



Research article

Gentle remediation options for soil with mixed chromium (VI) and lindane pollution: biostimulation, bioaugmentation, phytoremediation and vermiremediation

Rafael G. Lacalle^{a,*}, Juan D. Aparicio^{b,c,1}, Unai Artetxe^a, Erik Urionabarrenetxea^{d,e}, Marta A. Polti^{c,f}, Manuel Soto^{d,e}, Carlos Garbisu^g, José M. Becerril^a^a Department of Plant Biology and Ecology, Faculty of Science and Technology, University of the Basque Country (UPV/EHU), B° Sarriena s/n, E-48940, Leioa, Spain^b Planta Piloto de Procesos Industriales Microbiológicos (PROIMI), CONICET, Av. Belgrano y Pasaje Caseros, 4000, Tucumán, Argentina^c Facultad de Bioquímica, Química y Farmacia, Universidad Nacional de Tucumán, Ayacucho, 491. 4000, Tucumán, Argentina^d Department of Zoology and Animal Cell Biology, Faculty of Science and Technology, University of the Basque Country (UPV/EHU), B° Sarriena s/n, 48940, Leioa, Spain^e Department of Zoology and Animal Cell Biology, Research Centre for Experimental Marine Biology and Biotechnology, University of the Basque Country (UPV/EHU), Areatza Z-G, E-48620, Plentzia, Spain^f Facultad de Ciencias Naturales e Instituto Miguel Lillo, Universidad Nacional de Tucumán, Miguel Lillo 205, 4000, Tucumán, Argentina^g NEIKER, Department of Conservation of Natural Resources, c/Berreaga 1, E-48160, Derio, Spain

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ABSTRACT

Gentle Remediation Options (GROs), such as biostimulation, bioaugmentation, phytoremediation and vermiremediation, are cost-effective and environmentally-friendly solutions for soils simultaneously polluted with organic and inorganic compounds. This study assessed the individual and combined effectiveness of GROs in recovering the health of a soil artificially polluted with hexavalent chromium [Cr(VI)] and lindane. A greenhouse experiment was performed using organically-amended vs. non-amended mixed polluted soils. All soils received the following treatments: (i) no treatment; (ii) bioaugmentation with an actinobacteria consortium; (iii) vermiremediation with *Eisenia fetida*; (iv) phytoremediation with *Brassica napus*; (v) bioaugmentation + vermiremediation; (vi) bioaugmentation + phytoremediation; and (vii) bioaugmentation + vermiremediation + phytoremediation. Soil health recovery was determined based on Cr(VI) and lindane concentrations, microbial properties and toxicity bioassays with plants and worms. Cr(VI) pollution caused high toxicity, but some GROs were able to partly recover soil health: (i) the organic amendment decreased Cr(VI) concentrations, alleviating toxicity; (ii) the actinobacteria consortium was effective at removing both Cr(VI) and lindane; (iii) *B. napus* and *E. fetida* had a positive effect on the removal of pollutants and improved microbial properties. The combination of the organic amendment, *B. napus*, *E. fetida* and the actinobacteria consortium was the most effective strategy.

1. Introduction

The intensification and expansion of human activity caused by industrial growth has increased environmental pollution, threatening human and ecosystem health. Pollution and its negative effects are enhanced when organic pollutants (herbicides, pesticides, petroleum

hydrocarbons, etc.) and inorganic compounds (metals, metalloids, etc.) coexist, a phenomenon known as mixed pollution or co-pollution. This leads to dangerous and unpredictable situations resulting from the toxicity of each compound and the interactions among compounds and with soil organisms (Batty and Dolan, 2013).

* Corresponding author.

E-mail address: rafael.gomezl@ehu.eus (R.G. Lacalle).¹ Both authors have contributed equally to the work.<https://doi.org/10.1016/j.heliyon.2020.e04550>

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The presence of both organic and inorganic pollutants in soil is a widespread problem, since more than a third of polluted sites contain more than one type of pollutant (Politi et al., 2014). In particular, mixed pollution with the metal hexavalent chromium [Cr(VI)] and the pesticide lindane has been detected recently in different parts of the world, where both compounds have been reported in concentrations that exceeded the allowed maxima (Aparicio et al., 2018a, 2018b; Arienzo et al., 2013).

Cr(VI) is found in a wide variety of sites, due to its use in many industries such as metallurgy or tanning (Bankar et al., 2009). Cr(VI) has been reported to be 1000-fold more cytotoxic and mutagenic than Cr(III) (Biedermann and Landolph, 1990). Moreover, Cr(III) tends to precipitate, while Cr(VI) is more soluble (Zayed and Terry, 2003). The gamma isomer of hexachlorocyclohexane (γ -HCH), commercially known as lindane, is a highly chlorinated, recalcitrant organochlorine pesticide with toxic effects on animals, including humans. Lindane is accumulated in biological tissues and biomagnified through the food chain, and has been reported in soil, water, air, plants, animals, food, and humans, among others (Fuentes et al., 2011). The hazardous nature of these pollutants, along with their wide distribution and relatively common simultaneous presence, make this combination of pollutants a problem of particular scientific interest.

Gentle Remediation Options (GROs) such as bioaugmentation, phytoremediation, vermiremediation, and biostimulation have received considerable attention in recent years as effective risk-management strategies to reduce the transfer of contaminants to local receptors, through *in-situ* stabilization or extraction of pollutants (Cundy et al., 2013). These biological treatments can provide a cost-effective, environmentally friendly solution to soil co-pollution (Agnello et al., 2016), and are increasingly employed in place of the traditional remediation technologies.

Bioaugmentation attempts to improve the degradation capacity in polluted areas by introducing into the soil microorganisms capable of degrading pollutants or transforming pollutants into non-toxic or less-toxic species, and has been used for both chromium and lindane remediation (Alvarez et al., 2012; Bajaj et al., 2017; Gutiérrez-Corona et al., 2016). While several bacterial strains have been identified as Cr(VI) or lindane bioremediators, few studies have examined their effects on mixtures of these pollutants. Recently, Aparicio et al. (2018b) found that an actinobacteria consortium was effective in reducing high concentrations of Cr(VI) and lindane from polluted soils. Actinobacteria are a group of bacteria commonly found in soil, and their physiological diversity allows them to degrade a wide variety of substances and to play an important role in recycling (Goodfellow et al., 1988; Kieser et al., 2000). However, bioaugmentation has limitations, since the survival of inoculated bacteria is affected by soil characteristics and the existing microbial communities (Cycoń et al., 2017).

Metal phytoremediation includes phytostabilization (reduction of pollutant mobility and bioavailability) (Epelde et al., 2009; Galende et al., 2014a, 2014b) and phytoextraction (metal accumulation in plant shoots) (Barrutia et al., 2010; Epelde et al., 2010). Phytoremediation may also be suitable for the rhizoremediation of organic pollutants (Liu et al., 2017; Montpetit and Lachapelle, 2017), and organic compounds that roots exude to the rhizosphere create a nutrient-rich environment that stimulates microbial communities, enhancing the degradation of organic pollutants (Kuiper et al., 2004). Canola or oilseed rape (*Brassica napus* L.) has been reported to be a suitable candidate for metal phytoremediation (Belouchrani et al., 2016), rhizoremediation of organic pollutants such as diesel fuel (Lacalle et al., 2018a), and for polychlorinated compounds (Javorská et al., 2009). *B. napus* has attracted attention from scientists and industries, due to its potential for oil production from polluted soils (Cundy et al., 2016; Dhiman et al., 2016). These characteristics make *B. napus* a good candidate for phytomanagement, which envisages remediation of the soil while also generating social, environmental and economic benefits (Burgess et al., 2018; Evangelou et al., 2015). Ontañón et al. (2014), in a rhizoremediation study using *B. napus*, found a reduction of Cr(VI) to Cr(III) and phenol degradation in a co-polluted

hydroponic system. To date, no studies have examined remediation of soils co-polluted with lindane and chromium. Previous studies have shown that *B. napus* is moderately tolerant to mixed metal and organic pollution (Lacalle et al., 2018a, 2018b). Nevertheless, the capacity of *B. napus* to reduce the toxicity of this kind of mixed pollution has not been tested.

Another biological-remediation technology that has recently attracted attention from the scientific community is vermiremediation (Sinha et al., 2008). This technology uses earthworms to remediate soils containing metals (Suthar, 2008) and organic pollutants, including some chlorinated compounds (Shi et al., 2020). Earthworms burrow through the soil, mixing it, affecting its structure, and altering its nutritional profile and bacterial and fungal communities (Rodríguez-Campos et al., 2014). Vermiremediation has been used in combination with other GROs, such as bioremediation and phytoremediation (Ekperusi and Aigbodion, 2015; Lemtiri et al., 2016). These combined technologies open new possibilities for soil remediation in a holistic approach, considering soil-earthworm-plant-microbial interactions in the ecological context of the polluted soil. *Eisenia fetida* is a good candidate as a vermiremediator (Chachina et al., 2016; Suthar, 2008) and has also been widely used as an indicator of soil health (Irizar et al., 2015a; Shin et al., 2007).

Applications of GROs commonly include modification of polluted-soil conditions and/or application of amendments that enhance the biological activity of soil organisms, a process known as biostimulation. Organic amendments are a good choice for this purpose and have been widely used (Kästner and Miltner, 2016), as they add nutrients and carbon sources to the soil, promoting plant growth and microbial activity (Galende et al., 2014b) as well as the soil fauna (Dubey et al., 2019). They can also impact the oxidation status of metals and their bioavailability (Park et al., 2011). Pyrogenic carbonaceous materials such as engineered carbons and carbon nanomaterials have also been used to adsorb several metals, decreasing their availability (Zhang et al., 2019).

Each biological technology for soil remediation has certain limitations, and the simultaneous presence of inorganic and organic pollutants poses its own particular problems. These restrictions could be counteracted by a combination of technologies to remediate soil pollution, together with recovery of soil health. Accordingly, the aim of this study was to assess the individual and combined effectiveness of *B. napus* plants, and/or an actinobacteria consortium, and/or *E. fetida* earthworms as remediation strategies for soil polluted with Cr(VI) and lindane, in the presence or absence of an organic amendment.

2. Materials and methods

2.1. Experimental design

For this study, two soil samples were collected from a peri-urban area near the city of Vitoria-Gasteiz (42°50'N; 02°40'W, northern Spain), where a pre-treatment had already been applied, *i.e.* addition of an organic amendment. Actually, one sample was taken from a soil previously amended (four months before) with 100 t ha⁻¹ of an organic amendment consisting of recycled urban organic wastes from the city of Vitoria-Gasteiz (A). The other sample, without the abovementioned pre-treatment, was taken from an unamended (U) soil near the amended plot. Both samples were collected from the topsoil (0–15 cm), sieved to <2 mm, and air-dried prior to physicochemical characterization (Table 1).

In order to artificially contaminate the soils, a stock solution of 5 g L⁻¹ of Cr(VI) was prepared as K₂Cr₂O₇. The solution was sterilized by filtration, using Millipore filters with 0.22 μ m pore size. A lindane stock solution was prepared at 5 g L⁻¹ using acetone as the solvent. The soils were artificially polluted with both Cr(VI) and lindane solutions and mixed to homogenize them, establishing three conditions for the experiment: (i) control (C), with no pollution; (ii) moderate pollution (M), with 100 mg kg⁻¹ of Cr(VI) and 15 mg kg⁻¹ of lindane; and (iii) high pollution (H), with 300 mg kg⁻¹ of Cr(VI) and 15 mg kg⁻¹ lindane. All assays were

Table 1. Soil physicochemical properties.

	Unamended soil	Amended soil
Texture class (USDA)	Loam	Loam
Coarse sand (%)	17.9	14.5
Fine sand (%)	21.3	25.1
Total silt (%)	37.5	44.0
Total clay (%)	23.4	15.7
Carbonates (%)	54.7	44.0
Organic Matter (%)	1.0	19.5
Total C organic (% DW)	0.6	7.3
Total N (% DW)	0.1	0.9
C organic/N organic	6.7	8.6
Total S (% DW)	<0.05	<0.05
pH (1:2.5)	7.9	8.0
[Cr] (C) (mg kg ⁻¹)	25.2	25.5
[Cr] (M) (mg kg ⁻¹)	125.2	124.9
[Cr] (H) (mg kg ⁻¹)	325.9	324.9
[Lindane] (C) (mg kg ⁻¹)	0	0
[Lindane] (M) (mg kg ⁻¹)	13.6	15.3
[Lindane] (H) (mg kg ⁻¹)	14.0	13.3

Pollution level: control (C), moderate (M), high (H).

carried out in pots with 1 kg of soil and kept in a greenhouse to allow the pollutants to interact with the soil components. Samples were taken weekly to monitor the concentration of Cr(VI), which stabilized one month after it was added to the soil (data not shown). The greenhouse conditions were: photoperiod 14/10 h day/night, temperature 25/18 °C day/night, relative humidity 60/70% day/night.

After the one-month stabilization period, each soil sample was homogenized and the following treatments were applied: (i) no treatment (NT); (ii) inoculation of actinobacteria consortium (Ac); (iii) addition of 10 *Eisenia fetida* adult individuals (Ef); (iv) sowing of 20 *Brassica napus* seeds (Bn); (v) *E. fetida* + actinobacteria consortium (Ac + Ef); (vi) *B. napus* + actinobacteria consortium (Ac + Bn); and (vii) *E. fetida* + *B. napus* + actinobacteria consortium (Ac + Ef + Bn). The actinobacteria consortium was applied first, to allow the bacteria to colonize the soil, and the other biological treatments were applied 14 days later. After the *B. napus* seeds germinated, the seedlings were thinned to leave 5 seedlings per pot. The pots were kept in the greenhouse under the above environmental conditions for two months after the treatments were applied.

Inoculation was carried out according to Aparicio et al. (2018b). The actinobacteria *Streptomyces* sp. M7, *Streptomyces* sp. A5, *Streptomyces* sp. MC1, and *Amycolaptosis tucumanensis* DSM 45259 were used. They had been isolated from environments polluted with pesticides and metals, and were selected for their compatibility and effectiveness in reducing Cr(VI) and lindane concentrations simultaneously in soil in a previous study (Polti et al., 2014). The bacterial inoculum was prepared as follows: spores of the four actinobacteria species were inoculated individually in flasks with 30 mL of Tryptic Soy Broth (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. After the stabilization period, soils were inoculated with 2 g kg⁻¹ of the quadruple actinobacteria consortium. Equal proportions of biomass of each strain were added in order to reach the desired final concentration.

Seeds of *B. napus* (v. Expower) were provided by the commercial supplier Dekalb® (Barcelona, Spain). Specimens of *E. fetida* 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 weekly addition of horse manure as a nutrient source. In order to guarantee the homogeneity of the

earthworms used in the assays, healthy, sexually mature (clitellated) individuals weighing between 350–450 mg were selected.

During the course of the experiment, the soil was maintained at water-holding capacity (WHC) by irrigating the pots when necessary. Fifty-six days after the plants and worms were introduced, the plants were harvested, the worms were collected and counted, and soil samples were taken and immediately stored at 4 °C for preservation until analysis.

2.2. Physicochemical determinations

2.2.1. Total Cr and soluble Cr(VI) determination in soil

The soil was oven-dried at 35 °C for 72 h and sieved to <0.125 mm before analysis. In order to determine the total Cr content in the soil, samples were acid-digested (HCl and HNO₃), according to the method recommended by the US Environmental Protection Agency (US-EPA Method 3051A, 2007), using a Mars V microwave digestion oven. The total concentration of Cr was determined by Inductively-Coupled Plasma-Mass Spectrometry (ICP-MS) (Agilent 7700) with a limit of quantification (LOQ) of 0.03 µg L⁻¹. Accuracy was ensured using NIST Standard Reference Material 1640. The soluble fraction of Cr, composed of Cr(VI), was extracted following the method described by Jiang et al. (2015). Briefly, a 1:25 (p/v) mixture of soil and Milli-Q water was shaken at 200 rpm for 24 h, centrifuged at 10,000 × g for 15 min, and then filtered (0.45 µm) to remove the soil from the aqueous solution. The extract was analyzed using the same method as for total chromium.

2.2.2. Cr concentration in plants

Plants were oven-dried at 35 °C for 72 h, milled, and digested in a mixture of HNO₃ and HClO₄ (Zhao et al., 1994). Cr concentration was analyzed by Inductively-Coupled Plasma-Mass Spectrometry (ICP-MS) (Agilent 7700) (LOQ: 0.03 µg L⁻¹), with accuracy ensured using NIST Standard Reference Material 1640.

2.2.3. Lindane concentration in soil

Lindane concentration was determined according to Fuentes et al. (2011). Ten mL of a water-methanol-hexane (4:1:5) solution was added to 5 g of soil. The mixture was agitated using a vortex mixer and centrifuged to separate the organic phase, which was removed and evaporated to dryness. The lindane residue was resuspended in n-hexane and quantified as in Fuentes et al. (2011), using a gas chromatograph (Agilent 7890A; LOQ: 170 µg L⁻¹).

2.2.4. Lindane determination in plants

Lindane concentration in plants was determined using the “QuEChERS” method for extraction and analysis of pesticides, as described by Quintero et al. (2005). This method has two phases: i) lindane extraction from dry, milled plant samples with acetonitrile, magnesium sulfate and sodium acetate; ii) cleansing of the extract by solid-phase extraction with an Agilent kit (QuEChERS AOAC). Lindane was quantified as in Fuentes et al. (2011), by gas chromatography (Agilent 7890A; LOQ: 170 µg L⁻¹).

2.3. Soil microbial properties

Soil microbial properties were determined as described by Galende et al. (2014a): (i) microbial activity was determined by basal respiration (BR), following ISO 16072 (2002); (ii) potentially active microbial biomass was determined by substrate-induced respiration (SIR), following ISO 17155 (2002); and (iii) number of metabolized substrates (NUS) was determined using Biolog EcoPlates™.

2.4. Phytotoxicity bioassay with *Cucumis sativus*

In order to evaluate soil phytotoxicity, a root-elongation bioassay was performed. Briefly, pre-germinated seeds of *Cucumis sativus* were exposed for 72 h to 10 g of the soil under controlled conditions, and root elongation was measured after the exposure time, following the method

described by Lacalle et al. (2018a). Roots were measured using the software ImageJ (Schneider et al., 2012).

2.5. Toxicity bioassay with *Eisenia fetida*

Soil toxicity was assessed using a bioassay with *Eisenia fetida* worms, as described by Irizar et al. (2015b). Healthy clitellated earthworms of similar size, kept at 19 °C in constant humidity, were transferred to a non-polluted soil for 24 h for acclimation. Then, the earthworms were cleaned, and 10 individuals were weighed and placed in each jar containing the soils collected from the experiment, at 40% of the soil water-holding capacity. Three replicates per soil were established. After 14 days, the worms were removed from the jars and their mortality and weight were recorded.

2.6. Photosynthetic pigment profile and tocopherols

Prior to plant harvesting, six discs, each with a diameter of 3 mm, were collected from the youngest fully expanded leaf, frozen in liquid nitrogen, and stored at -80 °C until analysis. Leaf discs were homogenized using a tissue-tearor (Model 395; Dremel, México, D.F., Mexico) in 1 mL cold acetone. Then, samples were centrifuged at $13200 \times g$ for 20 min at 4 °C; the supernatant was collected, adjusted to a volume of 1.5 mL and filtered through a 2- μm PFTE filter (Teknokroma, Barcelona, Spain) and refrigerated until analysis.

A new and ultra-rapid uHPLC method was developed for quantification of photosynthetic pigments and tocopherols. This method is less time- and solvent-consuming, generates less residue, and provides a higher resolution for all compounds than traditional HPLC methods. Samples were injected into an Acquity™ uHPLC H-Class system (Waters®, Milford, MA, USA), using a reversed-phase column (Acquity UPLC® HSS C18 SB column, 100Å, 1.8 μm , 2.1 mm \times 100 mm) and a Vanguard™ pre-column (Acquity UPLC HSS C18 SB, 1.8 μm). The mobile phase had two components: solvent A, acetonitrile: water: methanol: Tris-HCl 1 M (84:12.6:2:1.4); and solvent B, methanol: ethyl acetate (68:32). Tocopherols and pigments were eluted using a linear gradient from 100% of solvent A to 100% of solvent B for the first 2.5 min, followed by an isocratic elution of solvent B for 1 min, and the initial conditions (100% solvent A) were restored with a linear gradient of 0.5 min. This isocratic elution with 100% of solvent A was maintained for 2.5 min to re-equilibrate the column prior to the next injection. The flow of the mobile phase was 0.5 mL/min, with a working pressure of around 5000 psi. The column was maintained at 45 °C, in an oven. The volume of the injected sample was 2 μL . The column was preserved overnight with 100% acetonitrile at 0.02 mL/min.

Photosynthetic pigments were detected with a photodiode detector (Acquity PDA uHPLC; Waters) in a range of 400–700 nm for their identification, and were quantified by (usually) integration at 445 nm. Tocopherols were detected by fluorescence, using FLR uHPLC Acquity (Waters), setting an excitation wavelength of 295 nm and an emission wavelength of 340 nm.

Pigments and tocopherols were identified and quantified by spectral characteristic and retention time (RT), using known concentrations of standards as described by García-Plazaola and Becerril (1999). Under our experimental conditions, photosynthetic pigments were detected and integrated at 445 nm and showed the following RT (in min): neoxanthin (1.72), violaxanthin (1.98), antheraxanthin (2.27), lutein (2.47), zeaxanthin (2.52), chlorophyll b (2.63), chlorophyll a (2.85), α -carotene (3.29), and β -carotene (3.32). For tocopherols under the fluorimetric conditions described above, the RT (in min) were: δ -tocopherol (2.53), β + γ tocopherol (2.67), and α -tocopherol (2.81). Under the chromatographic characteristics described above, the conversion factors (pmol per injection/area unit) were: neoxanthin (1.19×10^{-4}), violaxanthin (7.84×10^{-5}), antheraxanthin (8.00×10^{-5}), lutein (8.00×10^{-5}), zeaxanthin (8.38×10^{-5}), chlorophyll b (1.56×10^{-4}), chlorophyll a (2.58×10^{-4}), α -carotene (6.95×10^{-5}), and β -carotene (8.39×10^{-5}). For tocopherols

under the fluorimetric conditions described above the RT (in min) were: δ -tocopherol (7.30×10^{-7}), β + γ tocopherol (3.30×10^{-7}), and α -tocopherol (2.22×10^{-6}).

2.7. Statistical analysis

The statistical analyses used IBM SPSS Statistics for Windows, Version 24. Normality was checked with a Shapiro-Wilk test. Data were tested by means of a 2-way ANOVA (post-hoc: Tukey/Duncan). A Principal Components Analysis was performed, using The Unscrambler Version 9.2.

3. Results and discussion

3.1. Soil physicochemical properties

Table 1 lists the physicochemical properties of both the unamended and amended soils. Both are loamy soils with alkaline pH and a high carbonate content. The unamended soil was poor in quality, with very low organic-matter content (1%) compared to the amended soil (2.6%), and also contained low levels of other nutrients such as N and total organic carbon, which can reduce plant and microorganism growth.

3.2. Chromium and lindane concentration in soil

At the end of the experiment, the total chromium content remained unaltered in all treatments (data not shown), indicating that there were no leaching processes or significant Cr extraction by *B. napus* plants. Soluble chromium [Cr(VI)] showed very different concentrations, depending on the treatment; however, in all test conditions, the final soluble-chromium concentration was lower than the initial concentration (Figure 1A, B). This could be due to the reactivity of Cr(VI), which reacts with organic matter or inorganic minerals present in the soil and is reduced to Cr(III). The resulting Cr(III) could be precipitated as hydroxides or interact with clay minerals, which have a high metal-binding capacity (Sandrin and Maier, 2003). Here, the largest effect was due to the organic amendment, which significantly decreased the concentration of soluble chromium (Figure 1B). Organic matter plays an important role in the bioavailability of Cr in soil through its potential to reduce Cr(VI) to Cr(III). Addition of amendments rich in organic matter presumably accelerates the reduction of Cr(VI) to inert chromite [Cr(III)] (Antoniadis et al., 2018). Similarly, a decrease in soil redox potential can favor Cr(VI) reduction (Lacalle et al., 2020; Xia et al., 2019). In the polluted soils without amendment and with no biological treatment (NT), soluble Cr(VI) was 15.7% and 37.8% of total Cr for moderate and high pollution levels, respectively; while in soils with the organic amendment, the soluble fraction of chromium was less than 1% of the total Cr in both cases.

Bioaugmentation attempts often fail due to the incapacity of the inoculated microorganisms to survive in the soil, due to edaphoclimatic conditions, pollutant toxicity, competition with native microbial communities or lack of tolerance to compounds derived from the degradation of the parent contaminant (Cycoñ et al., 2017). However, in our study, the actinobacteria consortium (Ac) was highly effective in reducing Cr(VI) to Cr(III) (Karthik et al., 2017), as it significantly decreased the soluble Cr concentration in non-amended polluted soils in comparison with non-bioaugmented non-amended soils (Figure 1A). In fact, in amended soils, none of the biological treatments applied had any effect in decreasing soluble chromium concentration (Figure 1B). The reason may be that an equilibrium concentration (threshold) was reached, and/or the concentration was so low (1 mg kg^{-1}) that it is very difficult to stimulate biological reduction of the metal (Simón Solá et al., 2019). These findings agree with those of previous studies (Aparicio et al., 2018a, 2018b; Polti et al., 2014, 2009).

The other biological treatments with *E. fetida* and *B. napus* significantly reduced the concentration of soluble Cr in non-amended soils (Figure 1A), but less than the reduction caused by the consortium (Figure 1A). Nevertheless, in these soils the combination of the three

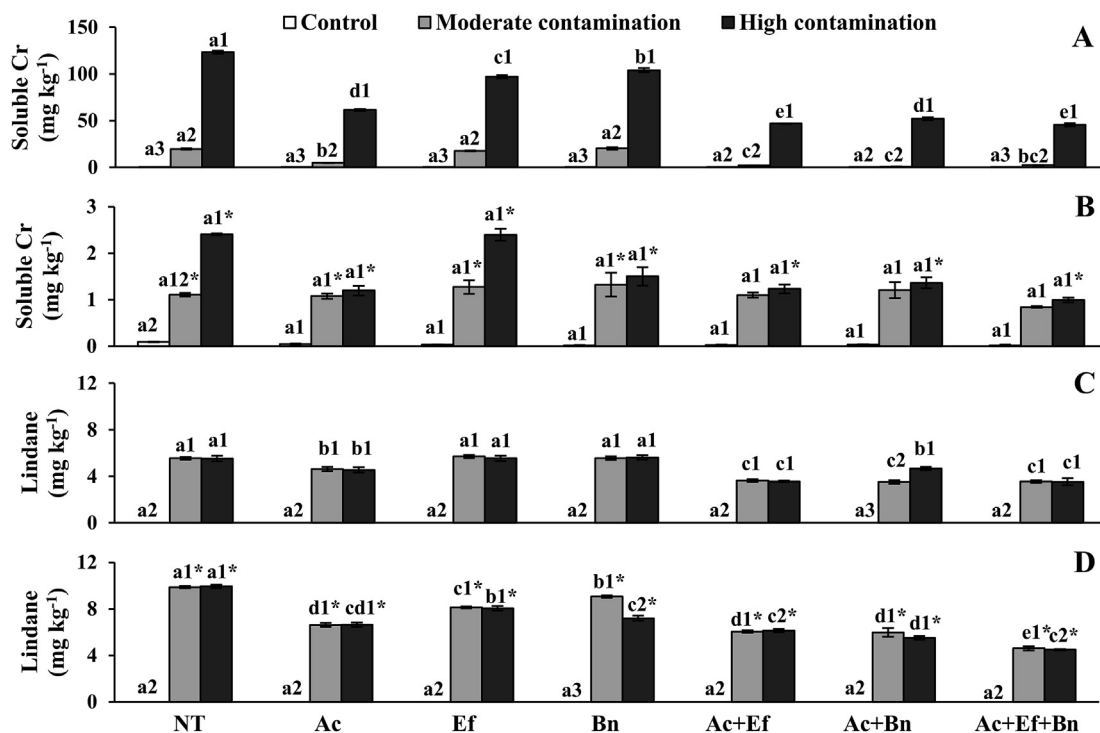


Figure 1. Pollutant concentrations. Soluble Cr concentration in unamended (A) and amended (B) soils. Lindane concentration in unamended (C) and amended (D) soils. Different letters indicate statistical significance ($P < 0.05$) between biological treatments, and numbers indicate statistical significance ($P < 0.05$) between pollution levels. Asterisks refer to statistical significance ($P < 0.05$) between homologous treatments with and without organic amendment.

biological treatments resulted in the lowest Cr(VI) levels (Ac + Bn + Ef). In any case, the concentration of Cr(VI) in the Ac + Bn + Ef treatment in the unamended soil was higher than the concentration of Cr(VI) produced by any treatment in amended soil (Figure 1B), which demonstrates the high effectiveness of the organic amendment in reducing the most toxic species of Cr.

Lindane residual concentrations are shown in Figures 1C and 1D. Natural attenuation occurred; in both soils with no biological treatment, the concentration decreased compared to the initial values. Organic matter inhibited the natural attenuation of lindane, as the concentration in amended soils (Figure 1D) remained significantly higher than in the unamended ones (Figure 1C). To our knowledge, this effect has not been previously reported; it could be explained by the bacteria metabolizing the more easily degradable organic compounds provided by the organic matter instead of the lindane. Another possible explanation could be the adsorption of lindane to soil organic matter. There is a wide range of both natural (e.g., fulvic acids) and artificial substances that can adsorb organic contaminants (Hofman et al., 2014; Tian et al., 2020). The actinobacteria consortium (Ac) was the most effective individual biological treatment in degrading lindane, since the concentration in bioaugmented soils was significantly lower than in the non-bioaugmented ones (Figures 1C, 1D). Degradation by actinobacteria was more pronounced in the amended soils, which had a higher concentration in the untreated soils at the end of the experiment. *B. napus* (Bn) significantly increased lindane degradation, probably by stimulating the microorganisms through exudates from the plant roots (Simon Sola et al., 2017). Concomitantly, *E. fetida* (Ef) also stimulated lindane degradation, probably by improving the aeration of the soil, increasing lindane availability by mineralizing soil organic matter (Rodríguez-Campos et al., 2014), and/or stimulating soil microbial communities (Rodríguez-Campos et al., 2019). The stimulation of lindane degradation caused by *B. napus* and *E. fetida* was accompanied by an increase of microbial functional diversity, suggesting the possibility of soil microorganisms having a key role in the degradation of this contaminant. Other authors have pointed out the relationship between microbial functional diversity and the

degradation of organic compounds (Segura and Ramos, 2013). The combination of the actinobacteria consortium with *E. fetida* (Ac + Ef) or with *B. napus* (Ac + Bn) increased the degradation more than when applied individually, and the effect on degradation of the three combined (Ac + Ef + Bn) was significantly higher than the binary treatments (Figs. 1C, D). Our results indicate that organic matter reduced soluble Cr, but it may have interfered with the optimal degradation of lindane. When the organic-matter content in the soil was low, the microorganisms metabolized lindane more efficiently, showing a synergistic effect in the presence of plants and worms.

3.3. Status of plants and worms

As mentioned before, the unamended soil was a low-quality soil for plant and worm growth, since in the absence of contaminants the shoot biomass was very low, but improved after amendment with organic matter and actinobacteria (Table 2). In addition, the pollutants present in the soil highly impacted the plants and worms. None of them survived in the unamended soils spiked with the highest concentrations of pollutants (Tables 2 and 3). At moderate pollution levels, the plants survived only in the treatment with the lowest soluble Cr(VI) concentration, reached in the treatment Ac + Bn (Table 2). Soluble chromium, rather than total chromium, was toxic to the plants and worms. In contrast, in amended soils, with lower concentrations of Cr(VI), both types of organisms survived in all treatments. The importance of organic matter should be highlighted, since the plants and worms survived in all the treatments, which the toxicity reduction by the actinobacteria consortium (Ac + Bn, Ac + Ef, Ac + Ef + Bn) did not achieve in unamended soils (Tables 2 and 3). The plants and worms benefited not only from the reduction of soluble Cr, but also from their better performance in the enriched soil.

Chromium phytotoxicity has been widely studied (Han et al., 2004; Samantaray et al., 1998), and its impact on *B. napus* was clearly revealed through the plant mortality (Table 2). It has been described that Cr(VI) toxicity can cause *B. napus* plants to develop less biomass and present lower contents of photosynthetic pigments (Ulhasan et al., 2019). In

Table 2. Plant parameters. Shoot dry biomass (DW), total chlorophyll (Chl a+b), ratio of antheraxanthin + zeaxanthin: violaxanthin + antheraxanthin + zeaxanthin (AZ:VAZ), and total carotenoid content (Carot).

	Soil type	Treatment	C	M	H	
Shoot DW (g)	U	Bn	1.2 ± 0.1 b	∅	∅	
		Ac + Bn	1.4 ± 0.1 b1	0.61 ± 0.03 2	∅	
		Ac + Bn + Ef	2.1 ± 0.2 a	∅	∅	
	A	Bn	3.2 ± 0.2 c3*	7.4 ± 0.2 a1	5.29 ± 0.23 a2	
		Ac + Bn	6.1 ± 0.2 a2*	8.0 ± 0.1 a1*	5.31 ± 0.06 a3	
		Ac + Bn + Ef	4.5 ± 0.2 b1*	4.7 ± 0.2 b1	4.64 ± 0.02 b1	
	Chl a+b (pmol mm ⁻²)	U	Bn	103.9 ± 11.8 a	∅	∅
			Ac + Bn	104.2 ± 11.5 a2	344.4 ± 20.8 1	∅
			Ac + Bn + Ef	157.7 ± 3.13 a	∅	∅
A		Bn	196.6 ± 9.8 a2*	301.8 ± 35.9 a1	311.09 ± 15.89 a1	
		Ac + Bn	220.2 ± 27.1 a1*	258.3 ± 60.8 a1*	169.63 ± 6.24 b1	
		Ac + Bn + Ef	258.2 ± 20.2 a1*	300.5 ± 23.5 a1	296.63 ± 43.41 a1	
AZ:VAZ		U	Bn	0.37 ± 0.03 a	∅	∅
			Ac + Bn	0.25 ± 0.03 b1	0.04 ± 0.00 2	∅
			Ac + Bn + Ef	0.20 ± 0.04 b	∅	∅
	A	Bn	0.15 ± 0.04 a*	0.13 ± 0.04 a	0.11 ± 0.03 b	
		Ac + Bn	0.10 ± 0.01 a2*	0.08 ± 0.03 a2	0.27 ± 0.02 a1	
		Ac + Bn + Ef	0.10 ± 0.01 a*	0.09 ± 0.02 a	0.11 ± 0.01 b	
	Carot (pmol mm ⁻²)	U	Bn	35.1 ± 3.9 a	∅	∅
			Ac + Bn	36.3 ± 2.1 a2	101.6 ± 6.7 1	∅
			Ac + Bn + Ef	51.5 ± 8.1 a	∅	∅
A		Bn	63.03 ± 2.6 a2*	92.9 ± 9.8 a1	93.9 ± 5.8 a1	
		Ac + Bn	72.2 ± 7.8 a12*	82.7 ± 17.1 a1	53.2 ± 1.8 b2	
		Ac + Bn + Ef	78.6 ± 5.7 a1*	90.2 ± 6.9 a1	91.8 ± 12.3 a1	

Soil types: unamended (U), amended with organic matter (A).

Pollution level: control (C), moderate (M), high (H).

Treatments: *Brassica napus* (Bn), actinobacteria + *B. napus* (Ac + Bn), actinobacteria + *B. napus* + *Eisenia fetida* (Ac + Bn + Ef).

∅ indicates that no specimen survived the treatment.

Different letters indicate statistical significance ($P < 0.05$) between biological treatments, and numbers indicate statistical significance ($P < 0.05$) between pollution levels.

* indicates statistical significance ($P < 0.05$) between homologous treatments with and without organic amendment.

unamended soils, the surviving plants reached a significantly lower biomass compared to controls, and only the presence of actinobacteria made the survival of plants possible (Table 2). The organic amendment highly stimulated plant development in the control soils, but even more in the polluted ones. This indicates that levels of Cr(VI) as low as 1–3 mg kg⁻¹ were not phytotoxic and might even have had a certain hormetic effect on plant growth, as reported for several species, including some

Table 3. Weight loss of *Eisenia fetida* worms in the pots during the experiment.

Soil type	Treatment	C	M	H
U	Ef	44.2 ± 0.7 b2	77.2 ± 0.9 a1	∅
	Ac + Ef	42.5 ± 2.8 b2	61.4 ± 2.3 b1	∅
	Ac + Bn + Ef	54.8 ± 1.6 a2	63.3 ± 2.5 b1	∅
A	Ef	34.5 ± 0.9 a2*	34.4 ± 2.1 a2*	52.6 ± 2.0 b1*
	Ac + Ef	8.35 ± 2.0 b3*	19.4 ± 2.2 b2*	38.2 ± 1.5 c1*
	Ac + Bn + Ef	38.8 ± 0.6 a2*	38.9 ± 3.7 a2*	64.9 ± 1.9 a1*

Soil types: unamended (U), amended with organic matter (A).

Pollution level: control (C), moderate (M), high (H).

Treatments: *Eisenia fetida* (Ef), actinobacteria + *E. fetida* (Ac + Ef), actinobacteria + *Brassica napus* + *E. fetida* (Ac + Bn + Ef).

∅ indicates that no specimen survived that treatment.

Different letters indicate statistical significance ($P < 0.05$) between biological treatments, and numbers indicate statistical significance ($P < 0.05$) between pollution levels.

* indicates statistical significance ($P < 0.05$) between homologous treatments with and without organic amendment.

members of Brassicaceae (Morkunas et al., 2018). On the other hand, the level of lindane used in our study was far below phytotoxic levels, and the tolerance of species of *Brassica* to lindane makes these species suitable for phytoremediation. Regarding the photosynthetic pigment content of the plants, the organic amendment also mimicked the observed stimulation of biomass in the presence of Cr, significantly increasing the content of Chl a+b and carotenoids (Table 2). The plants that survived in the moderately polluted unamended soils (only in those combined with the actinobacteria consortium) showed similar values to those in amended soils which indicates that the addition of actinobacteria allowed the plants to maintain normal physiological activity. Conversely, the presence of the organic amendment had a larger positive impact on photo-protective mechanisms, as the de-epoxidation ratio seemed to be similar to or lower than control values. The proportions of individual carotenoids (neoxanthin, violaxanthin, lutein, antheraxanthin, zeaxanthin, and β-carotene) to total chlorophyll did not change, as shown in Table 2. Total carotenoid content was not affected, following the same pattern as other photosynthetic pigments such as chlorophyll.

As mentioned above, *E. fetida* was also affected by the presence of Cr(VI) and lindane levels in the soils. Although toxic effects of lindane on *E. fetida* have been reported, most were at concentrations much higher than that used in our experiment (Lock et al., 2002; Shi et al., 2007). Therefore, we presume that the observed toxicity was due mainly to the effect of chromium. The earthworms in our experiment survived in all types of soils except in unamended soil with a high pollution level. In these conditions, not even the beneficial effects of the actinobacteria consortium were enough to make the soil survivable, although they positively affected mortality and weight loss (%) of the worms in the

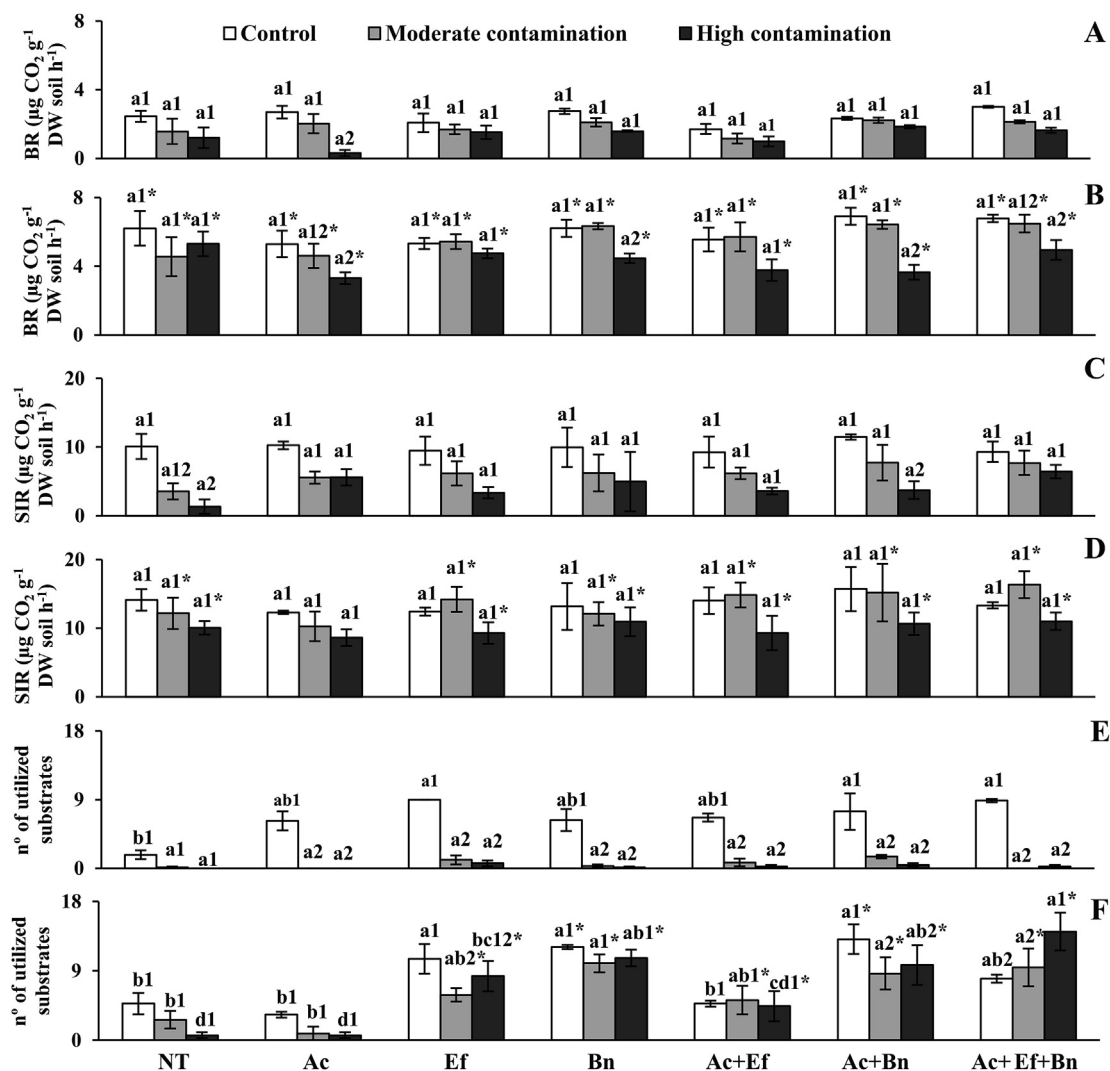


Figure 2. Microbial properties. Soil functional microbial diversity expressed by Number of Utilized Substrates (NUS) in the Biolog EcoPlates (A, B); soil basal respiration (BR) (C, D); and soil substrate-induced respiration (SIR) (E, F) in unamended and amended soils. Different letters indicate statistical significance ($P < 0.05$) between biological treatments, and numbers indicate statistical significance ($P < 0.05$) between pollution levels. Asterisks refer to statistical significance ($P < 0.05$) between homologous treatments with and without organic amendment.

other soils (Table 3). In the Ac + Ef treatment, weight loss of *E. fetida* in soils with moderate pollution was significantly lower than in the treatment without actinobacteria, and the beneficial effect of actinobacteria was marked in the control amended soils. As described by Irizar et al. (2015b), organic matter significantly reduced metal toxicity to *E. fetida*, through an improvement of nutritional status, which is essential to trigger protective mechanisms. This improvement, along with the reduction of Cr(VI) caused by the organic amendment, positively affected worm health, significantly reducing weight loss in all treatments, especially when combined with actinobacteria (Table 3).

However, a slight negative effect on mortality and weight loss was found when actinobacteria, *B. napus* and *E. fetida* were combined (Ac + Ef + Bn). The presence of *B. napus* resulted in a significant weight loss by the worms, especially in the amended soils (Table 3). The reason could be that the plants were able to survive and develop a larger biomass (Table 2). Similarly, the biomass of plant shoots was significantly smaller when they were sharing the pot with the *E. fetida* worms. This antagonistic effect was not observed by other authors (Ghavidel et al., 2018; Wen et al., 2004). However, Lemtiri et al. (2016) found that *E. fetida* worms weighed less when growing in planted pots. This weight loss might be due to competition for space in the pot.

The concentrations of Cr and lindane (data not shown) in plant shoots and worms were very low. *Brassica napus* has been considered as a good candidate for the phytoextraction of chromium (Brunetti et al., 2011) but, under our experimental conditions, this capacity was not observed. Although the concentration of Cr in *E. fetida* individuals collected from unamended soils was higher (30–80 mg kg⁻¹), the total concentration in the biomass of all worms was still low. In any case, the benefits of vermiremediation are not the extraction of pollutants through the worms, but rather the reduction of pollutant ecotoxicity and improvement of soil health.

The ability of the *B. napus* plants to develop a high biomass in amended polluted soils, combined with the low accumulation of Cr in their shoots, indicates the possibility of phytomanagement of co-polluted soils with chromium and lindane. It may be possible to obtain economic benefits from highly polluted soils during their remediation, and there is no risk that the pollutants might enter the food chain through the cultivation of rapeseed.

3.4. Microbial parameters

Microbial communities are excellent indicators of soil health, and parameters such as microbial activity, biomass and functional diversity

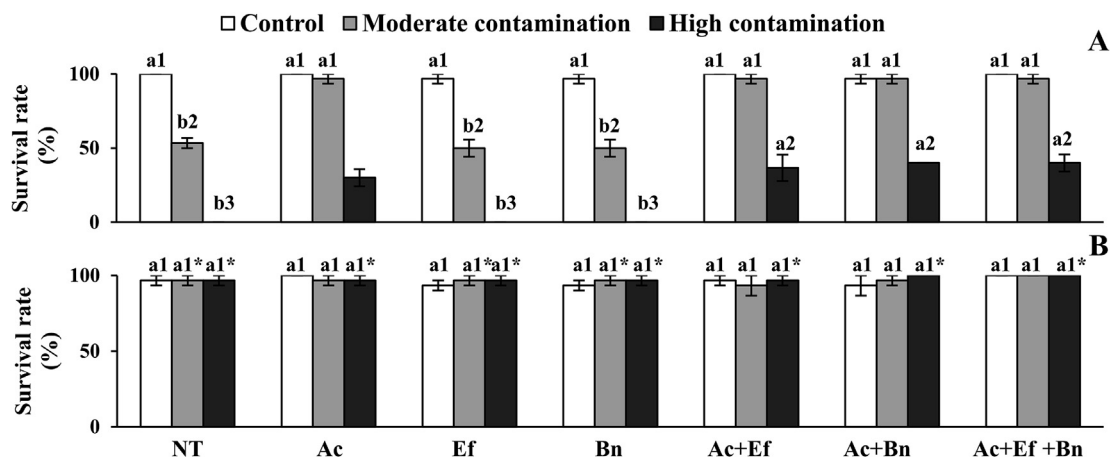


Figure 3. Survival rate of *Eisenia fetida* individuals in the ecotoxicity bioassays, in soils without (A) and with (B) organic amendment. Different letters indicate statistical significance ($P < 0.05$) between biological treatments, and numbers indicate statistical significance ($P < 0.05$) between pollution levels. Asterisks refer to statistical significance ($P < 0.05$) between homologous treatments with and without organic amendment.

have been used as bioindicators in studies of polluted soil (Epelde et al., 2014, 2010; Galende et al., 2014b). In particular, Cr(VI) has been reported to be toxic for soil microbial communities (Pradhan et al., 2019). In this study, basal respiration, which is a good indicator of microbial activity in soil, decreased in the presence of the pollutants in both the unamended (Figure 2A) and amended soils (Figure 2B). The organic amendment significantly increased this metabolic activity in all soils, including controls, due to the input of easily degradable nutrient sources (Galende et al., 2014a; Lacalle et al., 2018a). As shown in Table 1, total organic carbon was 12-fold higher in amended soils. This effect also contributed to the reduction of Cr(VI) due to the organic matter, as

discussed above. Previous studies have observed that a decrease of chromium availability can lead to a stimulation of soil microbial activity (Bashir et al., 2018). Consequently, basal respiration levels in soils with moderate pollution were similar to the control in most cases (Figure 2B). Moreover, in the highly polluted soils, basal respiration levels in amended soils (Figure 2A) increased, compared to the unamended soils. Biological treatments were not as effective as the organic amendment, but overall, the best treatments were Ac + Bn and Ac + Ef + Bn. In conclusion, *B. napus* plants and the inoculation of the actinobacteria consortium seemed to play a crucial role in reinforcing soil microbial activity.

Soil microbial biomass, as assessed by the substrate-induced respiration (SIR), increased in the presence of organic matter (Figure 2D), compared with the biomass in unamended soils (Figure 2C). Organic matter had a slight effect on the controls, but significantly increased SIR in almost all the polluted soils, which indicated that the alleviation of soil toxicity allowed the microbial biomass to increase. The biological treatments resulted in no significant differences in SIR. In any case, the most successful treatments were the combination of the actinobacteria consortium and *B. napus*, with or without *E. fetida*.

The selected indicator of the functional microbial diversity of the soils was the number of utilized substrates (NUS) of the Biolog Ecoplates. Figure 2E shows that in unamended soils, the pollutants had a strong negative impact on functional microbial diversity. The biological treatments increased the NUS of the unamended control soils, but were not very successful in increasing the number of substrates utilized in the polluted soils. This result may be due to the death or poor performance of most of the remediator organisms in the polluted soils (Tables 2 and 3). The situation was very different in the amended soils, which showed higher values of NUS overall, although the pollutants still had a negative effect (Figure 2F). Organic amendments provide a wide range of easily degradable substrates (Jones et al., 2010), which can then enhance microbial functional diversity. Moreover, some of the biological treatments were effective in stimulating the functional diversity of the amended polluted soils. So, soils treated with *E. fetida* worms, and especially *B. napus* plants, increased the NUS values in polluted soils, reaching values similar to the control. The addition of organic matter and stimulation by plants increase functional microbial diversity, improving the health of polluted soils (Borges et al., 2016). The actinobacteria consortium, conversely, did not increase this parameter, or even seemed to reduce it, as in the Ac and Ac + Ef treatments (Figure 2F). This could indicate that the actinobacteria consortium might be more competitive than the autochthonous microbial communities, reducing the microbial functional diversity. Actinobacteria usually can compete and produce antibiotics with antagonistic effects on other microorganisms (Polti et al., 2014). As mentioned for other microbial, plant and worm parameters,

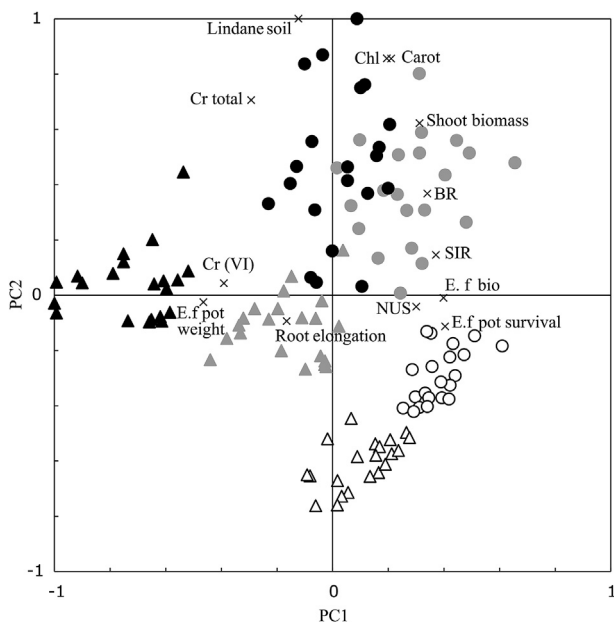


Figure 4. Principal Components Analysis, including lindane concentration in soil (Lindane soil), total (Cr total) and soluble [Cr(VI)] chromium in soil, soil basal respiration (BR), substrate-induced respiration (SIR), Number of Utilized Substrates, the survival rate of *Eisenia fetida* worms in both the pots (*E.f* pot survival) and the bioassay (*E.f* bio), weight loss of *E. fetida* individuals in the pots (*E.f* pot weight) and *B. napus* shoot biomass (Shoot biomass), total chlorophyll content (Chl) and carotenoid content (Carot). White, gray and black icons indicate control, moderate and high pollution, respectively. Triangles indicate unamended soils and circles indicate amended soils. Crosses indicate the position of variables.

the consortium of the three organisms was the best treatment to improve microbial functional diversity.

3.5. Ecotoxicity bioassays with *E. fetida*

Ecotoxicity bioassays with *E. fetida* have proved to be effective tools for the assessment of the toxicity caused by Cr(VI) and lindane co-contamination in soil (Aparicio et al., 2019). In our study, the ecotoxicity bioassays with *E. fetida* reflected a high toxicity in terms of mortality (Figure 3A, B). For worms in the untreated (NT) unamended soil, exposed to the moderate level of pollution, survival was reduced to 53%, and to 0% at the high pollution level (Figure 3A). The phytoremediation and vermiremediation treatments did not improve these mortality levels. In contrast, bioremediation by the actinobacteria consortium (Ac, Ac + Ef, Ac + Bn, Ac + Ef + Bn) significantly alleviated the soil toxicity to the worms, increasing their survival rates at the moderate pollution level to 97% and to 30–40% at the high pollution level. This effectiveness was directly related to the levels of Cr(VI) in the soils, more than to the levels of lindane. It appears that the toxicity in these soils was due to Cr, which agrees with the results for toxicity to the worms used as remediator organisms (Table 3). In any case, the addition of organic matter was the most effective treatment in reducing the toxicity in earthworms, since their survival rates in all the amended soils were close to 100%, even in the highly polluted soils (Figure 3B). Therefore, differences between biological treatments were not observed in the case of amended soils (Figure 3B). These results agree with observations in other studies with earthworms (Irizar et al., 2015b; Rüdell et al., 2001) and are congruent with the present observations on the earthworms in the pots (Table 3), whose status was significantly improved by the higher concentration of organic matter and lower levels of Cr(VI).

3.6. Principal Components Analysis

In the Principal Components Analysis (PCA), the first two principal components explained 66% of the variance, and the samples were clearly segregated in the bi-plot (Figure 4). The first principal component accounted for 44% of the variance and segregated the soils across the x axis by the toxicity of hexavalent chromium. Hence, the higher the pollution, the higher the impact on the indicators of soil health. The second principal component accounted for 22% of the variance and separated the soils by the presence or absence of the organic amendment, which, as mentioned above, was key for reducing chromium toxicity in this experiment and is related to many biological indicators. The results indicated that the presence of organic matter attenuates or nullifies the differences caused by the biological treatments, due to its capacity to reduce Cr(VI) to Cr(III) almost completely, and therefore alleviating the ecotoxicological effects of Cr.

Most of the parameters determined in the soil (BR, SIR, NUS) or in the remediator plants or worms are closely related to each other and to the organic amendment, and opposed to the toxic soluble Cr(VI). Weight loss of *E. fetida* individuals in the pots was highly correlated with soluble Cr(VI), due to the toxicity of hexavalent chromium. Root elongation in the *C. sativus* bioassay (data not shown), on the other hand, appeared around the middle of the PCA. This was due to the lack of acute phytotoxicity to the seedlings in the bioassay. Regarding the parameters measured for the potted plants, shoot biomass, total chlorophyll, and carotenoid content were closely related to each other as plant wellness indicators. Biological parameters, especially those relative to the *B. napus* plants, were strongly influenced by the organic amendment.

4. Conclusions

In the soils spiked with both Cr(VI) and lindane, the concentration of hexavalent chromium was the main component of toxicity. After application of the organic-matter amendment and/or several bioremediation techniques (alone or in combination), most of the hexavalent chromium

was reduced to its less-toxic form, trivalent chromium. The most effective treatment was the addition of organic matter, followed by the bioaugmentation treatment with the actinobacteria consortium, which was composed of species that were originally isolated in a medium with Cr and lindane. The consortium was able both to degrade part of the lindane and to reduce the levels of Cr(VI). This reduction of Cr(VI) lowered the toxicity of the soils, as reflected in many biological indicators of soil health, such as the improvement in the growth and health of *Brassica napus* and in the survival of *Eisenia fetida* individuals. Combined with the organic amendment, especially with the added actinobacteria and *E. fetida*, *B. napus* proved to be suitable for phytomanagement of soils with this kind of pollution. To our knowledge, the combination of organic matter with the actinobacteria consortium, *B. napus* and *E. fetida* has not been reported previously. Our results showed that this was the most successful treatment overall and would be a suitable strategy to reduce contamination and improve the health of soils co-polluted with hexavalent chromium and lindane.

Declarations

Author contribution statement

Rafael G. Lacalle, Juan D. Aparicio: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Unai Artetxe: Performed the experiments; Wrote the paper.

Erik Urionabarrenetxea: Performed the experiments.

Marta A. Polti: Contributed reagents, materials, analysis tools or data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Manuel Soto: Conceived and designed the experiments; Analyzed and interpreted the data; Wrote the paper.

Carlos Garbisu: Analyzed and interpreted the data; Wrote the paper.

José M. Becerril: Conceived and designed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

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Competing interest statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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