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Ecotoxicological assessment of soils polluted with chemical waste from lindane production: Use of bacterial communities and earthworms as bioremediation tools

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ABSTRACT

An ecotoxicological survey of soils that were polluted with wastes from lindane (γ-HCH) production assessed the effects of organochlorine compounds on the metabolism of microbial communities and the toxicity of these compounds to a native earthworm (*Allolobophora chlorotica*). Furthermore, the bioremediation role of earthworms as facilitators of soil washing and the microbial degradation of these organic pollutants were also studied. Soil samples that presented the highest concentrations of ε-HCH, 2,4,6-trichlorophenol, pentachlorobenzene and γ-HCH were extremely toxic to earthworms in the short term, causing the death of almost half of the population. In addition, these soils inhibited the heterotrophic metabolic activity of the microbial community. These highly polluted samples also presented substances that were able to activate cellular detoxification mechanisms (measured as EROD and BFCOD activities), as well as compounds that were able to cause endocrine disruption. A few days of earthworm activity increased the extractability of HCH isomers (e.g., γ-HCH), facilitating the biodegradation of organochlorine compounds and reducing the intensity of endocrine disruption in soils that had low or medium contamination levels.

1. Introduction

Lindane (γ-HCH) is an organochlorine insecticide that has neurotoxic action; during its synthesis, other hexachlorocyclohexane (HCH) isomers, α-, β-, δ- and ε-HCH, are formed. Lindane production is very inefficient; to produce a ton of insecticide, 8–12 t of other HCH isomers are generated that becomes waste that accumulates in landfills without control measures since it is believed that they are not dangerous. When HCHs reach the environment, they disperse and can be found in the air, surface and ground water, soil and living beings (Vijgen et al., 2006).

In Spain, a lindane factory that is located in Sabiñánigo (Huesca, northern Spain) was one of the leading producers worldwide. During its activity, waste was deposited in Sardas and Bailín landfills (1 and 4 km distant from the factory) plus other scattered locations yet to be determined (Fernández et al., 2013). Currently, the abandoned factory itself is a source of environmental pollution. The contamination of surface and ground water, in addition to a dense, non-aqueous liquid (DNAPL) that contains HCHs, benzene, chlorobenzenes and chlorophenols, has been detected under both landfills and the factory. This pollution is the most serious case of soil contamination in Spain and one of the most important in the world (Fernández et al., 2013). Recently, thousands of tons of soil have been moved from the Bailín landfill to a security cell. However, soil contamination in the surrounding area remains unknown and unsolved. In a study that was conducted around the factory, the five HCH isomers were found in all samples of water, soil, earthworms and plants that were analyzed (Hernández et al., 1991).

Apart from insects, lindane is also neurotoxic to other organisms,

Abbreviations: HCH, hexachlorocyclohexane; γ-HCH, lindane; DNAPL, dense non-aqueous phase liquid; AWCD, average well color development; EROD, ethoxyresorufin-*O*-deethylase; BFCOD, benzyloxy-4-[trifluoromethyl]-coumarin-*O*-debenzyloxylase; PCBs, polychlorinated biphenyls; PAHs, polyaromatic hydrocarbons

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including humans (Nolan et al., 2012). Neurological symptoms include ataxia, disorientation, tremors, seizures and death. Chronic exposure to lindane has adverse effects on other systems and organs such as the heart or liver. In addition, because of its lipophilicity, it bioaccumulates in the brain and other organs, as well as in the placenta (Luo et al., 2016), and is excreted in breast milk (Hajjar and Al-Salam, 2016). It also acts as a carcinogen (Westin, 1993) and an endocrine disruptor (Maranghi et al., 2007). However, not only lindane but all HCHs are toxic. Chronic exposure to them has been linked to immunosuppression, neurological problems and cancer in humans (Nolan et al., 2012). Additionally, the α-, β- and γ-HCH isomers are considered to be persistent organic pollutants (POPs) by the Stockholm Convention. Production, sale and use of lindane in agriculture have been banned in most of Europe, the United States and other countries (Nolan et al., 2012; Sang et al., 1999; Vijgen et al., 2011; Walker et al., 1999).

Several studies have evaluated the potential use of earthworms to monitor the levels of organochlorine compounds in soils (e.g., as bioindicators of soil contamination by dioxins as a result of the chemical accident in Seveso in 1976 (Edwards and Bohlen, 1996)). Moreover, earthworms influence the fate and toxicity of soil contaminants (Muñiz et al., 2014) because of their key role as ecosystem engineers (Wright and Jones, 2006). The soil that is influenced by earthworm activity is called the drilosphere and includes the soil in their gut, galleries and casts (Edwards, 1997). By releasing digestive enzymes, earthworms mobilize HCH residue that is retained in the soils (Verma and Pillai, 1991). Additionally, their feeding and burrowing activities affect the distribution, composition and metabolic activity of microbial communities (Edwards, 1997). Although many microorganisms survive in soils that are contaminated by HCHs and are able to degrade them (Phillips et al., 2005), the earthworms can facilitate the biodegradation of organochlorine compounds since they improve soil conditions, aid the dispersal of microorganisms and modify microbial communities (Singer et al., 2001). Nevertheless, HCHs can also be toxic to earthworms (Lock et al., 2002).

The objective of this work was to determine the soil concentrations of HCHs and other organochlorine compounds near the Bailín landfill, to analyze their effect on the metabolic activity of the heterotrophic microbial community (using Biolog ECO MicroPlates), and to analyze their toxicity to earthworms (using an avoidance test). Additionally, the possible role of earthworms as facilitators of extraction and/or microbial degradation of these organic pollutants was studied. To accomplish this goal, the endogeic earthworm *Allolobophora chlorotica* was used. *Allolobophora*, a very common genus in agricultural soils of temperate regions, is tolerant to environmental variability and resistant to chemical stress (Edwards and Bohlen, 1996). In addition, the presence of endocrine disruptors (hormonal agonists or antagonists) or enzymatic inducers in the soils was evaluated by using in vitro cellular assays. In particular, (anti-) estrogenic, (anti-) androgenic and (anti-) thyroidal activities, as well as enzymatic activity that is associated with cytochromes P450 (CYP1A and CYP3A), were studied. Lastly, the potential relations of these biological effects to the pollutants that were detected in soils was studied using a "stepwise selection" approach.

2. Material and methods

2.1. Soil collection

Six locations were selected in the study area (see Fig. S1 at Supplementary Information). L1 and L2 sites were located downstream from the landfill; L1 was directly exposed to a leachate upwelling during high water table episodes, whereas L2 corresponded to soil that was covered with herbaceous vegetation that was located 4 m from L1. L3 received superficial runoff and leachates and had been affected by a spill of the DNAPL under the landfill (because of geotechnical drilling). L4, L5 and L6 were situated in an area that acted as a topographic barrier to airborne particles that were dispersed during the operation of the landfill. L4 was nude soil that had been exposed to atmospheric deposition, L5 was nude soil that had been exposed to atmospheric deposition and "clean" runoff from naturally vegetated surrounding soils, and L6 corresponded to soil that was completely covered by shrubby and herbaceous vegetation.

Soil samples were taken during the period from September-November 2013 at depths of 0–10 cm (superficial, superficial, S) and 10–20 cm (deep, P). Once at the laboratory, the soil was kept at 4 °C in the dark. At L2, L4 and L5 sites, only superficial samples were collected. The soil was sieved through a 2 mm mesh in the field and placed in glass jars that remained refrigerated. These soil samples were physically and chemically characterized in subsequent days; the concentration of organic compounds was analyzed by n-hexane and water extractions, and they were used in Biolog 1 tests (see Section 2.4). In addition, part of the sieved soil was introduced into polystyrene boxes for experiments with earthworms (Avoidance and Exposure).

2.2. Soil physic and chemical characterization

The pH value, conductivity and amount of organic matter were determined as described previously (Sousa et al., 2008). Bulk density was calculated by weighing a known volume of dried and sieved soil. To estimate moisture content, soil was dried in an oven at 65 °C until its weight was constant (Liu et al., 2011). The moisture percentage was calculated as [(wet weight - dry weight) / dry weight] × 100. To estimate the field capacity, the formulas in Fernández (1979), Silva et al. (1988) were used. The texture was determined by aerometry (Bouyoucos, 1936). To determine the amount of total nitrogen, a variation of the Kjeldahl method with salicylic acid to transform nitrites and nitrates into ammonium was performed. The amount of chlorides were determined by using potentiometry with $AgNO_3$ on an extract of saturated paste.

2.3. Extraction and analysis of organic soil pollutants

The procedure for the extraction of organic compounds from soil was based on a previously described methodology (Singer et al., 2001). Extractions with organic solvents (n-hexane, acetone and Triton X-100) were performed in order to determine the total load of contaminants in soil and extractions with double distilled water (Milli-Q) were performed to distinguish the bioavailable fraction. Water allows the extraction of dissolved contaminants and those that are most weakly bound to the soil, providing information on their environmental bioavailability (i.e., their potential to be absorbed by organisms). Extractions with water also describe the potential transfer of contaminants from soil to groundwater (Kahru et al., 2005; Prokop et al., 2016). The composition of the extractant consisted, per 15 mL, of 10 mL of n-hexane (Panreac PAR-PAI 95%), 1 mL of acetone (Panreac, PA-ACS-ISO) and 4 mL of 1% (v/v) Triton X-100 (Sigma-Aldrich). In total, 15 mL of extractant were used per 15 g of soil. The glass materials that were used for the extractions were cleaned with the n-hexane extractant mix and rinsed with distilled water. Then, it was dried in an oven.

Extractions with the n-hexane extractant mix were done by mixing 15 g of wet soil with 15 mL of extractant in glass Falcon tubes (Ø = 35 mm). The tubes were closed with silicone caps and shaken on an orbital shaker at 250 rpm for 24 h. After this step, tubes were centrifuged at 500 rpm for 15 min and frozen (−20 °C). The supernatant was scratched off and put into other tubes, which were then centrifuged at 3000 rpm for 10 min. By freezing these tubes, three layers formed inside them, corresponding (from the bottom upwards) to soil, an aqueous phase and an organic phase. Only the organic phase remained in a liquid state at −20 °C. This organic phase was poured into glass jars that contained Na_2SO_4 powder (Panreac PAR-PAI) in order to eliminate the residual H_2O. To perform the extractions with distilled water, the same steps that were described above were followed (except that distilled water was used as the extractant), except that the samples