earthworm coelomocytes.

2.5.1.1. Coelomocyte extrusion and counting. Coelomocytes of the earthworms exposed during 3 days to landfill (H, M, L) and control soils were extruded through the dorsal pores by electrical stimulation (9 V) in extrusion solution (1 ml, PBS% 0.02 EDTA / worm) (Irizar et al., 2014a). The obtained solution was introduced in falcon tubes to be centrifuged at 530*g* and 10 °C for 10 min (Allegra™ 17 25R Centrifuge Beckman Coulter). The supernatant was obtained, removed, and the pellet resuspended in PBS cleaning solution (Saline phosphate buffer, pH 7.0–7.2). Subsequently, cell counting was carried out by using a haemocytometer, in order to determine cell density and adjust the same final concentration to 1×10^6 cells / ml (200,000 cells / well) in all the treatments. Cell solutions were placed in 96-well microplates and incubated at 18 °C (Sanyo Incubator MIR-152) for 30 min so they could adhere to the bottom of the wells.

2.5.1.2. Calcein-AM viability assay. For the Calcein-AM viability assay the plate was centrifuged (530 *g*, 5 min, 10 °C), supernatant was discarded and the cells were incubated for 40 min with 2.5 M Calcein-AM ($n = 4$, 100 μl per well). Celomocytes were washed twice (centrifugation, supernatant discard and addition of 100 μl of PBS) and the fluorescence was measured on a FLx fluorimeter for plates at 490 nm wavelength for excitation and 520 nm wavelength for emission. Additionally, basal fluorescence derived from riboflavin containing eleocytes was measured in wells without Calcein and subtracted to the wells incubated with the later.

2.5.2. *Eisenia fetida* reproduction test (OECD-222, 2016)

E. fetida adult earthworms ($n = 10$) between 0.3 and 0.6 g were introduced in containers with 500 g of soil, 3 replicates per group. Earthworms were maintained under 19 °C constant temperature for 28 days under dark-light (8:16 h) cycles. 5 g of horse manure per container was weekly added as organic matter input. Moisture content was weekly controlled until last day of experimentation, while food input was only made for the first 28 days following protocol recommendations. After 28 days of experiment, adult earthworms were removed, weighed, and samples were taken for chemical analysis by inductively coupled plasma spectrometry (ICP-MS) (see 2.6), leaving the cocoons and juveniles in the same experimental conditions already mentioned. At day 56, juveniles were counted, and juveniles' biomass was measured.

2.6. Sample treatment for chemical analysis

For plants chemical analyses, seedlings were previously dried at 36 °C for 72 h in oven, milled and digested with HNO_3. All analyses were carried out by ICP-MS.

Five earthworms per replicate ($n = 15$ per experimental group, H, M and L) from each test (Short term and reproduction test) were used to perform chemical analysis. After voiding their gut content, earthworms were dried in an oven at 120 °C (WTC Binder) for 48 h up to dry. Once dried, earthworms were weighted and rinsed in HNO_3 (Tracepur®, 69% 1 M). After digestion, acid dissolutions were placed on a hot plate until the acid was evaporated. Then, 6 ml of HNO_3 0.01 M were added to be maintained at 4 °C in covered test tubes until IPC-MS analysis was performed (SGiKER General Services of EHU/UPV Leioa).

2.7. Statistical analysis

Data obtained was studied by SPSS software vers. 22. Data normality was checked through Kolmogorov–Smirnov and Shapiro-Wilk tests. For the parameters with normal distribution, a unidirectional variance analysis (ANOVA) was performed. Possible significant differences between the control and the contaminated samples were studied by post-hoc Dunnet and Tukey tests ($p < 0.05$). The non-parametric data was processed by carrying a Kruskal-Wallis test (Dunn's test multi-comparison test).

3. Results

3.1. Experiments with *Lactuca sativa*

3.1.1. Chemical analysis of the leachates

The leachates coming from the landfill soils showed different levels of metals according to the metal contents in soils. The group H showed the highest values of heavy metals, group M presented intermediate values and the lowest heavy metal concentrations were observed in L group (Table 2).

3.1.2. Germination test (modified from EPA 850.4100)

After 3 days of exposure, germination showed similar values in the control and in seeds exposed to leachates coming from landfill soils. Lowest germination values were measured in the groups M and H, with a 66% and 70% of germination respectively (Fig. 1). This was not the case for the groups exposed to public park use limit values (positive control), where no seed germination was observed (Fig. 1). After 7 days of exposure to leachates, germination continued showing similar trends as at day 3 (Fig. 1). Seeds exposed to leachates coming from M and H soils showed the lowest germination rates, while the highest rates were quantified in the control group (88%) and in those exposed to L for 3 days.

3.1.3. Root elongation test (modified from EPA 850.4230)

After pre-germination and two days of exposure to leachates, no significant differences on root elongation were observed among groups; however, trends of highest values in M group and lowest in H group were recorded.

The chemical analysis of the lettuce seedlings exposed for two days to leachates coming from polluted soils showed different trends on metal accumulation despite no significant differences were observed among groups (Table 3). Seedlings exposed to H leachates showed higher Pb and Ni accumulations (13.89 and 8.79 μg/mg, respectively). However, low Cd (<0.16 μg/mg) and Cr (<0.26 μg/mg) accumulations were observed in the four experimental groups.

Table 2
Contents of Cr, Ni, Cd and Pb (ng/ml) on the leachates obtained from Landfill 17 soils. * Metal values on the leachate of the Control group (LUFA 2.3) showed values below detection limit.

	Control	L	M	H
Cr	<LOD	4.7	12.6	31.5
Ni	<LOD	4	2.9	17.7
Cd	<LOD	0.5	1	2
Pb	<LOD	2.7	5.8	12.4

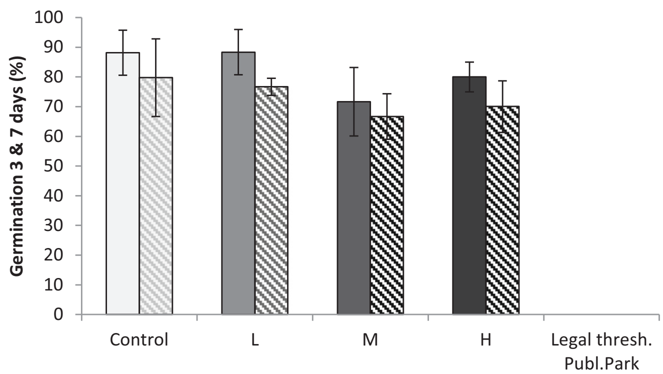

Fig. 1. Germination percentages of *Lactuca sativa* seeds maintained at 20 °C and exposed to leachates coming from landfill soils with different metal concentrations for 3 and 7 days. Bars without and with lines represent 3 and 7 days respectively. *No germination was observed (0%) on seeds exposed to (soil) limit values for public park use. Error bars represent standard deviation.

Table 3
Cr, Ni, Cd and Pb concentration (μg / mg) in *Lactuca sativa* seedlings, after exposure to leachates of different study soils for 2 days. * In control, Cr, Ni, Cd and Pb concentrations in lettuce tissues were found below detection limit.

	Control	L	M	H
Cr	<LOD	<LOD	<LOD	0.26
Ni	<LOD	4.64	3.29	8.79
Cd	<LOD	0.08	0.12	0.16
Pb	<LOD	3.56	3.9	13.89

3.2. *Eisenia fetida exposure to polluted soils*

3.2.1. *Short term exposure (modified from OECD- 207, 1984)*

Weight loss after 3 days of exposure did not show a clear pattern between groups of exposure. In fact earthworms exposed to different landfill soils for 3 d lost similar weight (<10%). No mortality was observed in any of the replicates.

3.2.1.1. Coelomocyte number. The cell counting after three days of exposure showed a lower concentration of extruded coelomocytes on those organisms exposed to landfill soils comparing to the LUFA soils (Fig. 2).

The highest number of cells was counted in the control group (Fig. 2) while the lowest concentration of cells was quantified in those earthworms exposed to M and H soils, with 72% and 62% fewer cells than in the control group. In L soils, 40% less cells than in control group were measured.

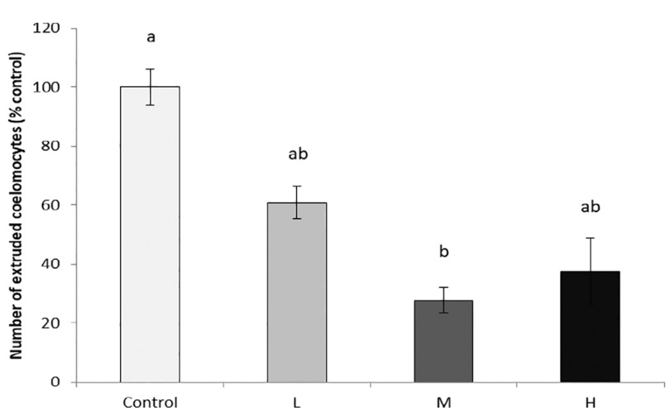

Fig. 2. Coelomocyte of *E. fetida* earthworms exposed to landfill soils with different metal concentrations during 3 days. Significant differences marked with letters (ANOVA test, $p < 0.05$). Error bars represent standard deviation.

3.2.1.2. Calcein AM viability test. Coelomocytes of earthworms exposed to landfill soils during 3 days showed less capacity to retain Calcein (53–54% less) than control soils, especially in M and H exposure groups where statistical differences were found comparing to control (Fig. 3).

3.2.2. *Earthworms reproduction test (OECD-222, 2016)*

3.2.2.1. Earthworms chemical analysis. Earthworms removed at day 28 of the test showed significant different metal accumulation trends between experimental groups (Fig. 4).

Individuals exposed to H and M soils showed an increase in the metals accumulated in tissues, while the control and L groups showed much lower levels (considered as basal accumulation). Earthworms maintained in H group showed significantly higher Cd, Cr, Ni and Pb concentrations than control and L group, while M soils showed significantly higher Cd and Ni values than control and L groups (Fig. 4).

3.2.2.2. Weight loss. After 28 days, the greatest weight losses were observed in control organisms and those exposed to L soil. On the contrary, the lowest weight loss was recorded in the group H, where the organisms lost less than the half (46.82%) comparing to control organisms (Fig. 5).

3.2.2.3. Number of juveniles. The number of juveniles counted after 56 days in L group showed significant differences in comparison with M and H groups (Fig. 6). Control, M and H groups showed average juvenile numbers around 50 decreasing in L group to 15. .

3.2.2.4. Total weight of the offspring. Although no statistically significant differences were found, the total weight of offspring increased in the H and M groups, doubling the average weight quantified in the control and L groups (in the case of H) (Fig. 7).

3.3. *Correlations*

After analysing statistically all data, strong correlations (Pearson >0.9) were found among soil parameters and measured endpoints.

Among the plant based studies, a negative effect of pH on root elongation was observed, while a positive strong correlation (Pearson >0.9) between leachates metal content and soil organic matter was noted (Table 4).

Regarding the endpoints measured on *E. fetida* earthworms (Table 5), a positive correlation between the number of cells extruded and calcein test results, or OM content and offspring weight were observed. Similarly, a negative correlation between OM content and earthworm weight

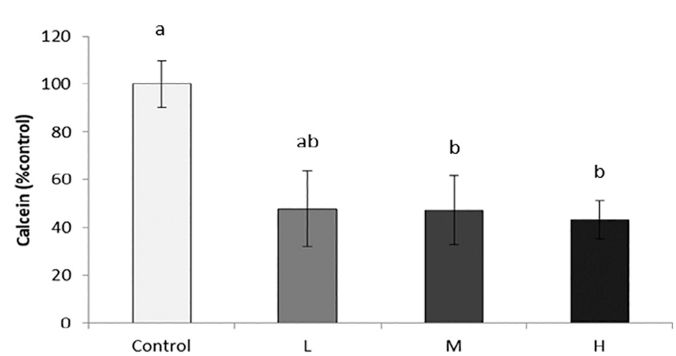

Fig. 3. Fluorescence signal after Calcein-AM Viability Test on coelomocytes extruded from earthworms exposed to landfill soils with different metal concentrations during 3 days. Significant differences marked with letters (ANOVA test, $p < 0.05$). Error bars represent standard deviation.

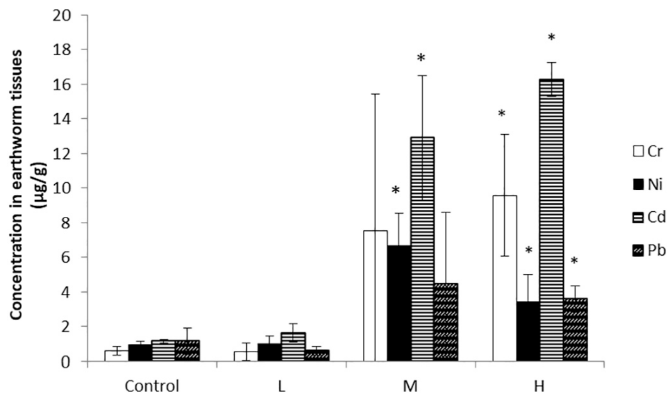

Fig. 4. Concentration of Cr, Ni, Cd and Pb (μg /mg earthworm) on tissues of *Eisenia fetida* earthworms exposed to different landfill soils for 28 days. Significant differences between metal concentrations on each group respect to control value for each metal marked with asterisk (ANOVA test, $p < 0.05$). Error bars represent standard deviation.

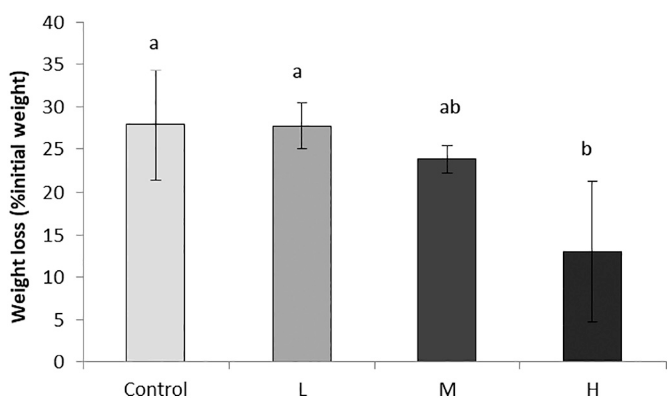

Fig. 5. Weight loss (% respect to initial weight) of *Eisenia fetida* earthworms exposed to different landfill soils for 28 days. Significant differences marked with letters (ANOVA test, $p < 0.05$). Error bars represent standard deviation.

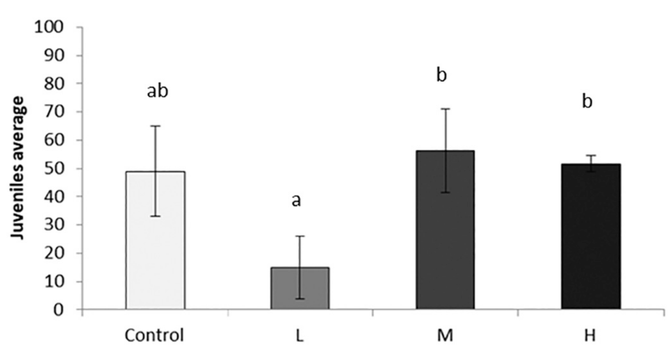

Fig. 6. Number of juveniles counted at day 56 of experiment after exposing *Eisenia fetida* adult earthworms to different landfill soils for 28 days. Significant differences marked with letters (Kruskal Wallis test, $p < 0.05$). Error bars represent standard deviation.

loss after 28 days, or earthworm weight loss and offspring weight was noted.

The statistical analysis showed also different Pearson correlations between the concentrations of metals accumulated in earthworms and different parameters (Table 6). Strong positive correlations between Cr and Cd contents were observed with OM amount and the total weight of the offspring.

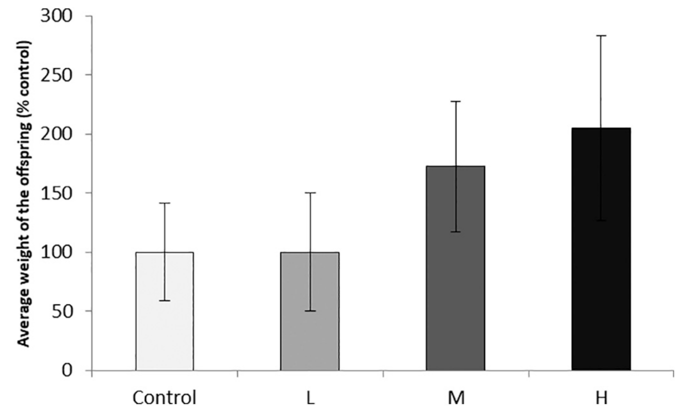

Fig. 7. Total weight of the offspring at day 56 of experiment after exposing *Eisenia fetida* adult earthworms to different landfill soils for 28 days. Error bars represent standard deviation.

4. Discussion

4.1. Physicochemical and metal characterization in soils and leachates

Metal availability, and toxicity in soil, is fully conditioned by soil properties. Among key factors, clay content, cation exchange capacity, pH or OM content must be highlighted (Spurgeon and Hopkin, 1995; Smit and Van Gestel, 1998; Gondek and Kopeć, 2006; Leduc et al., 2007). In soils used in the present experiments different pH trends were measured between experimental groups (Table 1). This data, along with OM contents, could explain differences on metal bioavailability observed between experimental groups (Visioli et al., 2013). The strong bounding between OM and contaminants, provided by organometallic ligands, fixes the contaminants to the ground, so, preventing contaminants migrate to aquatic phase and other media. Thus, time spent by metals in soil is a factor to be considered when analysing toxicity, indeed, metal bioavailability decreases as precipitates are formed (Martinez and McBride, 2001). So, lower bioavailability and lower toxicity is expected in aged soils, comparing to newly doped soils (Smolders et al., 2009). Thus, high OM content present on landfill aged soils (up to 19% dw in H group) would reduce metal bioavailability, reducing pollutants toxicity and buffering potential effects. This metal availability conditioners are also well reflected in leachate metal contents, with metal contents in leachates three orders of magnitude lower (ppb vs ppm) than in landfill soils. The strong correlation (0.947) between leachates metal content and soils OM content reinforced this trend, suggesting that heavy metals present in soil, are strongly bounded to OM, making them less available or mobile.

4.2. Effects on Lactuca sativa

Germinations quantified after 3 and 7 days did not show any significant differences among treatments. However, a slight decrease in germination was observed in seed exposed to leachates coming from H and M soils after 3 and 7 days exposure.

Regarding root elongation test, similar results were recorded among different experimental groups, suggesting that pollutants in lixiviates are not toxic enough to interrupt lettuce growth. Two days root exposure seemed not to be enough exposure time to appreciate significant correlations between metal content and elongation values. In fact a strong negative correlation (−0.999) was found between roots elongation and soils pH, suggesting that elongation trends are mainly driven by pH differences among soils. Although highest metal accumulations were detected in H group seedlings (highest Cr, Ni, Cd and Pb accumulations), no impacts reflecting this accumulation trend was recorded. This lack of response could be explained by the low exposure concentrations

Table 4
Pearson correlations estimated between root elongation, soil pH, elutriates metal content, OM content and germination rates. Strong (> 0.900) negative (red) and positive (green) correlations marked in colours.

Correlations

	Root elongation	pH	Elutriates metal cont.	OM content	Germination
Root elongation	1	-.999**	-.486*	-.384*	-.433*
pH		1	.514**	.241	.402*
Elutriates metal			1	.947**	-.532**
OM content				1	-.665**
Germination					1

**. 0.01 level significant correlation (2 tails).
*. 0.05 level significant correlation (2 tails).

Table 5
Pearson correlations estimated between calcein results, counted cells, OM content in soils, weight loss after 28 days of exposure, weight of the offspring and juveniles after 56 days. Strong (> 0.900) negative (red) and positive (green) correlations marked in colours.

Correlations

	Calcein	Cells counted	OM content	Weight loss 28d	Weight offspring	Juveniles
Calcein	1	.913**	-.790**	.592**	-.667**	.246
Cells counted		1	-.810**	.610**	-.798**	-.199
OM content			1	-.954**	.955**	.231
Weight loss 28d				1	-.928**	-.366*
Weight offspring					1	.492**
Juveniles						1

**. 0.01 level significant correlation (2 tails).
*. 0.05 level significant correlation (2 tails).

Table 6
Pearson correlations estimated between Cd, Cr, Ni and Pb respect to calcein results, cells counted, soils pH, weight loss of the earthworms after 28 days, soils OM content, weight of the offspring and juveniles after 56 days. Strong (> 0.900) positive (green) correlations marked in colours.

Correlations

	Calcein	Cells counted	pH	Weight loss 28d	OM content	Weight offspring	Juveniles
Chromium	-.605**	-.817**	-.182	-.887**	.931**	.997**	.639**
Niquel	-.510**	-.808**	-.670**	-.391*	.547**	.722**	.613**
Cadmium	-.629**	-.832**	-.164	-.888**	.939**	.997**	.615**
Lead	-.482*	-.791**	-.527**	-.659**	.739**	.899**	.756**

**. 0.01 level significant correlation (2 tails).
*. 0.05 level significant correlation (2 tails).

quantified in leachates. In fact, metal concentrations measured in lixiviates showed to be far from LOECs already published for lettuce plants: 2 ppb vs 14.38 ppm Cd, 12.4 ppb vs 53.04 ppm Pb, 17.7 ppb vs 6.939 ppm Ni (Di Salvatore et al., 2008) and 31.5 ppb vs 50 ppm Cr (Hou et al., 2013).

4.3. *Eisenia fetida exposure to polluted soils*

4.3.1. *Short term exposure effects*

Weight losses quantified after 3 days of exposure did not show any remarkable pattern among experimental groups. The short exposure time (3 days) could be the reason for the low weight loss and probably longer exposure periods would be more accurate to assess the impacts of soils receiving sludges. However, soils high OM content could also mask the effects upon earthworms biomass loss. It should be noted that, evaluating other sublethal effects is searched through this short term trials, for example, to assess damages at cellular or molecular level. This way, stress level can be evaluated in exposed organisms before weight loss, death or irreversible effects take place.

After coelomocytes extrusion, a clear decrease on cell number was measured in organisms exposed to landfill polluted soils, especially in M and H groups.

A notorious decrease on calcein retention was noted in coelomocytes of earthworms maintained in landfill soils, especially in H and M groups. This significant decrease in comparison to control suggested cell damage after 3 day exposure to polluted soils as previously described (Plytycz et al., 2007). Mentioned decrease on retention ability could be conditioned by an alteration in eleocyte/amoebocyte populations (Plytycz et al., 2009). In fact, the decrease on calcein signal could take place due to a decrease on eleocyte population (respect to amebocytes) which are more sensitive to metal pollution (Di Marzio et al., 2005; Olchawa et al., 2006; Homa et al., 2010; Plytycz et al., 2010); with amebocytes fagociting death eleocytes (Irizar et al., 2015b).

4.3.2. Earthworms reproduction test effects

After 28 days, a lower biomass loss in adult earthworms was observed in sewage sludge polluted soils. Highest weight losses were observed in the control and L groups, while H soil earthworms showed the less loss. However, all the groups lost more than 20% of their initial weight, and, this value is considered the threshold for severe weight loss as suggested by Garcia-Velasco et al. (2017). Unlike the results obtained after 3 days of exposure, weight losses obtained in H and M after 28 days could be explained by the OM present in soils. In fact, H and M were the experimental groups with highest OM content, with 19% and 11% respectively. Reinforcing this hypothesis, a strong negative correlation (−0.954) was observed between weight losses quantified after 28 days and soil OM contents. Thus, it can be stated that quantified weight loss was soil OM content dependant and was not a direct result of soil pollutants.

Chemical analyses of earthworms exposed for 28 days, showed higher metal accumulations at H and M groups comparing to control and L. In these two H and M groups remarkable concentrations were achieved, being quantified 18 and 16 Cd ppm in H and M groups respectively. As already demonstrated in other invertebrate species, this concentrations tends to increase significantly along time (Callahan et al., 1979). It is also remarkable the high correlation observed between soils OM content and Cr and Cd concentrations (0.931 and 0.939 respectively), suggesting the creation of metal-OM ligands responsible of heavy metal sorption in soil (Gondek et al., 2014). As different authors like Gondek and Kopeć (2006) suggested in case-studies about WWTP-OM and heavy metal interactions, around 25–59% of the Cd and 34–81% of Cr remains attached to the soil forming organo-mineral boundings. This was supported by the negative correlations found between 28 day weight losses and accumulated Cd (−0.888) or Cr (−0.887) contents. Thus, the low weight loss observed in organisms exposed to H and M soils could be explained by a higher metallically bounded OM uptake through the earthworms diet, that in turn would generate high accumulations of metal in tissues.

Besides, it must be highlighted also the negative correlations between coelomocyte counting and Cd (−0.832), Cr (−0.817) or Ni (−0.808) accumulations in earthworms, suggesting a potential cytotoxic effect caused by metals in tissues.

After 56 days from the beginning of the reproduction test, no reproductive damage that could suppose a significant change on population dynamics was observed since all exposed groups showed similar juvenile numbers respect to the control .Besides offspring biomass showed higher values in H and M groups comparing to L and control groups. In fact, H group doubled control and L group values. This trend could be explained by the high OM content in the mentioned H (19% d.w) and M (11% d.w) groups, where offspring could have had higher amounts of food from hatching. Corroborating this, a strong positive correlation (0.955) was observed between soils OM content and quantified offspring biomass.

5. Conclusion

Results obtained highlighted the necessity to integrate chemical results with toxicity bioassays when evaluating the health of a soil. Determining pH and OM quantities in soils receiving sewage sludges showed to be crucial to interpret properly the ecotoxicological results and explain differences among pollutants bioavailability.

While no differences were shown in the assays with L. *sativa* the short term analysis based on cellular endpoints in earthworms demonstrated cytotoxic effect exerted by soils from landfill. Furthermore, earthworms maintained in sewage sludge receiving soils exhibited high accumulations of Cd, Cr, Ni and Pb after 28 days of exposure. Hence, it was confirmed that organisms exposed to soils exhibiting intermedium and high pollutant levels are subjected to sublethal effect at short term that could be amplified at longer exposure times. Hence, the application of soil remediation technologies to minimise the risk was strongly recommended.

Declaration of competing interest

We declare that we do not have any conflict of interest.

Acknowledgements

Basque Government (IT810-13), CTM2017-87766-R from MINECO, PhytoSUDOE-SOE1/P5/E0189, and Gernika City Hall.

References

Adamo, P., Lavazzo, P., Albanese, S., Agrelli, D., De Vivo, B., Lima, A., 2014. Bioavailability and soil-to-plant transfer factors as indicators of potentially toxic element contamination in agricultural soils. Sci. Total Environ. 500–501 (2014), 11–22.

Bagur-González, M.G., Estepa-Molina, C., Martín-Peinado, F., Morales-Ruano, S., 2010. Toxicity assessment using Lactuca sativa L. bioassay of the metal(loid)s As, Cu, Mn, Pb and Zn in soluble-in-water saturated soil extracts from an abandoned mining site. J.SoilsSediments 11, 281–289.

Cajaraville, M.P., Bebianno, M.J., Blasco, J., Porte, C., Sarasquete, C., Viarengo, A., 2000. The use of biomarkers to assess the impact of pollution in coastal environments of the Iberian Peninsula: a practical approach. Sci. Total Environ. 247 (2–3), 295–311.

Callahan, M.A., Slimak, M.W., Gabel, N.W., 1979. Water-related Environmental Fate of 129 Priority Pollutants. In: Introduction And Technical Background, Metals And Inorganics, Pesticides And PCBs. Report to U.S. Environmental Protection Agency, Vol. 1. Office of Water Planning and Standards, Washington, D.C., by Versar Incorporated, Springfield, VA. EPA/ 440/4-79/029a.

Claiborne, A., 1985. Catalase activity. In: Greenwald, R.A. (Ed.), Handbook of Methods for Oxygen Radical Research, pp. 283–284. Boca Raton FL.

Curieses, S.P., García-Velasco, N., Urionabarrenetxea, E., Sáenz, M.E., Bilbao, E., Di Marzio, W.D., Soto, M., 2017. Responses to silver nanoparticles and silver nitrate in a battery of biomarkers measured in coelomocytes and in target tissues of Eisenia fetida earthworms. Ecotoxicol. Environ. Saf. 141, 57–63.

Deng, S., Wu, Y., Duan, H., Cavanagh, J.A.E., Wang, X., Qiu, J., Li, Y., 2021. Toxicity assessment of earthworm exposed to arsenate using oxidative stress and burrowing behavior responses and an integrated biomarker index. Sci. Total Environ. 800, 149479.

Di Marzio, W.D., Saenz, M.E., Lemiere, S., Vasseur, P., 2005. Improved single cell gel electrophoresis assay for detecting DNA damage in Eisenia foetida. Environ. Mol. Mutagen. 46, 246–253.

Di Salvatore, M., Carafa, A.M., Carratù, G., 2008. Assessment of heavy metals phytotoxicity using seed germination and root elongation tests: a comparison of two growth substrates. Chemosphere 73 (2008), 1461–1464.

EPA (United States Environmental Protection Agency), 1996. Ecological Effects Test Guidelines. Seed Germination/root Elongation Toxicity Test. OPPTS 850.4200.

Garcia-Velasco, N., Peña-Cearra, A., Bilbao, E., Zaldibar, B., Soto, M., 2017. Integrative assessment of the effects produced by Ag nanoparticles at different levels of biological complexity in Eisenia fetida mantained in two standard soils (OECD and LUFA 2.3). Chemosphere 181, 747–758.

Gómez-Sagasti, M.T., Epelde, L., Barrutia, O., 2019. Fitorremediazioa lurzoru kutsatuen kudeaketa iraunkorrerako estrategia gisa. Ekaia 35 (2019), 197–212.

Gondek, K., Kopeć, M., 2006. Heavy metal binding by organic substance in sewage sludge of various origin. Environ. Dev. 9, 3.

Gondek, K., Baran, A., Kopec, M., 2014. The effect of low-temperature transformation of mixtures of sewage sludge and plant material son content, leachability and toxicity of heavy metals. Chemosphere 117, 33–39.

Hertel-Aas, T., Brunborg, G., Jaworska, A., Salbu, B., Oughton, D.H., 2011. Effects of different gamma exposure regimes on reproduction in the earthworm Eisenia fetida (Oligochaeta). Science of The Total Environment 412–413, 138–147. https://doi.org/10.1016/j.scitotenv.2011.09.037.

H., Klimek, K., Cocquerelle, Vandenbulcke, Plytycz, 2010. Metal-specific effects on metallothionein gene induction and riboflavin content in coelomocytes of Allolobophora chlorotica. Ecotoxicology and Environmental Safety 73 (8), 1937–1943.

Hou, J., Liu, G.N., Xue, W., Fu, W.J., Liang, B.C., Liu, X.H., 2013. Seed germination, root elongation, root-tip mitosis, and micronucleus Induction of five crop plants exposed to chromium in fluvo-aquic soil. Environ. Toxicol. Chem. 33 (3), 671–676, 2014.

IHOBE, Sociedad Pública de Gestión Ambiental del Gobierno Vasco, 2015. Ley 4/2015, de 25 de junio, para la prevención y corrección de la contaminación del suelo. BOE (I. Disposiciones generales), 62274–62311. https://www.boe.es/eli/es-pv/l/2015/06/25/4.

Irizar, A., Duarte, D., Guilhermino, L., Marigómez, I., Soto, M., 2014a. Optimization of NRU assay in primary cultures of Eisenia fetida for metal toxicity assessment. Ecotoxicology 23 (7), 1326–1335.

Irizar, A., Izagirre, U., Diaz De Cerio, O., Marigómez, I., Soto, M., 2014b. Zonation in the digestive tract of Eisenia fetida: implications in biomarker measurements for toxicity assessment. Comp.Biochem.Physiol.C 160, 42–53.

Irizar, A., Rodriguez, M.P., Izquierdo, A., Cancio, I., Marigómez, I., Soto, M., 2015a. Effects of soil organic matter content on cadmium toxicity in Eisenia fetida: implications for the use of biomarkers and standard toxicity tests. Arch. Environ. Contam. Toxicol. 68, 181–192.

Irizar, A., Rivas, C., García-Velasco, N., Goñi de Cerio, F., Etxebarria, J., Marigómez, I., Soto, M., 2015. Establishment of toxicity thresholds in subpopulations of coelomocytes (amoebocytes vs. eleocytes) of Eisenia fetida exposed in vitro to a variety of metals: implications for biomarker measurements. Ecotoxicology 24 (5), 1004–1013.

Jalali, M., Khanlari, Z.V., 2008. Effect of aging process on the fractionation of heavy metals in some calcareous soils of Iran. Geoderma 143, 26–40.

Kooijman, S.A.L.M., Metz, J.A.J., 1984. On the dynamics of chemically stressed populations: the deduction of population consequences from effects on individuals. Ecotoxicol. Environ. Saf. 8, 254–274.

Kulikowska, D., Gusiatin, Z.M., Bułkowska, K., Klik, B., 2015. Feasibility of using humic substances from compost to remove heavy metals (Cd, Cu, Ni, Pb, Zn) from contaminated soil aged for different periods of time. J. Hazard. Mater. 300, 882–891.

Laycock, A., Díez-Ortíz, M., Larner, F., Dybowska, A., Spurgeon, D., Valsami-Jones, E., Rehkämper, M., Svendsen, C., 2016. Earthworm uptake routes and rates of ionic Zn and ZnO nanoparticles at realistic concentrations, traced using stable isotope labelling. Environ. Sci. Technol. 50 (1), 412–419.

Leduc, F., Whalen, J.K., Sunahara, G.I., 2007. Growth and reproduction of the earthworm Eisenia fetida after exposure to leachate from wood preservatives. Ecotoxicol. Environ. Saf. 69 (2008), 219–226.

Li, X., Wang, M., Chen, W., Jiang, R., 2019. Evaluation of combined toxicity of Siduron and cadmium on earthworm (Eisenia fetida) using Biomarker Response Index. Sci. Tot. Envi. 646, 893–901.

Lopes Alves, Nogueira Cardoso, 2016. Overview of the Standard Methods for Soil Ecotoxicology Testing. IntechOpen Limited, London.

Marigómez, I., Soto, M., Kortabitarte, M., 1996. Tissue-level biomarkers and biological effect of mercury on sentinel slugs, Arion ater. Arch.Environ. Contam. Toxicol. 31, 54–62.

Martinez, C.E., McBride, M.B., 2001. Cd, Cu, Pb and Zn coprecipitates in Fe oxide formed at different pH: aging effects on metal solubility and extractability by citrate. Environ. Toxicol. Chem. 20, 122–126.

Moriarty, F., 1983. Ecotoxicology. In: The Study of Pollutants in Ecosystems. Academic Press, London, UK.

Mtisi, M., Gwenzi, W., 2019. Evaluation of the phytotoxicity of coal ash on lettuce (Lactuca sativa L.) germination, growth and metal uptake. Ecotox. Environ. Saf. 170, 750–762. https://doi.org/10.1016/j.ecoenv.2018.12.047.

OECD, 1984. Terrestrial Plant: Growth Test OECD. Guideline for Chemicals, No., 208. Organization for Economic Cooperation and Development, Paris.

OECD, 2003. Guideline for the testing of chemicals. Proposal for updating guideline 208. In: Terrestrial Plant Test: 208: Seedling Emergence And Seedling Growth Test. Organisation for Economic Co-operation and Development.

OECD, 2016. Earthworm ReproductionTests-222. OECD Guideline for Testing of Chemicals, Section 2 Organization for Economic Cooperation and Development, Paris.

Olchawa, E., Bzowska, M., Sturzenbaum, S.R., Morgan, A.J., Plytycz, B., 2006. Heavy metals affect the coelomocyte-bacteria balance in earthworms: environmental interactions between abiotic and biotic stressors. Environ. Pollut. 142, 373–381.

Plytycz, B., Klimek, M., Homa, J., Grzegorz, T., Kolaczkowska, E., 2007. Flow cytometric measurement of neutral red accumulation in earthworm coelomocytes: Novel assay for studies on heavy metal exposure. European Journal of Soil Biology 43 (1), s116–s120. https://doi.org/10.1016/j.ejsobi.2007.08.050.

Plytycz, B., Lis-Molenda, U., Cygal, M., Kielbasa, E., Grebosz, A., Duchnowski, M., Andre, J., Morgan, A.J., 2009. Riboflavin content of coelomocytes in earthworm (Dendrodrilus rubidus) field populations as a molecular biomarker of soil metal pollution. Environ. Pollut. 157, 3042–3050.

Plytycz, B., Kielbasaa, G., Grebosz, A., Duchnowski, M., Morgan, A.J., 2010. Riboflavin mobilization from eleocyte stores in the earthworm Dendrodrilus rubidus inhabitating aerially-contaminated Ni smelter soil. Chemosphere 81 (2), 199–205. https://doi.org/10.1016/j.chemosphere.2010.06.056.

Sinha, R.K., Bharambe, G., Ryan, D., 2008. Converting wasteland into wonderland by earthworms—a low-cost nature's technology for soil remediation: a case study of vermiremediation of PAHs contaminated soil. Environmentalist 28, 466–475.

Smit, C.E., Van Gestel, C.A.M., 1998. Effects of soil type, pre percolation, and ageing on bioaccumulation and toxicity of zinc for the springtail Folsomia candida. Environ. Toxicol. Chem. 17, 1132–1141.

Smolders, E., Oorts, K., Van Sprang, P., Schoeters, I., Janssen, C.R., McGrath, S.P., McLaughlin, M.J., 2009. Toxicity of trace metals in soils affected by soil type and aging after contamination: using calibrated bioavailability models to set ecological soil standards. Environ. Toxicol. Chem. 28, 1633–1642.

Spurgeon, D.J., Hopkin, S.P., 1995. Extrapolation of the laboratory- based OECD earthworm toxicity test to metal-contaminated field sites. Ecotoxicology 4, 190–205.

Spurgeon, D.J., Hopkin, S.P., Jones, D.T., 1994. Effects of cadmium, copper, lead and zinc on growth, reproduction and survival of the earthworm Eisenia fetida (Savigny): assessing the environmental impact of point-source metal contamination in terrestrial ecosystems. Environ. Pollut. 84, 123–130.

Urionabarrenetxea, E., Garcia-Velasco, N., Anza, M., Artetxe, U., Lacalle, R., Garbisu, C., Becerril, T., Soto, M., 2021. Application of in situ bioremediation strategies in soils amended with sewage sludges. Sci. Total Environ. 20 (766), 144099.

Urionabarrenetxea, E., Garcia-Velasco, N., Marigómez, I., Soto, M., 2020. Effects of elevated temperatures and cadmium exposure on stress biomarkers at different biological complexity levels in Eisenia fetida earthworms. Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology 231, 108735. https://doi.org/10.1016/j.cbpc.2020.108735.

Verdú, I., Trigo, D., Martínez-Guitarte, J.L., Novo, M., 2018. Bisphenol A in artificial soil: effects on growth, reproduction and immunity in earthworms. Chemosphere 190, 287–295.

Visioli, G., Menta, C., Gardi, C., Delia Conti, F., 2013. Metal toxicity and biodiversity in serpentine soils: application of bioassay tests and microarthropod index. Chemosphere 90, 1267–1273.

Wijayawardena, M.A., Naidu, R., Megharaj, M., Lamb, D., Thavamani, P., Kuchel, T., 2015. Influence of ageing on lead bioavailability in soils: a swine study. Environ. Sci. Pollut. Res. 22, 8979–8988.