Ecotoxicology and Environmental Safety 181 (2019) 255–263

Contents lists available at ScienceDirect

Ecotoxicology and Environmental Safety

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

Evaluation of the effectiveness of a bioremediation process in experimental soils polluted with chromium and lindane

Juan Daniel Aparicio[a,b], Nerea Garcia-Velasco[c], Erik Urionabarrenetxea[c], Manu Soto[c], Analía Álvarez[a,d], Marta Alejandra Polti[a,d,*]

[a] Planta Piloto de Procesos Industriales Microbiológicos (PROIMI), CONICET, Av. Belgrano y Pasaje Caseros, 4000, Tucumán, Argentina
[b] Facultad de Bioquímica, Química y Farmacia, Universidad Nacional de Tucumán, Ayacucho 491, 4000, Tucumán, Argentina
[c] Faculty of Science and Technology and Research Centre for Experimental Marine Biology and Biotechnology, University of the Basque Country, Bilbao, E-48080, Basque Country, Spain
[d] Facultad de Ciencias Naturales e Instituto Miguel Lillo, Universidad Nacional de Tucumán. Miguel Lillo 205, 4000, Tucumán, Argentina

ARTICLE INFO

Keywords:
Co-contaminated soil
Actinobacteria
Bioremediation
Bioindicators
Biomarkers

ABSTRACT

Bioremediation using actinobacterium consortia proved to be a promising alternative for the purification of co-contaminated environments. In this sense, the quadruple consortium composed of *Streptomyces* sp. M7, MC1, A5, and *Amycolatopsis tucumanensis* AB0 has been able to remove significant levels of Cr(VI) and lindane from anthropogenically contaminated soils. However, the effectiveness of the bioremediation process could not be evaluated only by analytical monitoring, which is complex mainly due to the characteristics of the matrix, producing non-quantitative analyte recoveries, or interferences in the detection stage and quantification. However, the effectiveness of the bioremediation process cannot be evaluated only through analytical monitoring, which is complex due mainly to the characteristics of the matrix, to the recoveries of non-quantitative analytes or to interferences in the detection and quantification stage. For this reason, it is essential to have tools of ecological relevance to assess the biological impact of pollutants on the environment. In this context, the objective of this work was to establish the appropriate bioassays to evaluate the effectiveness of a bioremediation process of co-contaminated soils. For this, five model species were studied: four plant species (*Lactuca sativa*, *Raphanus sativus*, *Lycopersicon esculentum,* and *Zea mays*) and one animal species (*Eisenia fetida*). On plant species, the biomarkers evaluated were inhibition of germination (IG) and the length of hypocotyls/steam and radicles/roots of the seedling. While on *E. fetida*, mortality (M), weight lost, coelomocyte concentration and cell viability were tested. These bioindicators and the battery of biomarkers quantified in them showed a different level of sensitivity, from maximum to minimum: *E. fetida* > *L. esculentum* > *L. sativa* > *R. sativus* ≫ > *Z. mays*. Therefore, *E. fetida* and *L. esculentum* and their respective biomarkers were selected to evaluate the effectiveness of the bioremediation process due to the capability of assessing the effect on the flora and the fauna of the soil, respectively. The joint application of these bioindicators in a field scale bioremediation process is a feasible tool to demonstrate the recovery of the quality and health of the soil.

1. Introduction

Environmental pollution is one of the main problems that cause global concern. The accumulation of toxic compounds in the ecosystems has led to alterations of biogeochemical cycles which has a negative effect on environmental health and living organisms (Rayu et al., 2012). The effects of pollution are intensified in areas where the presence of different organic pollutants is aggravated by the co-existence with metals and metalloids in concentrations that exceed the acceptable levels (Chen et al., 2015). This phenomenon, known as mixed pollution or co-contamination, is becoming a relevant problem since more than one-third of the affected places suffer with more than one type of pollutant. Moreover, xenobiotic compounds have even been detected in remote areas due to diffusion processes (Alvarez et al., 2017; Mansour et al., 2009; Tang et al., 2010).

In particular, co-contamination with Cr(VI) and lindane, an organochlorine pesticide, is quite common all over the world (Arienzo et al., 2013; Coatu et al., 2013; Maggi et al., 2012), including Argentina, where both compounds were recently reported in concentrations exceeding the maximum permitted (Aparicio et al., 2018a, 2018b; Simón

* Corresponding author. PROIMI-CONICET, Av. Belgrano y Pasaje Caseros, 4000, Tucumán, Argentina.
E-mail addresses: mpolti@proimi.org.ar, marpolti@hotmail.com, marpolti@gmail.com (M.A. Polti).

https://doi.org/10.1016/j.ecoenv.2019.06.019
Received 12 April 2019; Received in revised form 4 June 2019; Accepted 5 June 2019

Solá et al., 2019). To deal with this problem, eco-friendly remediation technologies using actinobacteria are promising tools to contribute successfully to the ecosystem restoring. In this sense, Aparicio et al. (2018b) demonstrated that an actinobacterial consortium was able to survive and grow under high concentrations of Cr(VI) and lindane, besides to remove both contaminants from soils under anthropogenic contamination.

The complete mineralization of organic contaminants or their transformation to non-toxic products is desirable, but not always possible. Numerous authors have reported that a decrease in pollutant levels may sometimes be accompanied by the transformation of the original contaminant into more toxic metabolites that could persist in soil and produce toxicity to certain species (Calvelo Pereira et al., 2010; Fuentes et al., 2013; Saez et al., 2014). Similarly, the physicochemical properties of soil influence metal speciation and consequently, its mobility, bioavailability, and toxicity (Olaniran et al., 2013). Thus, after any biological intervention for environmental purposes, the evaluation of soil health becomes one additional step of the bioremediation process, being an important indicator of the effectiveness of the biological treatment.

The evaluation of soil quality is a complex analytical process due mainly to the heterogeneity of the system that results in non-quantitative recovery of the analyte and lack of accuracy or reproducibility (Kumpiene et al., 2014). This limitation can be overcome by using ecotoxicity tests, highly sensitive to fluctuations in the system and allowing a more accurate assessment of soil health and quality (Hirano and Tamae, 2011; Silva et al., 2003). Results from ecotoxicity monitoring are highly dependent on the tested organisms. Only a few species that have relevant ecological functions, commonly called model species or bioindicators, can be used for these purposes (Garcia-Velasco et al., 2017; Nikinmaa and Nikinmaa, 2014; Ronco et al., 2004; Sobrero and Ronco, 2004). Therefore, it is important to evaluate several assays/species together (Moradas et al., 2008). Only a few species that have relevant ecological functions, commonly called model species or bioindicators, can be used for these purposes (Garcia-Velasco et al., 2017; Nikinmaa and Nikinmaa, 2014; Ronco et al., 2004; Sobrero and Ronco, 2004). On the other hand, the responses or effects displayed by the model species are evidenced by specific biomarkers which can be registered at different levels of biological complexity, from cellular structures or enzyme systems, to complete organisms, populations or communities (Ronco et al., 2004; Sobrero and Ronco, 2004).

The transference of bioremediation technologies requires a full demonstration of its effectiveness. For instance, after the biological treatment not only the contaminant concentration decrease should be proven, but also the significant reduction in toxicity. In this context, the objective of this work was to evaluate the performance of different bioindicators and select the more appropriate/s ecotoxicological test to validate the effectiveness of a soil bioremediation process performed by an actinobacterial consortium. The usefulness of four plants (*Lactuca sativa*, *Raphanus sativus*, *Lycopersicon esculentum,* and *Zea mays*) and one animal species (*Eisenia fetida*) was evaluated.

2. Materials and methods

2.1. Obtaining and preservation of the quadruple actinobacterial consortium

The bioremediation assays were performed using an actinobacterial consortium. The strains *Streptomyces* sp. M7, *Streptomyces* sp. MC1, *Streptomyces* sp. A5 and *Amycolatopsis tucumanensis* AB0 were previously isolated from different contaminated areas of Argentina (Albarracín et al., 2005; Benimeli et al., 2003; Fuentes et al., 2011; Polti et al., 2007) and selected for their ability to remove lindane and Cr(VI) from liquid culture medium and from soil samples (Aparicio et al., 2018b).

Starch Casein Agar medium (SCA, composition in g L^{-1}: starch, 10; casein, 1; K_2HPO_4, 0.5; agar, 15; pH 7.0) was used for the preservation of the four actinobacteria and for obtaining spores (Hopwood, 1985).

Tryptone Soy Broth (TSB, composition in g L^{-1}: triptein, 17; soy peptone, 3; NaCl, 5; K_2HPO_4, 2.5; glucose, 2.3; pH 7.0) was used for bacterial biomass production.

Both culture media were sterilized in an autoclave at 121 °C and 1 atm of overpressure, for 15 min.

All other chemicals used during the study were analytical grade and purchased from certified manufacturers.

2.2. Soil spiking and bioremediation assay

Non-contaminated soil samples were collected from a recreational area of San Miguel de Tucumán, Argentina (26° 48'36.6 "S 65° 14'28.0" W). The samples were taken from the surface (5–15 cm depth). The soil was conditioned according to Aparicio et al. (2015). The physicochemical characteristics of these surface soils are listed in Supp. Table 1.

In order to artificially contaminate the soils, a stock solution of 5 g L^{-1} of Cr(VI) was prepared as $K_2Cr_2O_7$ (Cicarelli, Argentina). The solution was sterilized by filtration, using Millipore 0.22 µm pore size filters. Lindane stock was prepared (Sigma-Aldrich, United States) at 10 mg mL^{-1} using acetone as a solvent. The assays were carried out in glass pots containing 1 kg of soil. Five treatments were tested:

I) Non-contaminated soil
II) Soil contaminated with lindane 25 µg kg^{-1}
III) Soil contaminated with Cr(VI) 50 mg kg^{-1}
IV) Soil contaminated with Cr(VI) 50 mg kg^{-1} and lindane 25 µg kg^{-1}
V) Soil contaminated with Cr(VI) 50 mg kg^{-1} and lindane 25 µg kg^{-1} and after 14 days of stabilization, inoculated with the quadruple actinobacteria consortium.

The moisture of soils from all treatments was adjusted at 20%, then, soils were left to stabilize for 14 days, since dynamic physic-chemical phenomena usually occur between soil and contaminants (Aparicio et al., 2018b). After the stabilization period, soil from treatment V was inoculated with the bacterial consortium and incubated for another 14 days. Soils from treatments I to IV were also incubated for another 14 days but without inoculation.

Bacterial inoculum was prepared as follows: spores of the four actinobacteria were individually inoculated in flasks with 30 mL of TSB and then incubated in an orbital shaker at 30 °C, 180 rpm. After 72 h, microbial biomass was recovered by centrifugation (8385 × *g*), washed twice and resuspended in sterile distilled water to a final concentration of 100 g L^{-1}. The contaminated soils were inoculated with 2 g kg^{-1} of the quadruple actinobacteria consortium (treatment V). Equal proportions of each strain were added in order to reach the desired final concentration. The system was vigorously mixed to ensure a uniform distribution of soil components (Aparicio et al., 2018b). All pots were incubated for 14 days at 30 °C, and they were opened and mixed twice a week to allow O_2 exchange. In addition, the moisture of the soils was regularly monitored and adjusted with distilled water. At the end of the assay, soil samples were taken to determine the final concentrations of bioavailable Cr(VI) [$Cr(VI)_B$] and lindane and to carry out the ecotoxicological bioassays.

2.3. Determination of the sensitivity of the different bioindicators to Cr(VI) and lindane

Biological species tested were three dicotyledons *Lycopersicon esculentum* (tomato), *Lactuca sativa* (lettuce) and *Raphanus sativus* (radish), and the monocotyledonous *Zea mays* (corn). In addition, *Eisenia fetida* (earthworm) was used as a model of animal species.

However, in the particular case of chromium, due to its high toxicity, was first necessary to establish the “critical endpoint” (i.e. that

biological parameter that for being fully affected by the contaminant does not allow the evaluation of others biomarkers). On plants, the critical endpoint used was the IG, while for *E. fetida* the critical endpoint used was M.

To establish the sensitivity degree of the tested species against Cr (VI), NOEC (No Observed Effect Concentration), LOEC (Lowest Observed Effect Concentration) and EC_{50} (half maximal Effective Concentration) were determined using IG and M as critical endpoints. NOEC and LOEC were graphically obtained from the concentration-response curves. NOEC corresponds to the maximum metal concentration tested, which did not cause a significant effect on the critical endpoints, whereas LOEC corresponds to the minimum metal concentration tested which caused a significant effect on the critical endpoints. EC_{50} was determined by the Logit (P) statistical method.

Due to its low solubility and reactivity, the toxic effect of the pesticide is much less than that of the metal. For this reason, the bioassays to determine IG and M were carried out using a fixed lindane concentration: 1 mg L^{-1} or 1 mg kg^{-1}, depending on the matrix [filter paper for plant species or commercial soil (see below) for animal species]. In opposite, different Cr(VI) concentrations were tested, ranging from 10 to 150 mg L^{-1} (dicotyledons) or 10–150 mg kg^{-1} (earthworm), while for *Z. mays* [due to its high tolerance to Cr(VI)] the range of metal tested was from 10 to 500 mg L^{-1}.

Bioassays with plant species were carried out in Petri dishes that contained sterile filter paper moistened with 2 mL of each contaminant solution. Thirty seeds of dicotyledons or ten seeds of monocotyledons were located in plates, which were sealed with parafilm and incubated at 22 °C in the dark. After 5 days, the number of non-germinated seeds was recorded and the IG was calculated as a percentage.

For the toxicological bioassays using *E. fetida*, specimens were provided by the commercial supplier Lombricor S.C.A. (Córdoba-Spain). The earthworms were kept in the laboratory under controlled conditions (19 °C and 60% relative humidity) with the weekly contribution of horse manure as a nutrient source. In order to guarantee the homogeneity of the earthworms used in the bioassays, healthy, sexually mature (clitellated) individuals with a weight between 350 and 450 mg were selected.

Glass pots of 1.8 L capacity were filled with 750 g of standardized commercial LUFA 2.3 soil (Speyer, Germany) (Supp. Table 2) and then spiked with lindane and/or chromium at the concentrations previously specified for each one. The moisture of the system was adjusted at 20% and soils were left to stabilize for 14 days (stabilization period) (Aparicio et al., 2018a). After that, groups of ten specimens of *E. fetida* were weighed and placed into the pots, which were incubated at 18 °C for 14 days. The number of dead earthworms per pot was recorded and the M was calculated as a percentage.

2.4. Ecotoxicological bioassays using soil treated with an actinobacterium consortium

2.4.1. Bioassays using plants

The biomarkers evaluated on *L. esculentum*, *L. sativa* and *R. sativus* developments were IG and lengths of hypocotyls and radicles. Thirty seeds were placed in Petri dishes containing 15 g of soils coming from the different treatments evaluated in the bioremediation assay. The moisture was adjusted at 40% with sterile distilled water. Plates were sealed with parafilm and incubated at 22 °C for 5 days. Then, the number of non-germinated seeds was recorded, and the length of hypocotyls and radicles of the seedlings was measured.

The parameters evaluated on *Z. mays* development were IG and lengths of stems and roots. Ten seeds were placed in glass pots filled with 200 g of soil coming from different treatments evaluated in the bioremediation assay. The moisture was adjusted at 40% with sterile distilled water. After incubation at 22 °C for 7 days, the number of germinated seeds was recorded and the lengths of stems and roots of the seedlings were measured.

2.4.2. Bioassay using E. fetida

Bioassays using *E. fetida* were performed as explained in Section 2.2.1 but using the biologically treated soils from the bioremediation assay. The biomarkers evaluated were M, weight loss, coelomocyte concentration and cell viability (Garcia-Velasco et al., 2017).

The earthworm immune cells or coelomocytes were obtained according to Irizar et al. (2014b). Briefly, after the exposure to soil samples, each earthworm was submerged in extraction solution [1 mL of calcium and magnesium-free phosphate buffered saline (PBS)-EDTA 0.02%] and coelomocytes were obtained by extrusion through the dorsal pores by electrical stimulation (9 V). The obtained solution was centrifuged at 1000 × *g* for 10 min at 10 °C. Then, the supernatant was removed and the precipitated cells were washed twice with a cleaning solution (PBS, pH 7). Finally, the cells were resuspended in PBS and placed on a Neubauer cell counting chamber in order to obtain a stock solution of 10^6 cells mL^{-1} of coelomocytes.

For the evaluation of the cell viability, the Neutral Red Uptake (NRU) assay was used. A volume of 200 µL of the coelomocyte solution was placed in 96-well microplates (2×10^5 cells per well) and incubated at 18 °C for 30 min, to promote the adhesion of the cells to the bottom of the wells. Then, PBS was removed, previous centrifugation (1000 × *g*, 5 min, 10 °C) and 200 µL of 0.02% Neutral Red was added. The microplates were incubated at 18 °C for 30 min to allow the dye to enter and stain the living cells. Abiotic controls were performed in order to evaluate the colorant absorbed to the surfaces of the well.

Afterward, the microplates were centrifuged and the cells adhered were washed twice with PBS. The remaining dye inside the cells was removed by washing it with 100 µL of the extraction solution (50% ethanol, 49% water, and 1% acetic acid). Finally, absorbances were measured at 540 nm using a microplate spectrophotometer (Multiskan Spektrum Thermo Scientific) (Irizar et al., 2014).

2.5. Analytical determinations of contaminants in soils treated with the actinobacterium consortium

Residual lindane from soils was detected according to Aparicio et al. (2018b). Lindane concentration in extracts was quantified in a Gas Chromatograph (Agilent 7890A) equipped with ^{63}Ni micro-Electron Capture Detector (GC-µECD), HP5 capillary column (30 m × 0.53 mm × 0.35 m), a split/splitless Agilent 7693B injector and Agilent ChemStation software (Aparicio et al., 2018a).

$Cr(VI)_B$ was determined in soil by a physical method: 100 g of soil were centrifuged at 5050 × *g* for 60 min, to reproduce the maximal plant suction (soil water potential: 1500 kPa, conventional wilting point) (Aparicio et al., 2018b, 2018a; Csillag et al., 1999; Kim et al., 2015). After centrifugation, the supernatant was recovered and filtered at 0.45 mm. Soil extracts were analyzed by Atomic Absorption Spectrometry (AAS) using a PerkinElmer Analyst 400 for Cr content (characteristic concentration: 0.078 mg L^{-1}) (APHA, 2012).

2.6. Statistical analysis

All the assays and their respective controls were performed at least as three biologically independent replicates. For the statistical analysis of data, the Infostat software was used (version: 2018, Argentina). The data were subject to one-way variance analysis (ANOVA One way), considering a probability level of $p < 0.05$ as significant. They were also analyzed using the Tukey post-test ($p < 0.05$) in order to identify significant differences between treatments.

The EC_{50} values were calculated using the statistical method Logit (P) which analyze the IG values (for plants species) and the M values (for earthworms), obtained from each Cr(VI) concentration evaluated. Values of Cr(VI) concentrations were transformed to logarithms and IG and M values were expressed in Probit units (Supp. Table 3).

Measurement of hypocotyl and radicle lengths of tomato, lettuce and radish seedlings, as well as the lengths of stems and leaves of maize,

were determined using the Image Tools 3.0 software UTHSCSA (ITU).

3. Results and discussion

3.1. Toxicity evaluation of Cr(VI) and lindane

NOEC, LOEC, and EC_{50} were determined in order to establish the sensitivity degree of the tested species against chromium. For plants, EC_{50} was measured as IC_{50} (Inhibitory Concentration 50%), while for *E. fetida* EC_{50} was calculated as LC_{50} (Lethal Concentration 50%) (Nikolaeva and Terekhova, 2017).

In order to address the sensitivity of the bioindicators, the different species were exposed to a fixed concentration of lindane and a range of concentrations of Cr(VI).

Cr(VI) is known to be more toxic than lindane, due to its high solubility and reactivity and because the pesticide has agricultural use, and therefore, it has a less toxic effect on the plant species evaluated, even at high concentrations (Polti et al., 2014; Saez et al., 2014; Shahid et al., 2017). Sivakumar and Subbhuraam (2005) showed that 219 mg of lindane per kg of soil was necessary to cause the death of 50% of *E. fetida* specimens. In the same way, it has already been demonstrated that germination of the seeds of the four plants species evaluated was barely inhibited by the effect of lindane, even at concentrations up to 100 times higher than the one used in this study (Manonmani, 2011; Onogbosele and Scrimshaw, 2014; Saez et al., 2014). Therefore, the concentration of lindane employed in this study did not cause any loss of the selected plants. Conversely, Cr(VI) could completely affect the critical endpoint of each species evaluated, disabling a full assessment of the individual effect of each contaminant and the combined effect of both compounds on the studied parameters. Therefore, the NOEC, LOEC and EC_{50} values were calculated based on the toxicity of Cr(VI), ensuring the development of the selected species. This criterion allows evaluating the quality of the soils within the range of concentrations of metals used in this work without causing a total loss of experimental individuals.

Ecotoxicological tests on plants are generally carried out in two phases of their development: 1) germination and 2) growth of the seedlings, evaluating the elongation of roots and hypocotyls or stems, as markers of the effects of exposure of the plant to harmful substances (Munzuroglu and Geckil, 2002). However, in order to determine the NOEC, LOEC, and EC_{50} parameters, a specific critical endpoint must be established. Germination of the seed is the first physiological process affected by toxic compounds present in the soil, so the ability of the seeds to germinate under stress conditions would be indicative of its level of tolerance to contaminants (Peralta et al., 2001). Thus, the critical endpoint selected was the IG.

The IG was determined in the presence of different concentrations of Cr(VI) and a fixed concentration of lindane of 1 mg L^{-1} (Fig. 1) and the NOEC, LOEC and IC_{50} parameters were established. The IG for *L. esculentum* was 20% in the presence of 1 mg L^{-1} of lindane, without Cr (VI), while the pesticide did not cause a significant effect on the IG values of the other three plant species.

Cr(VI) showed a more pronounced effect on the germination of the *L. esculentum*, being the LOEC 30 mg L^{-1} and the NOEC 20 mg L^{-1}. On the other hand, the seeds of *L. sativa* and *R. sativus* were less sensitive to Cr(VI), with values of NOEC of 80 and 90 mg L^{-1}, respectively, and LOEC of 90 and 100 mg L^{-1}, respectively (Fig. 1A).

The tolerance of *Z. mays* to Cr(VI) was evaluated in two stages. In the first stage, the range of concentrations that had an effect on maize germination was determined (Fig. 1B). Subsequently, the region in which the IG increased significantly (350–450 mg L^{-1}) was re-evaluated. The NOEC and LOEC parameters were 370 and 380 mg L^{-1}, respectively.

The IC_{50} of Cr(VI), obtained from the Logit(P) statistical method, for *L. esculentum* was 73 ± 6 mg L^{-1}, while the IC_{50} values for *L. sativa* and *R. sativus* were 103 ± 6 and 119 ± 5 mg L^{-1}, respectively (Supp.

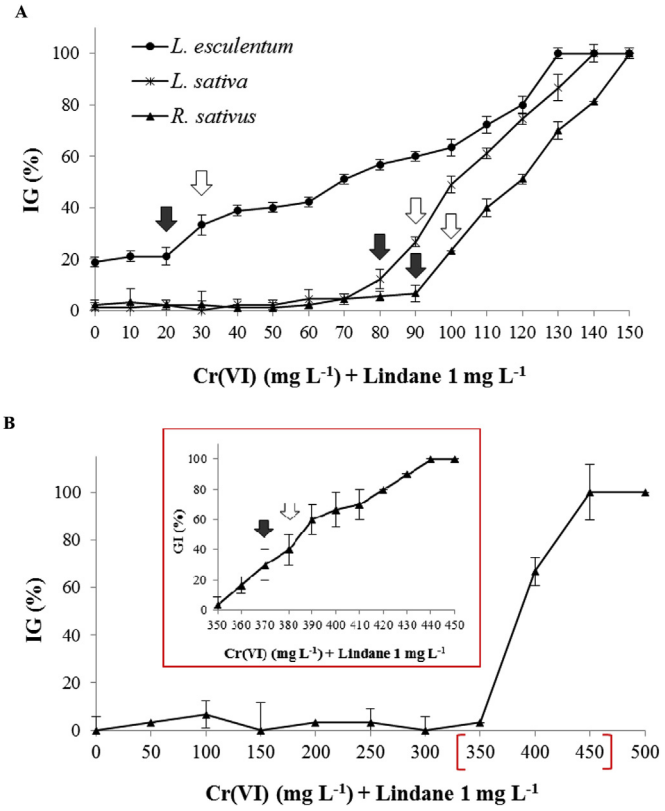

Fig. 1. Inhibition of the germination [IG (%)] in (A) *L. esculentum*, *L. sativa*, *R. sativus*, and (B) *Z. mays*, according to the concentrations of Cr(VI) tested plus a fixed lindane concentration. Grey arrow: NOEC (No Observed Effect Concentration); white arrow: LOEC (Lowest Observed Effect Concentration).

Figure 1). Although there was a marked inhibition of the germination in the three evaluated dicotyledons, *L. esculentum* was the most sensitive to the metal, followed by *L. sativa* and *R. sativus*.

As was expected, *Z. mays* was the most resistant species to Cr(VI) among the plant species tested, reaching an IC_{50} value of 387 ± 3 mg L^{-1} of Cr(VI).

The critical endpoint evaluated on *E. fetida* was M. The NOEC and LOEC values for *E. fetida* were 70 and 80 mg kg^{-1}, respectively (Fig. 2). Moreover, lindane did not inhibit key systems of the vital biochemical processes of earthworms, since no mortality was detected in the absence of Cr(VI).

The earthworms are excellent sentinels of the quality and health of the soil since they incorporate whole soil particles, so the contact with the contaminants is total, independently of the characteristics of the compound, including the different oxidation states (Irizar et al., 2014).

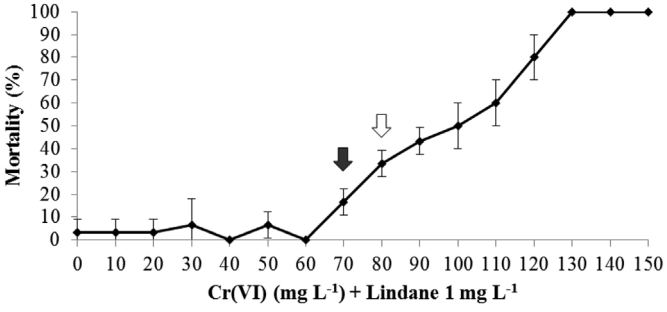

Fig. 2. Mortality of *E. fetida* [M (%)] according to the concentrations of Cr(VI) tested plus a fixed lindane concentration. Grey arrow: NOEC (No Observed Effect Concentration); white arrow: LOEC (Lowest Observed Effect Concentration).

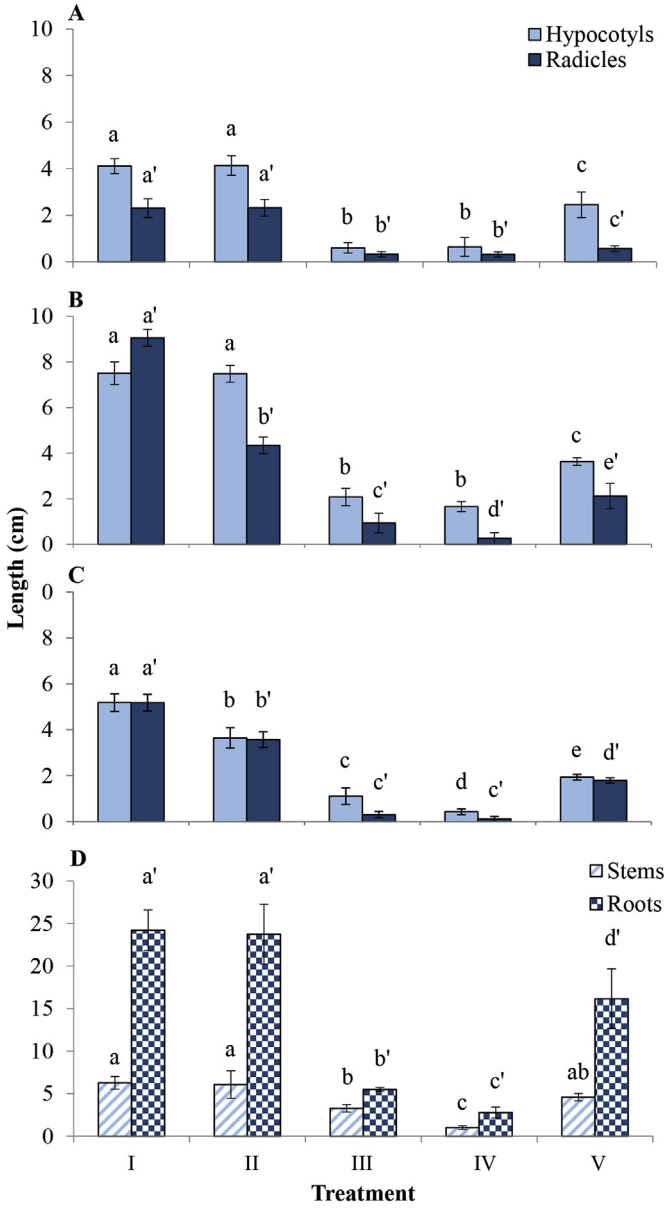

Fig. 3. Length (cm) of hypocotyls/steams and radicles/roots of (A) *L. sativa*, (B) *R. sativus*, (C) *L. esculentum*, and (D) *Z. mays*. Treatments: I) Non-contaminated soil; II) Soil contaminated with lindane 25 μg kg^{-1}; III) Soil contaminated with Cr(VI) 50 mg kg^{-1}; IV) Soil contaminated with Cr(VI) 50 mg kg^{-1} and lindane 25 μg kg^{-1}; V) Soil contaminated with Cr(VI) 50 mg kg^{-1} and lindane 25 μg kg^{-1} and after 14 days of stabilization, inoculated with the quadruple actinobacteria consortium. Bars showing different letters indicate they were significantly different ($p < 0.05$).

However, Cr(VI) is more toxic than Cr(III) for *E. fetida* as well as for eukaryotic organisms (Munzuroglu and Geckil, 2002).

Previously it was demonstrated that not all the Cr(VI) that is incorporated into the soil is bioavailable (Aparicio et al., 2018a), therefore it is necessary to determine the relationship between the total concentration of the metal [$Cr(VI)_T$] and the bioavailable fraction [$Cr(VI)_B$] (Supp. Figure 2A). In order to establish a mathematical relationship between the total concentration of Cr(VI) and the bioavailable fraction, the data were linearized (Supp. Figure 2B). From this linear approximation, the concentration of $Cr(VI)_B$ correlated with the concentration of Cr(VI) initially added was calculated. The values of NOEC and LOEC for *E. fetida* considering the $Cr(VI)_T$ were 70 mg kg^{-1} and 80 mg kg^{-1} (respectively), while NOEC and LOEC values corresponding to $Cr(VI)_B$ concentrations were 16 and 20 mg kg^{-1}, respectively.

The LC_{50} for *E. fetida*, obtained from the Logit(P) statistical method (Supp. Figure 3), was 97 ± 3 mg kg^{-1} of $Cr(VI)_T$, corresponding to 30 mg kg^{-1} of $Cr(VI)_B$.

An order of sensitivity against Cr(VI) was determined for all the species tested, considering the values of NOEC and EC_{50}, resulting *E. fetida* the most sensitive and *Z. mays* the most tolerant organism.

3.2. Ecotoxicological bioassays in soil

During the second phase, the most appropriate ecotoxicological bioassay for the evaluation of the bioremediation process was determined. For this purpose, five treatments were tested (Section 2.2). In addition to the critical endpoints, a battery of biomarkers was evaluated on each species.

3.2.1. Plant species

The IG, evaluated in *L. sativa* and *R. sativus*, did not show significant differences between the control (treatment I) and the soil contaminated with lindane (treatment II) (Table 1). Similarly, no statistically significant differences were found among IG values in the soil contaminated only with Cr(VI) (treatment III) and soil contaminated with the pesticide and the metal (treatment IV), indicating that the concentration of lindane used did not cause an appreciable toxic effect for these species. In opposite, IG values were higher in treatments III and IV ($p < 0.05$), in comparison with treatments I and II, evidencing the toxic effect of Cr(VI) at the concentrations used.

The IG of *L. esculentum* presented significant differences among the five treatments tested, demonstrating differential sensitivity to individual toxicants and to the mixture of both (Table 1).

On the other hand, in the case of *Z. mays*, no significant differences were observed between the IG obtained from the five treatments tested, showing that Cr(VI) and lindane did not cause a toxic effect on this biomarker in the concentrations tested, which was expected according to its values of NOEC, LOEC, and IC_{50}.

When the Cr(VI) effect on the germination of the dicotyledons seeds was evaluated, IG values higher than 50% were observed in all cases. This effect has already been previously reported on seeds of the weeds *Echinochloa colona* and alfalfa *Medicago sativa*, where the presence of Cr (VI) in the soil, at concentrations similar than those used in the present study, reduced the percentage of germination to 25 and 23%, respectively (Peralta et al., 2001; Rout et al., 2000). Previous studies shown that metals such as Cr(VI) reduce germination of seeds as they are able to penetrate said cover and/or attack the embryonic axis in germination, triggering a series of cellular alterations: interfere with cell division, increase the rigidity of cell walls through the cross-linking of pectins, decrease cellular respiration, modify the structure and functioning of plasma membranes, reduce water intake and interfere with the transport and metabolism of several essential nutrients (Casierra Posada and Cárdenas Hernández, 2007). In contrast, the germination of the seeds of *Z. mays* was not inhibited in presence of chromium, probably due to its characteristic coating that provides protection to the embryo against external contaminants, preventing the indiscriminate passage of substances (Munzuroglu and Geckil, 2002; Srivastava et al., 2007). The tolerance of *Z. mays* to high levels of contamination makes this species potentially useful in phytoremediation strategies (Alvarez et al., 2012; 2015; Simon Sola et al., 2017; 2019; Polti et al., 2011).

On the other hand, lindane did not affect the germination of lettuce, radish and corn seeds. Calvelo Pereira et al., (2010) showed that lindane was not able to inhibit the germination of nine species of plants commonly used in ecotoxicological tests. This could be attributed to the protective effect exerted by an organic matter of the soil by decreasing the bioavailability of the pesticide and the selective permeability exerted by the seed coatings, which is a characteristic of each species (Wierzbicka and Obidzińska, 1998). In this context, germination of the

Table 1
Inhibition of germination [IG (%)] in *L. esculentum*, *R. sativus*, *L. sativa* and *Z. mays*. Treatments: I) Non-contaminated soil; II) Soil contaminated with lindane 25 μg kg^{-1}; III) Soil contaminated with Cr(VI) 50 mg kg^{-1}; IV) Soil contaminated with Cr(VI) 50 mg kg^{-1} and lindane 25 μg kg^{-1}; V) Soil contaminated with Cr(VI) 50 mg kg^{-1} and lindane 25 μg kg^{-1} and after 14 days of stabilization, inoculated with the quadruple actinobacteria consortium. Values that sharing the same letter were not significantly different ($p < 0.05$).

Treatment	Residual concentrations		IG (%)[a]			
	$Cr(VI)_B$ (mg kg^{-1})[b]	Lindane (μg kg^{-1})[b]	*L. esculentum*	*L. sativa*	*R. sativus*	*Z. mays*
I	0	0	5 ± 2%^{a}	5 ± 2%$^{a'}$	4 ± 3%$^{a''}$	3 ± 3%$^{a'''}$
II	0	25	17 ± 3%^{b}	2 ± 1%$^{a'}$	2 ± 1%$^{a''}$	6 ± 2%$^{a'''}$
III	15	0	57 ± 1%^{c}	72 ± 4%$^{b'}$	65 ± 5%$^{b''}$	3 ± 1%$^{a'''}$
IV	15	25	64 ± 4%^{d}	75 ± 3%$^{b'}$	63 ± 3%$^{b''}$	8 ± 4%$^{a'''}$
V	6	5	26 ± 2%^{e}	48 ± 4%$^{c'}$	31 ± 4%$^{c''}$	0 ± 0%$^{a'''}$

[a] Media ± standard deviation.
[b] The values indicated in treatments **II**, **III** and **IV** corresponds to the concentration achieved after the stabilization period (14 days) and maintained after the incubation period (14 days); in **V** corresponds to the concentration determined after stabilization and bioremediation periods (28 days).

seeds of these three plants species would not be a sensitive biomarker to detect the phytotoxic effects of lindane in the initial stages of plant growth. However, on tomato seeds, the pesticide caused an IG of 17%, probably because of the reduction in the protease activity of the seeds, which was already reported by Ajithkumar et al. (1998) in tomato seeds exposed to 3-chloro- and 4-chlorobenzoate. Also, this effect could be a consequence of the non-selective permeability of the seeds of *L. esculentum* (Wierzbicka and Obidzińska, 1998), which allows the incorporation of lindane and its subsequent interaction with the embryonic axis in germination. According to these results, the germination of tomato seeds would be a sensitive biomarker to detect the phytotoxic effects of lindane and $Cr(VI)_B$ as single contaminants or as a mixture of them (treatment II, III and IV, respectively).

The lengths of hypocotyls and radicles of the *L. sativa* seedlings (Fig. 3A) grown in the soil contaminated with lindane (treatment II) did not show significant differences with those of the seedlings from the soil without contaminants (treatment I). Similarly, there were no significant differences between the values obtained for these biomarkers measured in the seedlings grown in soil contaminated only with Cr(VI) (treatment III) and the soil contaminated with both toxicants (treatment IV), indicating that the used concentration of lindane did not cause an effect appreciable on the growth of the seed, for this species. However, the toxic effect of the concentration of Cr(VI) was evidenced when comparing the seedlings from the non-contaminated soil and those contaminated with Cr(VI), both individually and as a mixture, where the values of both biomarkers were reduced up to 83 and 86%, respectively.

On *R. sativus* (Fig. 3B) the used concentration of lindane did not cause a significant effect on the elongation of hypocotyls, whereas Cr (VI) did show a significant effect. That is, this biomarker only allowed to evaluate the toxic effect of the metal. However, the lengths of radicles of the seedlings at the five treatments tested showed significant differences between them, which indicates that this biomarker, measured on *R. sativus*, detected both the individual effect of lindane and Cr (VI) and the effect of both pollutants, in the concentrations used.

L. esculentum (Fig. 3C) was the only dicotyledons that presented significant differences in the lengths of hypocotyls and radicles under all the tested treatments. In this way, both biomarkers measured on *L. esculentum*, allowed to detect the toxic effect, both individual and combined, of lindane and Cr(VI) in the concentrations tested.

It is remarkable that in the three dicotyledons evaluated, the lengths of the hypocotyls and radicles of seedlings from bioremediated soil were significantly higher than those from the untreated soil, demonstrating that the toxic effects caused by Cr(VI) and lindane were partially reversed after the bioremediation process (Fig. 3).

The lengths of stems and roots of *Z. mays* seedlings grown on soil without contaminants (treatment I) did not show significant differences with those of seedlings grown on soil contaminated only with lindane (treatment III) (Fig. 3D). In contrast, the values of these biomarkers measured on seedlings grown in soil contaminated only with Cr(VI) (treatment II) were significantly higher than the values obtained from soil seedlings contaminated with both toxicants [(treatment IV), ($p < 0.05$)]. This result could indicate that lindane, by itself, did not cause a significant toxic effect on the lengths of stems and roots of the corn seedlings in the tested concentration. However, the presence of the pesticide in soils contaminated with Cr(VI), increased the toxicity in the soil. The lengths of stems and roots of the *Z. mays* seedlings grown on the bioremediated soil (treatment V) were statistically higher than the values reached in the untreated soil (treatment I), demonstrating a partial recovery of the toxic effects caused by Cr(VI) and lindane.

The development of the root determines the capacity of the plant to acquire resources from the surrounding soil (water, nutrients). The toxic effect of the contaminants on the root development is reflected in the physiological performance, especially during the initial stages of growth, showing a reduction of the physiological capacity of the plants (Shanker et al., 2005). The decrease in root growth due to the presence of metals in general, and Cr, in particular, is well-documented (Tang et al., 2001). Prasad, Greger, and Landberg (2001) showed that the root length of new primordia of *Salix viminalis* was more affected by Cr than by other heavy metals studied such as Cd and Pb. Moreover, Chen et al. (2015) showed that 20 mg kg^{-1} of Cr(VI) inhibited the growth of roots of *Triticum dicoccum* (wheat) seedlings. Samantaray et al. (1999) noted that root growth was significantly affected by the presence of the metal in a soil of five mung bean cultivars contaminated with chromite. Accordingly, in the present study, the elongation of the radicles/roots of the four plant species evaluated was also inhibited by the presence of the metal, demonstrating the high toxicity of Cr(VI). This general response of decreased root growth could be due to the inhibition of the division/elongation of the cells of the radicle/root or to the prolongation of the cell cycle (Shanker et al., 2005).

The lindane effect on the growth rate of radicles of radish and tomato were significant. The way in which the plant explores its environment efficiently and how the translocation of carbohydrates from root to shoot occurs (Boot and Mensink, 1990) could be strongly affected by the presence of lindane (Blondel et al., 2016). In contrast, the elongation of the radicles/roots of the lettuce and corn seedlings were not inhibited by the presence of lindane. There is evidence that some plants in particular, in response to the stress caused by this pesticide, produce equal or greater root tissue, in order to conserve or increase the nutrition capacity (Fitter and Hay, 2002). At long-term, this strategy should favor the survival of the plant, since the damage caused during germination would be repaired and photosynthesis would be favored. However, *Z. mays* showed a particularity: although the concentration of lindane used did not cause a significant toxic effect on the elongation of the root of the corn seedlings, the toxic effect of the metal was enhanced in the presence of the pesticide. This effect was not observed on radicles

of lettuce seedlings, where the inhibition of the elongation was due exclusively to Cr(VI).

Adverse effects of Cr(VI) on the hypocotyls/stems length of the plants have also been reported, associated with the late growth of the outbreaks (Rout et al., 2000). In the present study, the elongation of hypocotyls/stems of the four plant species evaluated was inhibited by the concentration of Cr(VI) used. This was probably a consequence of the inhibition of radicle/root growth and decreasing in nutrients and water transported to the upper parts of the plant. Moreover, the transport of Cr(VI) to the aerial part can directly impact the cellular metabolism of the shoots that contribute to the reduction of the height of the seedlings (Shanker et al., 2005).

On the other hand, lindane can also affect the length of hypocotyls/stems of seedlings. Previously, Saez et al. (2015) demonstrated that the pesticide inhibited the elongation of the hypocotyls of *L. sativa* seedlings by up to 40% although the concentrations of the pesticide were 100 times higher (10–100 mg kg^{-1}) than those used in this work. In this sense, the length of the hypocotyl is a representative marker of the presence of lindane only when its concentration is extremely high. In the present work, the elongation of hypocotyl/stem of lettuce, radish and corn seedlings were not affected by the presence of lindane as a single contaminant. However, in soils co-contaminated with Cr(VI), lindane increased the toxic effect on the elongation of corn stems. On the other hand, the elongation of *L. esculentum* hypocotyls was inhibited by the pesticide. This species was the most sensitive to the presence of these contaminants. The biochemical and morphological alterations that take place during germination, lead to reduced root growth. In this way, the transport of nutrients towards the hypocotyl, and later towards the leaves, is scarce (Calvelo Pereira et al., 2010; Kısa et al., 2016), leading to seedlings of reduced length.

3.2.2. *E. fetida* earthworm

Lindane did not affect the survival of earthworms at the used concentration. However, Cr(VI) caused between 80 and 85% of mortality of the specimens, at a concentration of 50 mg kg^{-1} (Fig. 4). Even though changes in the mortality rate of earthworms can be used as a reliable endpoint of environmental contamination with pesticides. Kokta (1992) showed that pesticides used for decades, such as lindane, present elevated values of LC_{50}, so they are harmless to earthworms in terms of survival. Therefore, the concentration of the pesticide used in this study (1 mg kg^{-1}) did not affect the survival of the specimens (no mortality).

On the other hand, the effects of lindane and/or Cr(VI) were significant on the weight loss of the specimens (Fig. 4). In the non-contaminated soil, the weight loss was 14%, while in the presence of lindane or Cr(VI) it was 27 and 41%, respectively. In the presence of both

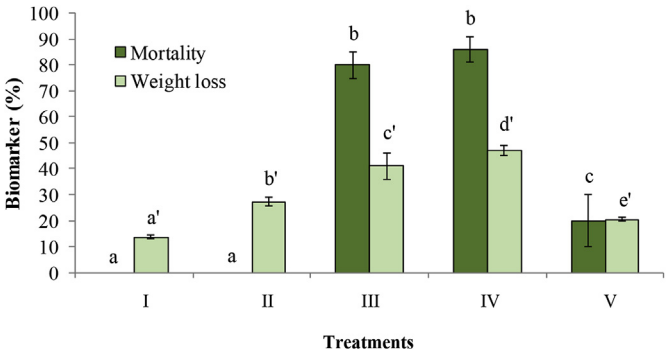

Fig. 4. Mortality [M (%)] and weight loss (%) of *E. fetida* individuals. Treatments: I) Non-contaminated soil; II) Soil contaminated with lindane 25 μg kg^{-1}; III) Soil contaminated with Cr(VI) 50 mg kg^{-1}; IV) Soil contaminated with Cr(VI) 50 mg kg^{-1} and lindane 25 μg kg^{-1}; V) Soil contaminated with Cr(VI) 50 mg kg^{-1} and lindane 25 μg kg^{-1} and after 14 days of stabilization, inoculated with the quadruple actinobacteria consortium. Bars showing different letters indicate they were significantly different ($p < 0.05$).

pollutants, the loss weight was up to 50%. M and weight loss observed in bioremediated soil (20% for both biomarkers) were significantly lower than the values reached in soils contaminated with Cr(VI) (single or as a mixture of contaminants).

The earthworm has become a well-established model for assessing the toxicity of soil contaminated with metals (Homa et al., 2003; Lukkari et al., 2004). In particular, Sivakumar and Subbhuraam (2005) showed that 250 mg kg^{-1} of Cr(VI) is highly toxic to *E. fetida*, affecting critical factors, such as the survival and growth of the specimens. In fact, in the present work, 50 mg Cr(VI)/kg produced the mortality of more than 80% of earthworms. This mortality was preceded by morphological and behavioral changes. The morphological changes observed were oozing of coelomic fluid and cliteral bulge, while the changes in behavior included slow movements and the formation of a structure similar to a knot in the anterior end of the worms.

Changes in biomass can be a good indicator of chemical stress, both by organic and inorganic contaminants (Shi et al., 2007). Specimens maintained in reference soils (treatment I) suffered a weight loss less than 15%. This slight loss suggested that soil nutrients were sufficient to maintain earthworm survival but insufficient to allow additional growth (Sibly and Calow, 1989).

In earthworms exposed to Cr(VI), the weight loss reached 40%. Similarly, some of the previously mentioned morphological and/or behavioral changes occurred anticipating the death of individuals. The toxic impact of Cr(VI) is the result of its action as an oxidizing agent After crossing cell membranes by diffusion facilitated using non-specific sulfate transporters (Costa, 2003), Cr(VI) is reduced to Cr(III) in the cytosol and reacts nonspecifically with the different cellular components (proteic and nucleic) causing their oxidation and the inhibition of some enzymatic systems (Bagchi et al., 2002). Therefore, this sequence of effects (morphological and behavioral changes, mortality) may be due to the higher entry rate of Cr(VI) and the ability to cause oxidative damage to the cells, and to the subsequent toxicity of its reduction product, Cr(III) (Sivakumar and Subbhuraam, 2005).

The growth inhibition of earthworms caused by lindane in this study was similar to those reported for other organic contaminants (Brown et al., 2004; Reinecke and Venter, 1985; Shi et al., 2007). Possibly this effect is correlated with the reduced intake of food to avoid the incorporation of toxic compounds (Burrows and Edwards, 2002; Shi et al., 2007). Similar adjustments were observed in the isopod *Porcellio dilatatus*, with a reduction in consumption rates when exposed to endosulfan (reduced feeding rate and efficiency) that leads to growth inhibition (Ribeiro et al., 2001).

The use of biomarkers at lower levels of biological complexity is very important in order to evaluate the toxic effects of contaminants in the soil before deleterious and irreversible changes can occur (M and weight loss). For instance, the number of coelomocytes in *E. fetida* and their viability can be used as early warning biomarkers of environmental risk caused by Cr(VI) and lindane (Uwizeyimana et al., 2017). Accordingly, a significant reduction in coelomocyte numbers was observed in the 3 spiked soils in comparison with controls (Fig. 5A): lindane 39%, Cr(VI) 70%, both compounds 81%. Interestingly, the number of coelomocytes extruded from earthworms maintained in the bioremediated soil was only 26% of control specimens. It can be concluded that coelomocyte numbers is a sensitive biomarker to detect the toxic effects produced by single contaminants or mixtures, and can accurately evaluate the cost-effectiveness of bioremediated strategies.

The evaluation of coelomocyte viability was evaluated by the Neutral Red Uptake (NRU) assay (Fig. 5B).

A significant reduction in coelomocyte viability was produced after exposure to lindane (20%), Cr(VI) (35%) and the mixture (> 50%) (Fig. 5B). Moreover, the cell viability was highly recovered (85%) after bioremediation. As in the case of coelomocyte number, coelomocyte viability can be used to detect the toxic effects produced by contaminants and to assess the degree of recovery after remediation.

The reactive oxygen species that are produced in the coelomocytes

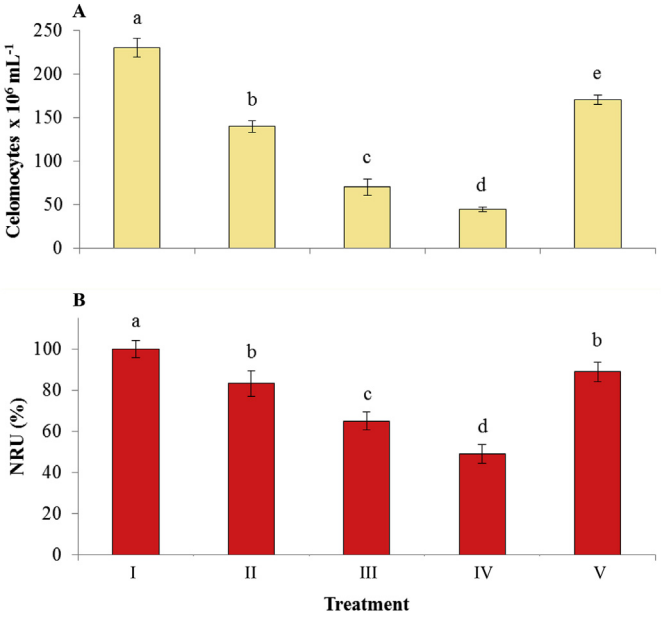

Fig. 5. (A) Number of coelomocytes of *E. fetida* specimens after extrusion on buffer PBS (cells x 10^6 mL^{-1}). (B) Cell viability evaluated as Neutral Red Capture (NRU) (% with respect to control) in coelomocytes of *E. fetida*. Treatments: I) Non-contaminated soil; II) Soil contaminated with lindane 25 μg kg^{-1}; III) Soil contaminated with Cr(VI) 50 mg kg^{-1}; IV) Soil contaminated with Cr(VI) 50 mg kg^{-1} and lindane 25 μg kg^{-1}; V) Soil contaminated with Cr(VI) 50 mg kg^{-1} and lindane 25 μg kg^{-1} and after 14 days of stabilization, inoculated with the quadruple actinobacteria consortium. Bars showing different letters indicate they were significantly different ($p < 0.05$). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

of the earthworms exposed to stress by the presence of Cr(VI) and lindane in the soil, could cause great oxidative damage (Liang and Zhou, 2003; Wang et al., 2015), which lead to functional reductions of the immune system and, ultimately, to cell death.

4. Conclusions

The biomarkers evaluated in each bioindicator showed different levels of sensitivity to the pollutants used. However, the biomarkers of two key species: *L. esculentum* and *E. fetida*, were the most sensitive to evaluate the toxic impact of Cr(VI) and lindane at the concentrations tested. In this sense, both bioindicators and their biomarkers were the most appropriated for evaluating the efficiency of the bioremediation process of co-contaminated soils under these conditions. Moreover, in soils with high levels of Cr(VI), the use of a less sensitive species, such as *R. sativus*, could be useful to complement the ecotoxicological evaluation of the soil quality.

Conflicts of interest

The authors declare that they have no conflict of interest.

Acknowledgments

This work was supported by the Agencia Nacional de Promoción Científica y Tecnológica (PICT 2016-0493), Universidad Nacional de Tucumán (PIUNT D626) and the Consejo Nacional de Investigaciones Científicas y Técnicas (PIP 0372).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ecoenv.2019.06.019.

Unlisted references

Dreyer Parr and Taylor, 1982.

References

Ajithkumar, P.V., Gangadhara, K.P., Manilal, P., Kunhi, A.A.M., 1998. Soil inoculation with *Pseudomonas aeruginosa* 3mT eliminates the inhibitory effect of 3-chloro- and 4-chlorobenzoate on tomato seed germination. Soil Biol. Biochem. 30, 1053–1059. https://doi.org/10.1016/S0038-0717(97)00249-6.

Albarracín, V.H., Amoroso, M.J., Abate, C.M., 2005. Isolation and characterization of indigenous copper-resistant actinomycete strains. Chem. der Erde - Geochem. 65, 145–156. https://doi.org/10.1016/j.chemer.2005.06.004.

Alvarez, A., Saez, J.M., Davila Costa, J.S., Colin, V.L., Fuentes, M.S., Cuozzo, S.A.S.A., Benimeli, C.S., Polti, M.A., Amoroso, M.J., 2017. Actinobacteria: Current research and perspectives for bioremediation of pesticides and heavy metals. Chemosphere 166, 41–62. https://doi.org/10.1016/j.chemosphere.2016.09.070.

Alvarez, A., Benimeli, C.S., Saez, J.M., Giuliano, A., Amoroso, M.J., 2015. Lindane removal using Streptomyces strains and maize plants: a biological system for reducing pesticides in soils. Plant Soil 395, 401–413. https://doi.org/10.1007/s11104-015-2575-5.

Alvarez, A., Yañez, M.L., Benimeli, C.S., Amoroso, M.J., 2012. Maize plants (Zea mays) root exudates enhance lindane removal by native Streptomyces strains. Int. Biodeterior. Biodegrad. 66, 14–18.

Aparicio, J.D., Raimondo, E.E., Gil, R.A., Benimeli, C.S., Polti, M.A., 2018a. Actinobacteria consortium as an efficient biotechnological tool for mixed polluted soil reclamation: Experimental factorial design for bioremediation process optimization. J. Hazard Mater. 342, 408–417. https://doi.org/10.1016/j.jhazmat.2017.08.041.

Aparicio, J.D., Saez, J.M., Raimondo, E.E., Benimeli, C.S., Polti, M.A., 2018b. Comparative study of single and mixed cultures of actinobacteria for the bioremediation of co-contaminated matrices. J. Environ. Chem. Eng. 6, 2310–2318. https://doi.org/10.1016/j.jece.2018.03.030.

Aparicio, J.D., Simón Solá, M.Z., Benimeli, C.S., Julia Amoroso, M., Polti, M.A., 2015. Versatility of *Streptomyces* sp. M7 to bioremediate soils co-contaminated with Cr(VI) and lindane. Ecotoxicol. Environ. Saf. 116, 34–39. https://doi.org/10.1016/j.ecoenv.2015.02.036.

APHA, 2012. Standard Methods for the Examination of Water and Waste Water, twenty-second ed. American Public Health Association, Washington, DC.

Arienzo, M., Masuccio, A., Ferrara, L., 2013. Evaluation of sediment contamination by heavy metals, organochlorinated pesticides, and polycyclic aromatic hydrocarbons in the Berre coastal lagoon (Southeast France). Arch. Environ. Contam. Toxicol. 65, 396–406. https://doi.org/10.1007/s00244-013-9915-3.

Bagchi, D., Stohs, S.J., Downs, B.W., Bagchi, M., Preuss, H.G., 2002. Cytotoxicity and oxidative mechanisms of different forms of chromium. Toxicology 180, 5–22. https://doi.org/10.1016/S0300-483X(02)00378-5.

Benimeli, C.S., Amoroso, M.J., Chaile, A.P., Castro, G.R., 2003. Isolation of four aquatic streptomycetes strains capable of growth on organochlorine pesticides. Bioresour. Technol. 89, 133–138. https://doi.org/10.1016/S0960-8524(03)00061-0.

Blondel, C., Khelalfa, F., Reynaud, S., Fauvelle, F., Raveton, M., 2016. Effect of organochlorine pesticides exposure on the maize root metabolome assessed using high-resolution magic-angle spinning 1H NMR spectroscopy. Environ. Pollut. 214, 539–548. https://doi.org/10.1016/J.ENVPOL.2016.04.057.

Boot, R.G.A., Mensink, M., 1990. Size and morphology of root systems of perennial grasses from contrasting habitats as affected by nitrogen supply. Plant Soil 129, 291–299. https://doi.org/10.1007/BF00032425.

Brown, P.J., Long, S.M., Spurgeon, D.J., Svendsen, C., Hankard, P.K., 2004. Toxicological and biochemical responses of the earthworm Lumbricus rubellus to pyrene, a non-carcinogenic polycyclic aromatic hydrocarbon. Chemosphere 57, 1675–1681. https://doi.org/10.1016/j.chemosphere.2004.05.041.

Burrows, L.A., Edwards, C.A., 2002. The use of integrated soil microcosms to predict effects of pesticides on soil ecosystems. Eur. J. Soil Biol. 38, 245–249. https://doi.org/10.1016/S1164-5563(02)01153-6.

Calvelo Pereira, R., Monterroso, C., Macías, F., 2010. Phytotoxicity of hexachlorocyclohexane: Effect on germination and early growth of different plant species. Chemosphere 79, 326–333. https://doi.org/10.1016/j.chemosphere.2010.01.035.

Casierra Posada, F., Cárdenas Hernández, J., 2007. Influencia del aluminio sobre el crecimiento de la raíz en coliflor (brassica oleracea l., var. Botrytis, hib. 'nevada f1'). Rev UDCA Actual Divulg Científica 10, 149–157.

Chen, M., Xu, P., Zeng, G., Yang, C., Huang, D., Zhang, J., 2015. Bioremediation of soils contaminated with polycyclic aromatic hydrocarbons, petroleum, pesticides, chlorophenols and heavy metals by composting: Applications, microbes and future research needs. Biotechnol. Adv. 33, 745–755. https://doi.org/10.1016/j.biotechadv.2015.05.003.

Coatu, V., Ţigănuş, D., Oros, A., Lazăr, L., 2013. Analysis of hazardous substance contamination of the marine ecosystem in the Romanian black Sea coast, part of the Marine Strategy Framework Directive (2008/56/EEC) implementation. Rech Mar 43, 144–186.

Costa, M., 2003. Potential hazards of hexavalent chromate in our drinking water. Toxicol. Appl. Pharmacol. 188, 1–5. https://doi.org/10.1016/S0041-008X(03)00011-5.

Csillag, J., Pártay, G., Lukács, A., Bujtás, K., Németh, T., 1999. Extraction of soil solution for environmental analysis. Int. J. Environ. Anal. Chem. 74, 305–324. https://doi.org/10.1080/03067319908031434.

Dreyer Parr, P., Taylor, F.G., 1982. Germination and growth effects of hexavalent chromium in Orocol TL (a corrosion inhibitor) on Phaseolus vulgaris. Environ. Int. 7,

197–202. https://doi.org/10.1016/0160-4120(82)90105-2.
Fitter, A., Hay, R.K.M., 2002. Environmental Physiology of Plants. Academic Press.
Fuentes, M.S., Briceño, G.E., Saez, J.M., Benimeli, C.S., Diez, M.C., Amoroso, M.J., 2013. Enhanced removal of a pesticides mixture by single cultures and consortia of free and immobilized *Streptomyces* strains. BioMed Res. Int. 2013. https://doi.org/10.1155/2013/392573.
Fuentes, M.S., Saez, J.M., Benimeli, C.S., Amoroso, M.J., 2011. Lindane biodegradation by defined consortia of indigenous *Streptomyces* strains. Water Air Soil Pollut. 222, 217–231. https://doi.org/10.1007/s11270-011-0818-5.
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 maintained in two standard soils (OECD and LUFA 2.3). Chemosphere 181, 747–758. https://doi.org/10.1016/J.CHEMOSPHERE.2017.04.143.
Hirano, T., Tamae, K., 2011. Earthworms and soil pollutants. Sensors 11, 11157–11167. https://doi.org/10.3390/s111211157.
Homa, J., Niklinska, M., Plytycz, B., 2003. Effect of heavy metals on coelomocytes of the earthworm Allolobophora chlorotica: The 7th international symposium on earthworm ecology · Cardiff · Wales · 2002. Pedobiologia 47, 640–645. https://doi.org/10.1078/0031-4056-00239.
Hopwood, D.A., 1985. Genetic Manipulation of *Streptomyces* : A Laboratory Manual. John Innes Foundation.
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, 1326–1335. https://doi.org/10.1007/s10646-014-1275-x.
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 Toxicol. Pharmacol. 160, 42–53. https://doi.org/10.1016/j.cbpc.2013.11.006.
Kim, R.-Y.Y., Yoon, J.-K.K., Kim, T.-S.S., Yang, J.E., Owens, G., Kim, K.-R.R., 2015. Bioavailability of heavy metals in soils: Definitions and practical implementation—a critical review. Environ. Geochem. Health 37, 1041–1061. https://doi.org/10.1007/s10653-015-9695-y.
Kısa, D., Elmastaş, M., Öztürk, L., Kayır, Ö., 2016. Responses of the phenolic compounds of Zea mays under heavy metal stress. Appl. Biol. Chem. 59, 813–820. https://doi.org/10.1007/s13765-016-0229-9.
Kokta, C., 1992. A laboratory test on sublethal effects of pesticides on Eisenia fetida. In: PW, G.-S., H, B., PJ, E., F.H. (Eds.), Ecotoxicology of Earthworms. Intercept, London, pp. 213–216.
Kumpiene, J., Bert, V., Dimitriou, I., Eriksson, J., Friesl-Hanl, W., Galazka, R., Herzig, R., Janssen, J., Kidd, P., Mench, M., Müller, I., Neu, S., Oustriere, N., Puschenreiter, M., Renella, G., Roumier, P.-H., Siebielec, G., Vangronsveld, J., Manier, N., 2014. Selecting chemical and ecotoxicological test batteries for risk assessment of trace element-contaminated soils (phyto)managed by gentle remediation options (GRO). Sci. Total Environ. 496, 510–522. https://doi.org/10.1016/j.scitotenv.2014.06.130.
Liang, J., Zhou, Q., 2003. Single and binary-combined toxicity of methamidophos, acetochlor and Cu on earthworm *Eisenia foetida*. J. Appl. Ecol. 14, 593–596.
Lukkari, T., Taavitsainen, M., Soimasuo, M., Oikari, A., Haimi, J., 2004. Biomarker responses of the earthworm *Aporrectodea tuberculata* to copper and zinc exposure: Differences between populations with and without earlier metal exposure. Environ. Pollut. 129, 377–386. https://doi.org/10.1016/j.envpol.2003.12.008.
Maggi, C., Ausili, A., Boscolo, R., Cacciatore, F., Bonometto, A., Cornello, M., Berto, D., 2012. Sediment and biota in trend monitoring of contaminants in transitional waters. TrAC Trends Anal. Chem. (Reference Ed.) 36, 82–91. https://doi.org/10.1016/j.trac.2012.01.006.
Manonmani, H.K., 2011. Bioremediation of hexachlorocyclohexane contaminated soil: Field trials. In: Pesticides in the Modern World - Pesticides Use and Management. InTech. https://doi.org/10.5772/20802.
Mansour, S.A., Belal, M.H., Abou-Arab, A.A.K., Gad, M.F., 2009. Monitoring of pesticides and heavy metals in cucumber fruits produced from different farming systems. Chemosphere 75, 601–609. https://doi.org/10.1016/j.chemosphere.2009.01.058.
Moradas, G., Auresenia, J., Gallardo, S., Guieysse, B., 2008. Biodegradability and toxicity assessment of trans-chlordane photochemical treatment. Chemosphere 73, 1512–1517. https://doi.org/10.1016/J.CHEMOSPHERE.2008.07.035.
Munzuroglu, O., Geckil, H., 2002. Effects of metals on seed germination, root elongation, and coleoptile and hypocotyl growth in *Triticum aestivum* and *Cucumis sativus*. Arch. Environ. Contam. Toxicol. 43, 203–213. https://doi.org/10.1007/s00244-002-1116-4.
Nikinmaa, M., Nikinmaa, M., 2014. Bioindicators and biomarkers. An. Introd. Aquat. Toxicol. 147–155. https://doi.org/10.1016/B978-0-12-411574-3.00012-8.
Nikolaeva, O.V., Terekhova, V.A., 2017. Improvement of laboratory phytotest for the ecological evaluation of soils. Eurasian Soil Sci. 50, 1105–1114. https://doi.org/10.1134/S1064229317090058.
Olaniran, A.O., Balgobind, A., Pillay, B., 2013. Bioavailability of heavy metals in soil : Impact on microbial biodegradation of organic compounds and possible improvement strategies. Int. J. Mol. Sci. 14, 10197–10228. https://doi.org/10.3390/ijms140510197.
Onogbosele, C.O., Scrimshaw, M.D., 2014. Hexabromocyclododecane and hexachlorocyclohexane: How lessons learnt have led to improved regulation. Crit. Rev. Environ. Sci. Technol. 44, 1423–1442. https://doi.org/10.1080/10643389.2013.782172.
Peralta, J.R., Gardea-Torresdey, J.L., Tiemann, K.J., Gomez, E., Arteaga, S., Rascon, E., Parsons, J.G., 2001. Uptake and effects of five heavy metals on seed germination and plant growth in alfalfa (*Medicago sativa* L.). Bull. Environ. Contam. Toxicol. 66, 727–734. https://doi.org/10.1007/s001280069.
Polti, M.A., Amoroso, M.J., Abate, C.M., 2007. Chromium(VI) resistance and removal by actinomycete strains isolated from sediments. Chemosphere 67, 660–667. https://doi.org/10.1016/j.chemosphere.2006.11.008.
Polti, M.A., Aparicio, J.D., Benimeli, C.S., Amoroso, M.J., 2014. Simultaneous bioremediation of Cr(VI) and lindane in soil by actinobacteria. Int. Biodeterior. Biodegrad. 88, 48–55. https://doi.org/10.1016/j.ibiod.2013.12.004.
Polti, M.A., Atjian, M.C., Amoroso, M.J., Abate, C.M., 2011. Soil chromium bioremediation: Synergic activity of actinobacteria and plants. Int. Biodeterior. Biodegrad. 65, 1175–1181. https://doi.org/10.1016/j.ibiod.2011.09.008.
Prasad, M.N.V., Greger, M., Landberg, T., 2001. *Acacia nilotica* L. Bark removes toxic elements from solution: Corroboration from toxicity bioassay using *Salix viminalis* L. In hydroponic system. Int. J. Phytoremediation 3, 289–300. https://doi.org/10.1080/15226510108500060.
Rayu, S., Karpouzas, D.G., Singh, B.K., 2012. Emerging technologies in bioremediation: Constraints and opportunities. Biodegradation 23, 917–926. https://doi.org/10.1007/s10532-012-9576-3.
Reinecke, A.J., Venter, J.M., 1985. The influence of moisture on the growth and reproduction of the compost worm *Eisenia fetida* (*Oligochaeta*) [clitellum development; cocoon production]. Rev d'Ecologie Biol du sol 22, 473–481.
Ribeiro, S., Sousa, J.P., Nogueira, A.J.A., Soares, A.M.V.M., 2001. Effect of endosulfan and parathion on energy reserves and physiological parameters of the terrestrial isopod *Porcellio dilatatus*. Ecotoxicol. Environ. Saf. 49, 131–138.
Ronco, A., Diaz Baez, M., Pica Granados, Y., 2004. Conceptos generales. In: Castillo Morales, G. (Ed.), Ensayos Toxicológicos y Métodos de Evaluación de Calidad de Aguas. Estandarización, Intercalibración, Resultados y Aplicaciones. IMTA, México, pp. 17–22.
Rout, G.R., Samantaray, S., Das, P., 2000. Effects of chromium and nickel on germination and growth in tolerant and non-tolerant populations of *Echinochloa colona* (L.). Chemosphere 40, 855–859. https://doi.org/10.1016/S0045-6535(99)00303-3.
Saez, J.M., Alvarez, A., Benimeli, C.S., Amoroso, M.J., Álvarez, A., Benimeli, C.S., Amoroso, M.J., 2014. Enhanced lindane removal from soil slurry by immobilized *Streptomyces* consortium. Int. Biodeterior. Biodegrad. 93, 63–69. https://doi.org/10.1016/j.ibiod.2014.05.013.
Saez, J.M., Aparicio, J.D., Amoroso, M.J., Benimeli, C.S., 2015. Effect of the acclimation of a *Streptomyces* consortium on lindane biodegradation by free and immobilized cells. Process Biochem. 50, 1923–1933. https://doi.org/10.1016/j.procbio.2015.08.014.
Samantaray, S., Rout, G.R., Das, P., 1999. Studies on the uptake of heavy metals by various plant species on chromite minespoils in sub-tropical regions of India. Environ. Monit. Assess. 55, 389–399. https://doi.org/10.1023/A:1005982915175.
Shahid, M., Shamshad, S., Rafiq, M., Khalid, S., Bibi, I., Niazi, N.K., Dumat, C., Rashid, M.I., 2017. Chromium speciation, bioavailability, uptake, toxicity and detoxification in soil-plant system: A review. Chemosphere 178, 513–533. https://doi.org/10.1016/j.chemosphere.2017.03.074.
Shanker, A.K., Cervantes, C., Loza-Tavera, H., Avudainayagam, S., 2005. Chromium toxicity in plants. Environ. Int. 31, 739–753. https://doi.org/10.1016/j.envint.2005.02.003.
Shi, Y., Shi, Y., Wang, X., Lu, Y., Yan, S., 2007. Comparative effects of lindane and deltamethrin on mortality, growth, and cellulase activity in earthworms (*Eisenia fetida*). Pestic. Biochem. Physiol. 89, 31–38. https://doi.org/10.1016/J.PESTBP.2007.02.005.
Sibly, R.M., Calow, P., 1989. A life-cycle theory of responses to stress. Biol. J. Linn. Soc. 37, 101–116. https://doi.org/10.1111/j.1095-8312.1989.tb02007.x.
Silva, J., Torrejon, G., Bay-Schmith, E., Larrain, A., 2003. Calibration of the acute toxicity bioassay with *Dapnia pulex* (crustacea: *Cladocera*) using a reference toxicant. Gayana 67, 87–96. https://doi.org/10.4067/S0717-65382003000100011.
Simon Sola, M.Z., Pérez Visñuk, D., Benimeli, C.S., Polti, M.A., Alvarez, A., 2017. Cr(VI) and lindane removal by Streptomyces M7 is improved by maize root exudates. J. Basic Microbiol. J. Basic Microbiol. (57). https://doi.org/10.1002/jobm.201700324.
Simón Solá, M.Z., Lovaisa, N., Dávila Costa, J.S., Benimeli, C.S., Polti, M.A., Alvarez, A., 2019. Multi-resistant plant growth-promoting actinobacteria and plant root exudates influence Cr(VI) and lindane dissipation. Chemosphere 222, 679–687. https://doi.org/10.1016/J.CHEMOSPHERE.2019.01.197.
Sivakumar, S., Subbhuraam, C.V., 2005. Toxicity of chromium(III) and chromium(VI) to the earthworm *Eisenia fetida*. Ecotoxicol. Environ. Saf. 62, 93–98. https://doi.org/10.1016/j.ecoenv.2004.08.006.
Sobrero, M., Ronco, A., 2004. Ensayo de toxicidad aguda con semillas de lechuga (*Lactuca sativa* L.). In: Castillo Morales, G. (Ed.), Ensayos Toxicológicos y Métodos de Evaluación de Calidad de Aguas. Estandarización, Intercalibración, Resultados y Aplicaciones. IMTA, México, pp. 71–79.
Srivastava, S., Ahmad, A.H., Thakur, I.S., 2007. Removal of chromium and pentachlorophenol from tannery effluents. Bioresour. Technol. 98, 1128–1132. https://doi.org/10.1016/j.biortech.2006.04.011.
Tang, S., Wilke, B.-M., Brooks, R.R., 2001. Heavy-metal uptake by metal-tolerant *Elsholtzia Haichowensis* and *Commelina Communis* from China. Commun. Soil Sci. Plant Anal. 32, 895–905. https://doi.org/10.1081/CSS-100103915.
Tang, X., Shen, C., Shi, D., Cheema, S.A., Khan, M.I., Zhang, C., Chen, Y., 2010. Heavy metal and persistent organic compound contamination in soil from Wenling: An emerging e-waste recycling city in Taizhou area, China. J. Hazard Mater. 173, 653–660. https://doi.org/10.1016/j.jhazmat.2009.08.134.
Uwizeyimana, H., Wang, M., Chen, W., Khan, K., 2017. The eco-toxic effects of pesticide and heavy metal mixtures towards earthworms in soil. Environ. Toxicol. Pharmacol. 55, 20–29. https://doi.org/10.1016/J.ETAP.2017.08.001.
Wang, Y., Chen, C., Qian, Y., Zhao, X., Wang, Q., 2015. Ternary toxicological interactions of insecticides, herbicides, and a heavy metal on the earthworm *Eisenia fetida*. J. Hazard Mater. 284, 233–240. https://doi.org/10.1016/j.jhazmat.2014.11.017.
Wierzbicka, M., Obidzińska, J., 1998. The effect of lead on seed imbibition and germination in different plant species. Plant Sci. 137, 155–171.