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Comprehensive study of acute toxicity using Microtox® bioassay in soils contaminated by lindane wastes

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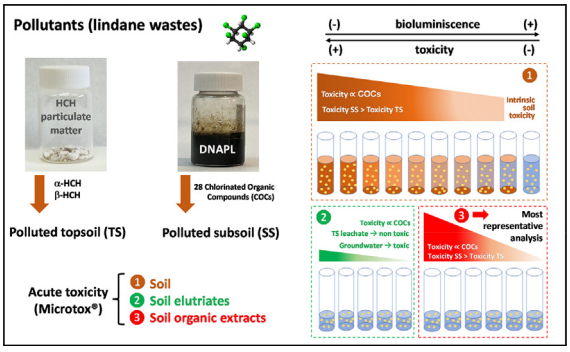
HIGHLIGHTS

- Acute toxicity evaluation of soils contaminated with lindane wastes.
- Three Microtox ® bioassays: Soils, soil elutriates and soil organic extracts.
- The higher the concentration of the pollutants, the higher toxicity.
- Acute toxicity caused by DNAPL (subsoil) is greater than that of HCHs (topsoil).
- Soils, organic extracts, and subsoil elutriates presented high toxicity.

GRAPHICAL ABSTRACT



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ABSTRACT

This research studies the acute toxicity of real contaminated soils (topsoil and subsoil) with hazardous chlorinated organic compounds (COCs) from lindane manufacturing wastes. The Microtox® bioassay was used to determine the toxicity of soils (*modified Basic Solid Phase Test*), soil elutriates (*Basic Test*), and organic extracts (*adapted Organic Solvent Sample Solubilization Test*), in which hydrophobic organic compounds are soluble. The acute toxicity of these persistent contaminants (hexachlorocyclohexanes, HCH isomers, as particulate matter in topsoil, and COCs, from dense non-aqueous phase liquid, DNAPL, in subsoil) and the commercial compounds were also measured. Soils tested showed different contaminant levels (topsoil: 0.9–1149 mg/kg and subsoil: 20–9528 mg/kg). Soil contaminants distribution, concentration and acute toxicity were highly related to the contamination source (HCHs or DNAPL). Soils, organic extracts, and subsoil elutriates presented high toxicity, highlighting the need for remediation of these sites. EC_{50} was calculated in the three-test applied for the soils tested. EC_{50} vs. COCs concentration in soils and soil elutriates showed an asymptotic trend, explained by the low pollutants solubility in the aqueous phase. Contrarily, EC_{50} vs. soil COCs concentration was more linear in the case of the organic extracts. This test was the most reliable from statistical analysis. The three methods reveal interesting and complementary information and are necessary for a complete overview of the acute toxicity of contaminated soils.

1. Introduction

Lindane (the gamma isomer of 1,2,3,4,5,6-hexachlorocyclohexane, γ-HCH) was a broad-spectrum insecticide extensively produced in Europe during the second half of the 20th century (Vega et al., 2016). For each tonne of lindane produced, approximately 6–10 t of other waste HCH-isomers without insecticidal properties were generated (mainly α-HCH and β-HCH). These wastes were usually dumped in the vicinity of production sites, originating an environmental problem of great magnitude (Santos et al., 2018a, 2018b). One of the most severe cases of lindane waste pollution is located in the province of Huesca (Sabiñánigo, Spain), in Sardas

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and Bailín landfills, where the company Inquinosa discharged approximately 115,000 tons of solid and liquid wastes with a high content of HCHs and other organochlorine compounds (Fernández et al., 2013), generating the contamination of soil and groundwater. These contaminated sites have aroused great interest due to their complexity and the risk associated with the proximity of the Gállego River (Santos et al., 2018a, 2018b; Dominguez et al., 2021a).

The disposal of lindane wastes (as solid and liquid waste) in these landfills, and the consequent types of associated pollution (topsoil (TS), superficial water, leachates, subsoil (SS), and groundwater), are schematized in Fig. 1. The topsoil pollution derives from the discharge of solid HCH-wastes generated during the production of lindane in non-secure or informal landfillds, mainly α-HCH and β-HCH (Fig. 1). This kind of pollution is found in the form of particulate matter and/or adsorbed into the soil particles, as described in previous works (Fernández et al., 2013; Dominguez et al., 2021a). The effect of rain on contaminated topsoil causes contamination of surface water, as well as leachates. The subsoil contamination was mainly originated from the dumping of liquid waste from failed chlorination reactions and distillation tails in unlined landfills (Santos et al., 2018a, 2018b). This waste is a dense non-aqueous phase liquid (DNAPL) consisting of a mixture of 28 Chlorinated Organic Compounds (COCs) from chlorobenzene to heptachlorocyclohexane (Santos et al., 2018a, 2018b; García-Cervilla et al., 2020). Due to its high density (1.5–1.8 g/mL), this phase migrated through the subsoil until impermeable bedrocks, causing a contaminant plume (Fig. 1). The DNAPL has been detected at very variable depths, up to 40 m depth (Fernández et al., 2013).

Different chemical oxidation treatments (Fenton, Persulfate (PS) activated by alkali, PS activated by T, PS activated by US, and PS activated by alkali and T) have been recently proposed to remediate superficial soil, subsoil and groundwater (Garcia-Cervilla et al., 2021; Checa-Fernández et al., 2021; Dominguez et al., 2021a; Dominguez et al., 2021b; Checa-Fernández et al., 2022; Garcia-Cervilla et al., 2022). These treatments were successfully applied and led to high contaminant degradation degrees, from 70 to 96 % depending on the soil particle diameter and the treatment time. However, to evaluate the effectiveness of the remediation treatments and assess their suitability, it is important to analyze the ecotoxicity of the soil before and after their application.

Ecotoxicological tests ensure the quantification of the overall toxic effect on selected test organisms (crustaceans, fish, rats, mice, algae, plants and bacteria, etc.) and reveal the bioavailable fraction of a contaminant in a selected matrix. The main disadvantages associated with animal and plant bioassays are the problems with organisms' standardization, requirements for special equipment and skilled operators, long duration assays and lack of reproducibility. On the contrary, bacterial bioassays are relatively quick and simple. The Microtox® bioassay, based on the measurement of the natural luminescence emitted by the marine bacterium *Vibrio fischeri*, is the most used. It is an ideal option to evaluate the acute toxicity of soils since it is a standardized method, it is fast (requires only between 5 and 30 min for toxicity prediction), simple and inexpensive, allowing the analysis of a large number of samples (Doe et al., 2005). Moreover, it is considered the most sensitive and versatile test, so it can be used for almost all kinds of toxicants. Finally, it has no ethical issues ensuing from the use of higher organisms such as fish and rats. Furthermore, considering that a substance that is toxic to an organism often demonstrates similar toxic effects on other organisms, the test results obtained with *Vibrio fischeri* show a good correlation with other tests (Parvez et al., 2006). Nonetheless, a comprehensive literature search retrieved no relevant studies at a global level on the *Vibrio fischeri* ecotoxicity of soils contaminated with lindane wastes (neither before nor after remediation treatments). Only Svenson (1996) analyzed the ecotoxicity (Microtox® *Solid-Phase Test*) of sediments where lindane had been applied as a pesticide (sediments were collected in the Stockholm harbor area and at the Swedish east coast in the Bothnian Sea), with very low concentrations of this pollutant. Moreover, these sediments also contained metals, polychlorinated biphenyls, and other chlorinated pesticides, so it was impossible to isolate the contribution of lindane on the measured toxicity.

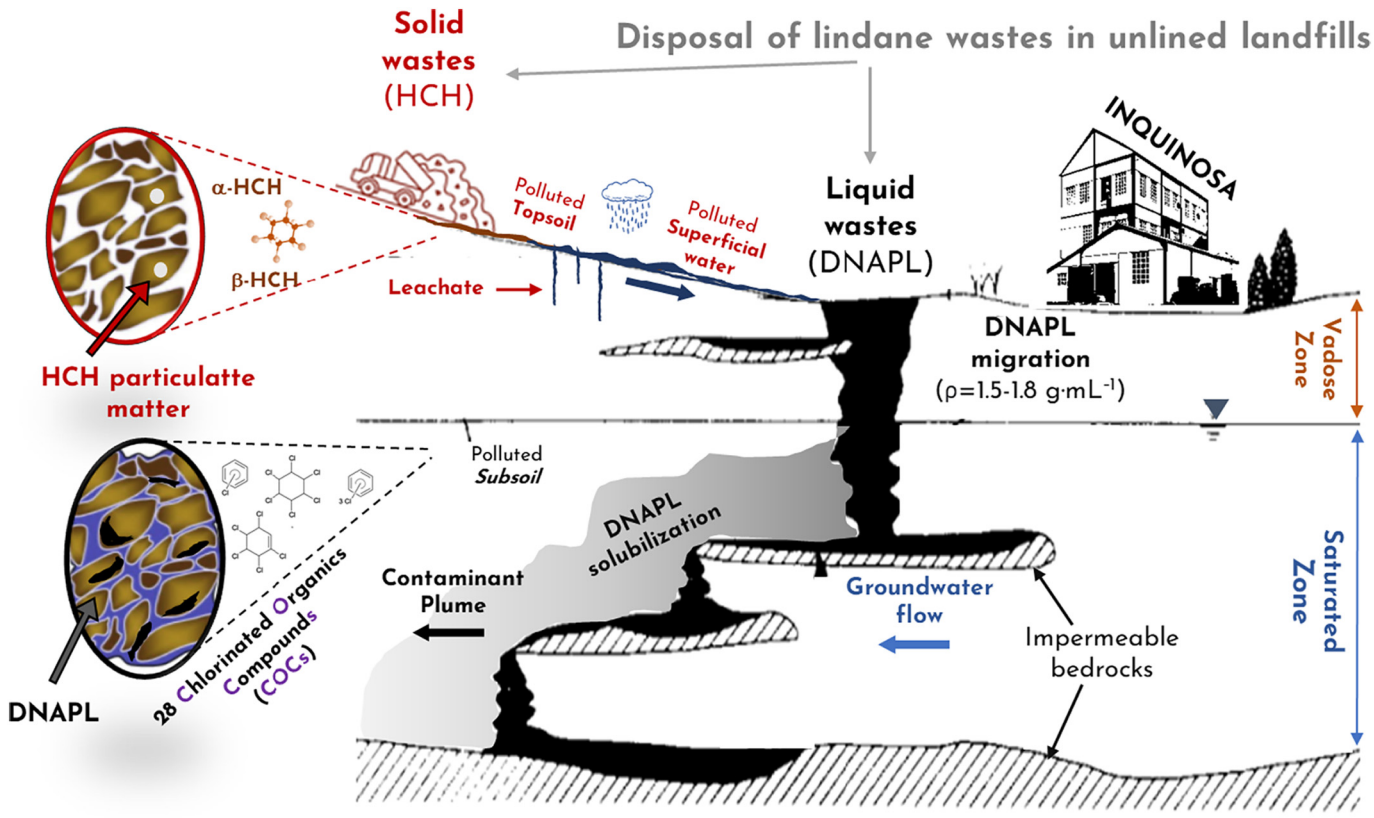

Fig. 1. Schematization of lindane wastes disposal by INQUINOSA and the further dispersion of contaminants in the topsoil and subsoil.

In this scenario, the Microtox® bioassay has been used here for the first time to comprehensively assess the acute toxicity of real soil samples highly polluted with lindane wastes (supported by statistical analysis) following three different procedures. The *modified Basic Solid-Phase Test* (mBSPT) has been applied to analyze the toxicity of an aqueous slurry of polluted soils in water at standard conditions. The *Basic Test* procedure has been used to study the toxicity of an aqueous phase in contact with the soil after partitioning equilibrium was reached (called elutriates). Finally, the *Organic Solvent Sample Solubilization Test* has been adapted to measure the acute toxicity of organic extracts from soils, after all the hydrophobic organic compounds in soil were dissolved in this extract. To the best of our knowledge, this is the first study that combines three different sample preparation approaches and measures for assessing the acute toxicity of real polluted soils using the Microtox® bioassay. Furthermore, the toxicity of soils highly contaminated with different lindane wastes (HCH particulate matter and DNAPL) has been determined for the first time, providing valuable information for the toxicity characterization of these real matrices, an essential aspect for the future application of remediation treatments.

2. Materials and methods

2.1. Soil samples

Two types of soils polluted with lindane wastes, topsoil (TS) and subsoil (SS), were collected at different points from Bailín and Sardas landfills (Sabiñánigo, Spain), respectively. TS, provided by SARGA (Sociedad Aragonesa de Gestión Agroambiental), was taken at a depth between 0 and 0.3 m in the most contaminated area of the Bailín landfill. Samples (moisture content <2 % (vol.)) were collected by stainless steel trowel, milled to size lower than 2 mm, and stored in plastic bags (4 °C, darkness) during transport and until analysis (15–30 days). Received TS samples were sieved into different particle-size fractions using an electromagnetic sieve shaker (BA-200-N). The fraction with a particle size between 2 and 0.25 mm of each sample (95 % of the received soil) was selected for the toxicity bioassays. SS samples were obtained from boreholes (drilled by the company Emgrisa) from one of the most contaminated areas in Sardas landfill. The soil used for SS samples corresponds to the permeable layer located at 12.5–15.5 m below ground level (moisture content of around 15 % (vol.)). It is composed of gravel sand with some interbedded clay, which constitutes the alluvial. Its lithological characteristics were described elsewhere (García-Cervilla et al., 2020). SS samples were dried (48 h at room temperature, 22 °C), stored in glass jars (4 °C, darkness, <30 days) and sieved (electromagnetic sieve shaker BA-200-N), and the soil fraction with a particle size >2 mm was rejected. The fraction with a particle size between 2 and 0.25 mm of each sample was named "coarse" (C), and the fraction <0.25 mm, which corresponds with fine sand, silt, and clay (Wentworth classification (Wentworth, 1922)), was named "fine" (F).

The distinction between physical interferences and effect of target pollutants on toxicity tests is usually achieved using reference soil samples, equivalent in appearance and composition to the test samples but with a substantially lower concentration of organic pollutants (Doherty, 2001). For this reason, additional soil samples with physicochemical characteristics equivalent to each soil type (topsoil (TS), subsoil coarse (SS-C), and subsoil fine (SS-F)) but with a significantly lower pollutants concentration have been used in this study. These samples were collected from the vicinity of the most contaminated areas in the landfills (Bailín and Sardas), outside the perimeter of the contaminated areas at similar depths to those used for respective contaminated samples.

Table 1 summarises the depth (TS, SS), pollutant type (HCH, DNAPL) and particle size of the soil samples analyzed by the acute toxicity bioassay (Microtox®). Samples were classified according to their pollution level (L) based on an arbitrary scale (L1 corresponds to the soil sample with the lowest COCs concentration, and increasing numbers (L2, L3, etc.) are used as COCs concentration increases, for both TS and SS) and reference soil samples were denoted by pollution level "L0". Thus, 4 contamination levels were obtained for TS (L1-L4, in addition to L0) and 3 for each fraction (coarse and fine) of SS (L1-L3, in addition to L0). The acronyms included in Table 1 have been used throughout the manuscript to simplify.

Table 1
Soil samples used in Acute Toxicity Tests with Microtox® bioassay.

Soil	Depth (m)	Pollutants type	Particle size (mm)	Pollution level	Acronym
Topsoil (TS)	0–0.3	HCH-particulate matter	0.25–2	L0	TS L0
				L1	TS L1
				L2	TS L2
				L3	TS L3
				L4	TS L4
Subsoil (SS)	12.5–15.5	DNAPL (Σ_{COCs} = 28)	0.25–2	L0	SS-C L0
			Coarse (C)	L1	SS-C L1
				L2	SS-C L2
				L3	SS-C L3
			<0.25	L0	SS-F L0
			Fine (F)	L1	SS-F L1
				L2	SS-F L2
				L3	SS-F L3

2.2. Chemicals

Microtox® Acute Reagent (freeze-dried preparation of a specially selected strain of the marine bacterium *Vibrio fischeri*, with around one hundred million test organisms) and Reconstitution Solution (nontoxic Ultra Pure Water) used in toxicity tests were purchased from ModernWater Inc., along with a positive quality control testing. Sodium chloride (NaCl), required for preparing the Diluent Solution (NaCl 2 %) and the Osmotic Adjustment Solution (OAS, NaCl 22 %), were supplied by Sigma-Aldrich. Phenol, used as a control compound for validating the performance of the Microtox® bioassay, was provided by Riedel-de Haën. Commercially available COCs (representative of TS and SS contamination: α-hexachlorocyclohexane (α-HCH), β-hexachlorocyclohexane (β-HCH), γ-hexachlorocyclohexane (γ-HCH), δ-hexachlorocyclohexane (δ-HCH), 1,2,3-trichlorobenzene (1,2,3-TCB), 1,2,4-trichlorobenzene (1,2,4-TCB) and 1,2,3,4-tetrachlorobenzene (1,2,3,4-TetraCB)) were supplied by Sigma-Aldrich. Methanol (CH_3OH) and n-hexane (C_6H_{14}), used to extract COCs from the soil and the aqueous phase, respectively, were provided by Fisher Chemical and Honeywell. Bicyclohexyl ($C_{12}H_{22}$, Sigma-Aldrich) and tetrachloroethane ($C_2H_2Cl_4$, Sigma-Aldrich) were used as internal standards (ISTDs) in gas chromatography analyses. All the reagents used in the current work were of analytical quality. Aqueous solutions were prepared with high purity water from a Millipore Direct-Q system with resistivity >18 MΩ cm (25 °C).

2.3. Analysis

The content of total organic carbon (TOC) and inorganic carbon (IC), as well as the concentration of metals in TS and SS samples have been measured following the procedures described in previous works (Dominguez et al. (2021b) and García-Cervilla et al. (2020)). The identification of COCs was performed by gas chromatography (GC, Agilent 6890) coupled with mass spectrometry detector (GC-MSD), whereas their quantification was accomplished by GC coupled with flame ionization detector (FID) and electron capture detector (ECD), the last one in case of low COCs concentrations, after soil extraction with an organic solvent. More details about the chromatographic method can be found in previous works (Santos et al., 2018a, 2018b; García-Cervilla et al., 2020; Dominguez et al., 2021b). The extraction of chlorinated organic pollutants (COCs) from soil was performed by mixing 15 g of soil with 15 g of methanol in 40 mL-PTFE sealed vials in an ultrasound bath (180 min, 45 °C, Power sonic 505). The PTFE vials were cooled (room temperature) and centrifuged (10 min, 9000 rpm, MEDTRONIC-BL-S, JP SELECTA®); and the organic phase was analyzed (Checa-Fernández et al., 2021; Dominguez et al., 2021a). This extraction method was previously optimized to select soil-solvent ratio, energy source (US, microwave), extraction time, soil-solvent separation procedure

(centrifugation, filtering), etc. A percentage between 95 and 98 % of COCs in soil are extracted to the organic phase with very reproducible results. The COCs extraction from aqueous phases was performed using n-hexane (1/1 mass ratio). The biphasic mixture was vigorously agitated, and the organic supernatant was analyzed by GC/FID/ECD (Checa-Fernández et al., 2021, Dominguez et al., 2021a). The limit of detection (LOD) and quantification (LOQ) in GC analysis for each individual compound are 0.1 and 500 mg/kg, respectively. The GC analyses were carried out in triplicate, and a standard deviation <5 % was found. The values shown are the average of these three measurements. The polluted soil pH was measured using a Basic 20-CRISON pH electrode from a soil-water suspension using an aqueous phase/soil ratio equal to 2.

2.4. Microtox® toxicity bioassays

Microtox® is a commercial toxicity bioassay based on measuring the natural luminescence emitted by the marine bacteria *Vibrio fischeri* (ISO 11348-3, 1998). The exposure of these bacteria to toxic substances causes the disruption of the bacteria's respiratory processes, resulting in a reduction of the emitted light (Bond and Martin, 2005). The sample dilution ratio that yields a 50 % reduction of bacteria light emission is named IC_{50} (Eq. (1)):

$$IC_{50}(\%) = \frac{V_{sample}}{V_{total}} 100 \quad (1)$$

where V_{sample} is the volume of the polluted sample in the total volume V_{total} that cause 50 % of inhibition of the light emitted at standard conditions (15 °C and corresponding time). The total volume is the sum of sample volume, the added volume of the diluent and the bacteria.

If the polluted sample contains only one contaminant of known concentration, the effective nominal concentration of toxicant that reduces the intensity of light emission by 50 % (EC_{50}) can be calculated following Eq. (2):

$$EC_{50_i}\left(\frac{mg}{L}\right) = \frac{IC_{50}}{100} C_i \quad (2)$$

where C_i is the concentration of the contaminant in the polluted sample (mg/L).

The Microtox® bioassay was used to measure the acute toxicity of the soils, soil elutriates, and soil organic extracts. Consequently, different sample preparation procedures and standard protocols have been used. Toxicity assessments were performed in triplicate using a Microtox® M500 Analyzer (Microbics Corporation, USA). Negative and positive controls were diluent (NaCl, 2 %) and 100 mg/L of phenol (EC_{50} = 13–26 mg/L). The criteria for judging the quality of the data obtained in a particular test followed the guideline: i) the variation coefficient of the light readings for the control solutions must be <12 %, ii) light loss decreases as test concentration decreases (approximately monotonic), and iii) the coefficient of determination (R^2) must be >0.9. If any of these conditions were not fulfilled, the test was discarded, and the sample was analyzed again. In the current work, samples have been considered nontoxic when bioluminescence inhibition did not exceed 20 % during analysis time, following the recommendations found in the literature (Baran et al., 2019). The fractions of soils with particle diameter above 0.25 mm (TS and SS-C) were crushed and sieved (<0.25 mm) before determining their toxicity and preparing the elutriates and organic extracts to increase the analyses representativeness and reproducibility. No pH adjustment was required before toxicity analyses since all samples were within the acceptable range for the bacteria, pH between 6.0 and 8.0 (Microbics Corporation, 1995).

The samples studied have been labelled according to the classifications found in the literature: nontoxic, moderately toxic and very toxic samples in the case of polluted soils (Kwan and Dutka, 1995; Libralato et al., 2010) and low, moderately and very toxic samples in the case of elutriates (Libralato et al. (2010)).

2.4.1. Toxicity of soil by modified basic solid-phase test

Soil toxicity was evaluated with the *modified Basic Solid-Phase Test* (mBSPT), proposed by Campisi et al. (2005), based on the analysis of an aqueous soil suspension (Slurry). The detailed procedure used in the mBSPT analysis, including the protocol followed to prepare the soil suspension, has been included in Scheme 1 of the Supplementary Material (Scheme S.M. 1). Soil suspensions (slurries) were prepared by mixing 7 g of soil (<0.25 mm) with 35 mL of a saline solution (diluent, NaCl 2 %) for 10 min under magnetic stirring. The concentration of soil in the slurry was 200 g/L. For each slurry, 9 dilutions and 3 blanks were analyzed.

Light emitted (I) by the bioluminescent bacteria was measured without filtration (bacteria were directly exposed to the soil suspension). Thus, turbidity and colour effects on light emission must be considered (Doherty, 2001). In the mBSPT, these aspects are taken into account by recording the initial bacterial light emission (named as $I_{0,soil}$) immediately after adding the sample. This is the main difference from the standard *Basic Solid-Phase Test* (BSPT) procedure, in which the initial light reading (I_0) is carried out before contacting the reagent with the sample, only with the bacteria suspension, and therefore sample turbidity represents an important limitation. Following the mBSTP procedure, the sample was added at different concentrations to the bacteria vials, where the reconstituted bacterial reagent (1 vial of Microtox® Acute Reagent mixed with 1 mL of the Reconstitution Solution, stored at 5 °C in the "Reagent well") is mixed with NaCl, 2 % (Scheme S.M. 1). The maximum concentration of soil in the analysis cuvette after the addition of the soil suspension (200 g/L) with the diluent and the reconstituted bacterial reagent was 99 g soil/L (9.9 % mass), with each successive dilution being 50 % of the previous one. The soil concentration was normalized for the sample's moisture content, <10 % (dry-weight basis). The bioluminescence inhibition of *Vibrio fischeri* was measured after 30 min of sample exposure ($I_{30,soil}$). Toxicity results have been expressed as $EC_{50,soil}$ (g/L), which indicates the concentration of soil in the cuvette (in g/L) that yields 50 % of light inhibition (Eq. (3)).

$$EC_{50,soil}(g\ soil/L) = \frac{mass_{soil}}{V_{total}} \quad (3)$$

2.4.2. Toxicity of aqueous phases in contact with soil (elutriates) by Basic Test

Toxicity of the aqueous phase after the partitioning equilibrium soil-water was reached (elutriates) was evaluated with the Microtox® *Basic Test*, according to the standard operating procedure (Microbics Corporation, 1995). Topsoil and subsoil elutriates were prepared to study the acute toxicity of aqueous phases in contact with topsoil and subsoil. The detailed procedure used for elutriates toxicity analysis, including the protocol followed to prepare the aqueous elutriates from soil samples, has been included in Scheme S.M. 2.

Soil elutriates (SE) were obtained after mixing 15 g of soil (<0.25 mm) and 30 g of Milli Q water in 40 mL-PTFE vials for 24 h (22 °C, 80 rpm, rotary agitator, RR80, LBX®), enough time to reach COCs desorption equilibrium between the soil and the aqueous phases (Garcia-Cervilla et al., 2021; Checa-Fernández et al., 2022). The vials were centrifuged (10 min, 9000 rpm, MEDTRONIC-BL-S, JP SELECTA®) to separate both phases, the aqueous phase constituting the Microtox® test sample: SE.

The osmotic control of the initial SE (2.5 mL) was reached by adding 250 μL of OAS (NaCl, 22 %). For each SE, 4 dilutions and a blank (without SE) were analyzed. Vials with the bacteria reagent were prepared by mixing 0.5 mL of the diluent and 10 μL of the reconstituted bacteria reagent, and the initial bacteria light emission ($I_{0,elutriate}$) was measured. Following, the diluted samples (0.5 mL) were added to the vials with the reconstituted bacteria. The maximum concentration of SE in the analysis cuvettes is 45 %, being the concentration 50 % of the previous one with each successive dilution. The SE dilution ratio that yields 50 % reduction of bacteria light emission is $IC_{50,elutriate}$. The bioluminescence inhibition of *Vibrio fischeri* was measured at 15 min of exposure ($I_{15,elutriate}$). Toxicity results have

been expressed as $EC_{50,elutriate}$ (g soil/L), which considers the soil/water ratio used (500 g/L) in elutriates preparation (Eq. (4)):

$$EC_{50,elutriate}(g\ soil/L) = \frac{IC_{50,elutriate}\ (\%)}{100} \cdot \frac{mass_{soil}}{V_{water}} \quad (4)$$

Thus, the soil concentration used in this test is in the range from 225 to 28.2 g_{soil}/L, in addition to the blank (without SE).

2.4.3. Toxicity of soil extracts by organic solvent sample solubilization test

Hydrophobic organic compounds (HOCs), as is the case of most of the COCs present in the soils under study, are poorly soluble in the aqueous phase; therefore, soil elutriates toxicity is not representative of toxic compounds in soil. The *Organic Solvent Sample Solubilization Test* (Microbics Corporation, 1995) dissolve (or extract) organic solvent-soluble compounds from soils and determine the toxicity of this organic extract. The Microbics Corporation (1995) manual recommended solvents are ethanol, methanol, and dimethyl sulfoxide. Methanol has been chosen in this study since this solvent is efficient in COCs extraction from these soils (García-Cervilla et al., 2020; Checa-Fernández et al., 2021; Dominguez et al., 2021a; Dominguez et al., 2021b).

The detailed procedure for soil organic extracts toxicity analysis (*adapted Organic Solvent Sample Solubilization Test*) including the protocol followed to prepare the soil organic extracts, has been summarized in Scheme S.M. 3. COCs extraction was performed using the following procedure: 15 g of soil (crushed and sieved) were put in contact with 15 g of methanol (soil/solvent mass ratio = 1/1) in 40 mL-PTFE sealed vials in an ultrasound bath (180 min, 45 °C, Power sonic 505). The PTFE vials were cooled (room temperature) and centrifuged (10 min, 9000 rpm, MEDTRONIC-BL-S, JP SELECTA®), the supernatant organic phase constituting the soil organic extract (SOE).

The bacterial reagent is sensitive to organic solvents. Therefore, the organic solvent's maximum allowable concentration (MAC) in the solution in contact with the microorganism must be previously determined to ensure a null effect of the solvent on the measured toxicity with this bioassay (Kwan and Dutka, 1990). A sensitivity analysis was carried out to determine the MAC of methanol with the Microtox® *Basic Test* (as summarized in Fig. S.M. 1) using known concentrations of methanol as toxicant. Methanol MAC found for the test was 4 % in volume, which agrees with that previously reported in the literature (Kwan and Dutka, 1990).

Therefore, the sample with the highest concentration of SOE was prepared by mixing 4 % in volume of SOE with 96 % of diluent. The solution used to prepare the progressive dilutions of this sample (5 dilutions including the blank), named "DS solution", should also contain 4 % of methanol (so that, all dilutions have the same percentage of methanol and differences in toxicity can be only attributed to organic contaminants). Therefore, DS was prepared by mixing 4 volumes of methanol and 96 volumes of eluent (NaCl, 2 %). To acclimatize the bacteria to methanol, the bacterial reagent in this protocol (adapted bacterial reagent) was prepared using the DS solution and stored in the read well (see Scheme S.M. 3). The measurement of the initial bacteria light emission ($I_{0,organic}$) is performed after adding 100 μL of the adapted reconstituted bacteria to 2 mL of DS solution. Following, the diluted samples (0.9 mL) were added to the vials with the reconstituted bacteria and the bioluminescence inhibition of *Vibrio fischeri* was measured after 15 min ($I_{15,organic}$). In this protocol, sample preparation and dilution (step 3 in Scheme S.M. 3) were performed in bigger vials and outside the Microtox® device at the same temperature conditions (15 °C).

The dilution ratio of the organic extract that yields 50 % reduction of bacteria light emission is $IC_{50,organic}$.Toxicity results have been expressed as $EC_{50,organic}$ (g soil/L), being calculated by (Eq. (5)), which considers the soil/methanol mass ratio (1000 g/kg) used for SOE preparation and the methanol density ($\rho_{methanol}$ = 0.792 kg/L)

$$EC_{50,organic}\left(g\frac{soil}{L\ MeOH}\right) = \frac{IC_{50,organic}\ (\%)}{100} \cdot \frac{mass_{soil}}{mass_{methanol}} \cdot \rho_{methanol} \quad (5)$$

2.4.4. Soil contaminants (HCH particulate matter and DNAPL) and standards

To delve into the relationship between soil toxicity measured and pollutants nature and concentration, the acute toxicity of soil contaminants: HCH particulate matter (topsoil) and DNAPL (subsoil), has also been determined. HCH particulate matter in the landfill is detectable with the naked eye as white grains in topsoil. These particles were separated by hand and crushed before analysis. DNAPL was found at 15 m in the contact between altered marls and the alluvial in Sardas Landfill (Santos et al., 2019) and was obtained by extraction in specific wells in the alluvial. A photograph of particulate matter and DNAPL can be found in Fig. S.M. 2 a) and b), respectively. In addition, the acute toxicity of the most representative COCs in HCH particulates and DNAPL, commercially available, was also determined. Aqueous and organic (methanol) solutions were prepared for that purpose. It should be highlighted that COCs under study are slightly soluble in water and highly soluble in methanol (Lorenzo et al., 2020).

Saturated aqueous solutions were obtained after contacting 100 mg of HCH particles with 100 mL of Milli Q water and 1 g of DNAPL with 100 mL of Milli Q water. Aqueous solutions of commercial standards were prepared individually by putting in contact Milli Q water with a mass of contaminant that ensured the saturation of the medium (ultrasound bath, 30 min, 22 °C, Power Sonic 505). The supernatant was filtered (syringe filters 0.45 μm) and COCs concentration of the aqueous samples was analyzed by GC. Acute toxicity of filtrates was measured using the *Basic Test* (Subsection 2.4.2). Moreover, these pollutants, as well as a known mass of HCH particles and DNAPL, were individually dissolved in methanol in the range of 400–4000 mg/kg (depending on their acute toxicity) using an ultrasound bath (30 min, 22 °C, Power Sonic 505). After filtering, COCs quantification was carried out by GC, and the acute toxicity of the organic sample was assessed using the *adapted Organic Solvent Sample Solubilization Test* (Subsection 2.4.3). Inhibition at 15 min was reported in both bioassays, using aqueous and organic phases. Since composition of COCs in the liquid phases was known, the EC_{50} values were expressed as the concentration of contaminant (pure compound or mixture) that produced a 50 % inhibition (Eq. (2)).

2.5. Statistical analysis

Statistical analyses using Pearson and Spearman correlation coefficients were carried out to determine if the concentration of total COCs and acute toxicity of samples are significantly associated. Pearson's correlation coefficient measures the linear dependence between two continuous and quantitative random variables, and Spearman's correlation coefficient measures the rank correlation between two random variables (both continuous and discrete). Both coefficients range between −1 and +1, indicating negative or positive associations, respectively. A value of 0 would indicate the absence of correlation between the two variables. The 95 % confidence limit range for each EC_{50} value was also calculated to check the quality results. The toxicity result of a sample was considered valid if the 95 % confidence interval was not >30 % of the EC_{50} and the variation between replicates was <20 % (Libralato et al., 2010).

3. Results and discussion

3.1. Soils characterization

The chemical characterization of the soils is summarized in Table S.M. 1. It has been performed globally for each soil type (and not for the different contamination levels) since TOC, IC, and pH hardly depends on COCs concentration. The natural organic matter content in soils is relatively low, and the concentration of inorganic species is similar in all soil types. The results agree with those previously reported for these soils (TS (Dominguez et al. (2021b)) and SS (García-Cervilla et al. (2020)). The calcium carbonate content is above 40 %, indicating that these soils are strongly buffered (pH ≈ 7.5). The total concentration of COCs in soils and the corresponding soil elutriates obtained (procedure described in Section 2.1.2) is provided in Table S.M. 2. COCs concentration in SS samples was significantly higher

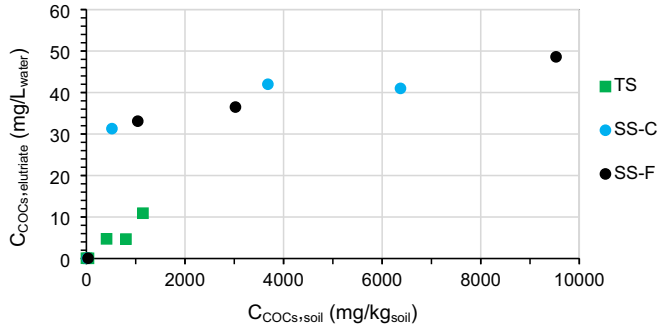

Fig. 2. COCs concentration in the elutriates vs. soils.

than in TS, which is attributed to the different sources of soil contamination (particulate matter of HCHs for topsoil and DNAPL for subsoil). DNAPL migration through the alluvial and COCs solubilization from the DNAPL to the groundwater flux resulted in high contamination levels in some areas of the alluvial. According to the COCs concentration in TS, only a small percentage of TOC (1.5 %) corresponds to chlorinated organic compounds. The rest of the organic matter is attributable to the presence of natural organic matter as humic acid-like compounds (Dominguez et al., 2021b). On the contrary, the carbon from the identified COCs in SS fits well with the measured TOC, and the presence of other organic compounds than those identified can be considered irrelevant.

COCs concentration in the elutriates has been represented vs. COCs concentration in the soil phase (Fig. 2). As expected, as COCs concentration in the soil increases, COCs concentration in the elutriates also increases. It should be noted that this increase was not linear. In the case of subsoil elutriates (SS-C and SS-F), a maximum value of COCs solubilized of around 40–50 mg/L was reached (asymptotic trend), which can be attributed to the saturation of COCs in the aqueous phase caused by the high pollutant concentration in this soil. Moreover, the asymptotic trend was similar both SS soils (SS-C and SS-F). However, this asymptotic trend was not reached in the case of topsoil. For similar COCs concentration in the soils (≈400–500 mg/kg), TS elutriates presented a significantly lower COCs concentration than SS elutriates (i.e. TS L2 vs. SS-C L1, Table S.M. 2). The origin of this result lies in the contamination source. HCH isomers (topsoil) are poorly soluble in water, while DNAPL (subsoil) contains a mixture of chlorinated organic compounds (among them, chlorobenzenes) with higher water solubility (Lorenzo et al., 2020).

The distribution of COCs in HCH particulate matter and DNAPL is depicted in Fig. 3a and b, respectively. The composition of HCH particulate matter can be explained by attending to the lindane (γ-HCH) manufacturing process. The distribution of HCH isomers in the technical-HCH was: α = 55–80 %, β = 5–14 %, γ = 10–15 %, δ = 2–16 %, ε = 3–5 % (Fernández et al., 2013; Wacławek et al., 2019). Consequently, after lindane purification process, the solid residue (HCH particulate matter) was mainly composed of α-HCH, followed by β-HCH. In the analyzed samples, the percentage of these isomers was 83.4 and 8.7 %, respectively. As expected, γ-HCH, the product of interest (only isomer with pesticide properties), was found in a smaller proportion (1.8 %). β-pentachlorocyclohexene (β-PCX) was detected in traces (below the LOD, <0.1 mg/kg). This compound was obtained as an impurity during the manufacture of lindane and from the alkaline dehydrochlorination of HCHs (Santos et al., 2018a, 2018b). Experimentally, it has been confirmed that β-PCX comes from α-HCH, the main compound in HCHs particles.

In the case of DNAPL, pollutants have been grouped by isomer families: chlorobenzene (CB), dichlorobenzenes (DCBs), trichlorobenzenes (TCBs), tetrachlorobenzenes (TetraCBs), pentachlorocyclohexenes (PCXs), hexachlorocyclohexenes (HCXs) and heptachlorocyclohexanes (HeptaCHs), whereas HCH isomers (α, β, γ, δ, ε-HCH) have been considered separately due to their interest (Fig. 3b). The concentration of each COC in the DNAPL is listed in Table S.M. 3. The main DNAPL compounds were HCHs (29 %), being lindane the major HCH isomer present (13.6 %), followed by δ-, α-, and ε-HCH, whereas β-HCH was the HCH isomer found in the lowest concentration (0.3 %). The percentage of the rest of COCs was: HeptaCHs (17 %) > TCBs (16 %) > TetraCBs (13 %) > DCBs (9 %) > CB ≈ PCXs (7 %) > HCXs (3 %). These results agree with those obtained in a previous work, in which different DNAPLs from Sabiñánigo landfills were characterized (Santos et al., 2018a, 2018b).

The distribution of COCs in TS samples (pollution levels L1-L4) vs. COCs concentration is shown in Fig. 4. The pollution level L0 has not been included since its low COCs concentration (<1 mg/kg) would lead to an erroneous interpretation of the results. COCs distribution was similar for the pollutant's levels studied, with a mass fraction of α-HCH and β-HCH of about 80–85 % and 10–15 %, respectively, except for L1, sample in which the proportion between these compounds was reversed. This can be attributed to the fact that the readily available pollutant fraction (easily extractable or bioavailable fraction, α-HCH) diminished in a biphasic manner, i.e. part of α-HCH, more soluble than β-HCH, was degraded or lost from the soil through leaching and part was transformed into more recalcitrant compounds (Semple et al., 2003). Moreover, β-HCH is the most recalcitrant HCH isomer because of the equatorial position of the six chlorines in the molecule, which makes it very stable and low biodegradable (Dominguez et al. (2021b)). Besides, the L1 sample was the only one where HCH particulate matter was not detected with the naked eye. This observation agrees with Dominguez et al. (2021b), who reported that the higher the percentage of β-HCH, the greater the contamination percentage

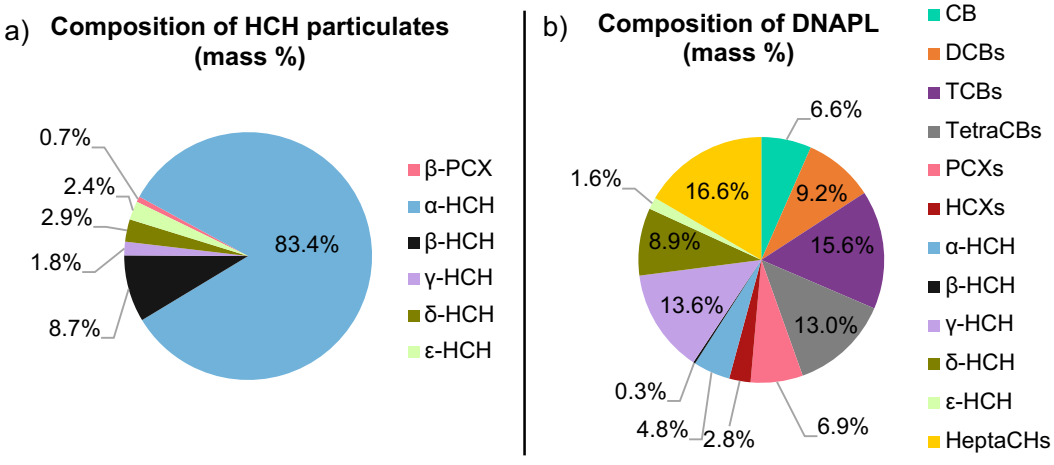

Fig. 3. COCs distribution in a) HCH particulates and b) DNAPL.

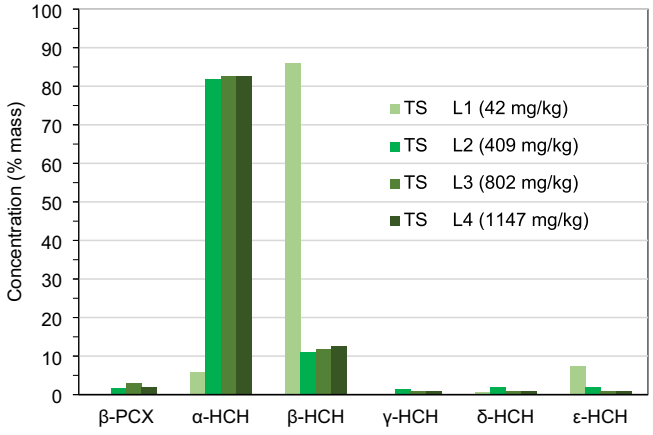

Fig. 4. COCs distribution in TS samples (COCs concentration is included in the legend).

adsorbed to the soil and the lower in the form of particulate matter. Thus, except for TS L1, the distribution of COCs in soil samples (Fig. 4) corresponds to that of HCH particulate matter (Fig. 3a), confirming that the contamination in these superficial soils is attributed to the presence of HCH particulates.

The distribution of COCs in SS samples is depicted in Fig. 5a and b. As in the case of topsoil, pollution levels L0 ($C_{COCs,\ SS\text{-}C}$ = 20 mg/kg, $C_{COCs,\ SS\text{-}F}$ = 33 mg/kg) have not been included to avoid erroneous interpretations of the results. COCs composition is very variable in the coarse fraction of SS (the contribution of γ-HCH is especially high in L1). In contrast, in the fraction F, pollutants distribution was similar regardless of the contamination level (γ-HCH percentage increases with COCs concentration). Similarities in COCs distribution in both fractions have been detected with the DNAPL composition (Fig. 3b), although CB and DCBs were not detected in significant amounts in the soils. It is likely that part of these contaminants, initially present in the soil, has been lost during soil storage, sieving, and drying, due to their high volatility.

3.2. Toxicity of soils

Soil toxicity values ($EC_{50,soil}$, Microtox® mBSPT) with the confidence interval and the concentration of COCs of each sample are given in Table 2. According to the classification proposed by Kwan and Dutka (1995): $EC_{50} > 10$ g/L nontoxic sample, 5 g/L < $EC_{50} \leq 10$ g/L moderately toxic sample and $EC_{50} \leq 5$ g/L very toxic sample, soils collected from Sardas and Bailín landfills are highly toxic ($EC_{50} \leq 5$ g/L). The fact that the reference samples (L0), with low COCs concentration, exhibit intrinsic toxicity would indicate that there are toxic compounds for the bacteria in the soil matrix.

The relationship between soil toxicity (expressed as $1/EC_{50,soil}$, in L/g_{soil}) and COCs concentration for each type of soil (TS, SS-C, and SS-F) and pollution level has been represented in Fig. 6a (COCs concentration has been considered as a single compound to simplify).

As can be seen in Fig. 6a, in general, acute toxicity increased with increasing COCs concentration. However, in the case of subsoil coarse fraction (SS-C), an almost constant toxicity is obtained at all the pollution levels. In the case of SS-F and TS, the toxicity increased with COCs concentration until it reached an asymptotic value. This asymptote can be partly explained because solubilized pollutants (more available to the bacteria) reached saturation values when COCs concentration in soil increases, as previously shown in Fig. 2 (COCs concentration in the elutriates vs. soils). Moreover, at high toxicity levels, the sensitivity of the bacteria is reduced, and the differences between highly toxic samples with different pollutant concentration are less significant (Marugán et al., 2012).

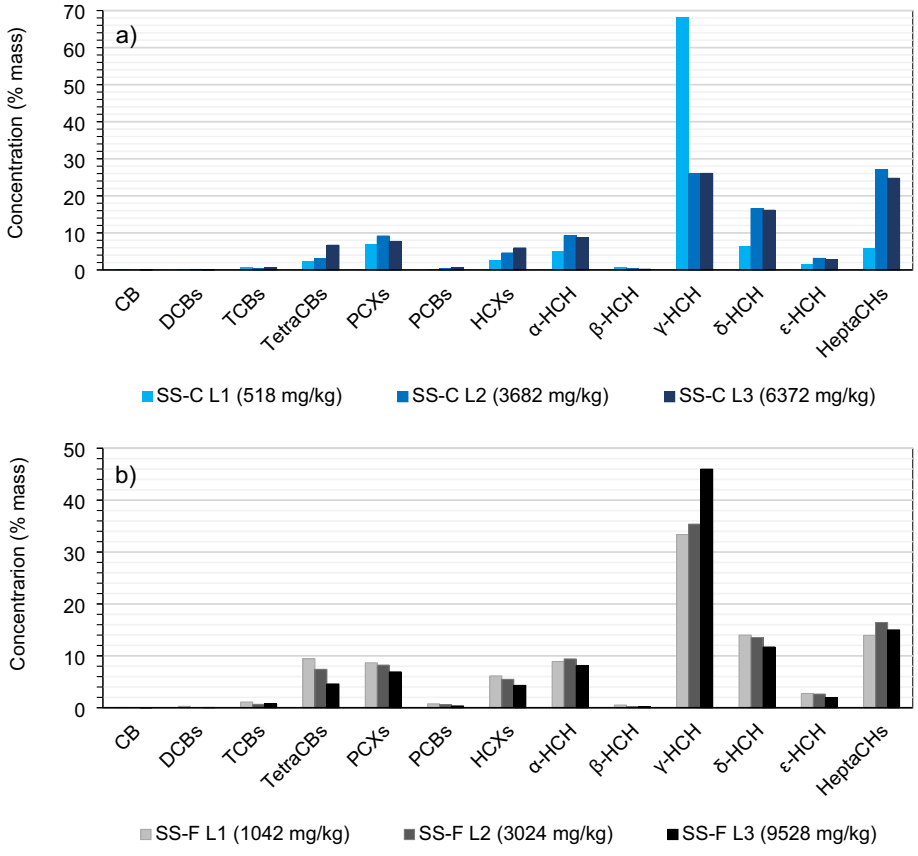

Fig. 5. COCs distribution in SS-C (a) and SS-F samples (b) (COCs concentration is included in the legend).

Table 2
Acute toxicity measured by Microtox® mBSPT (a), Microtox® Basic Test (b) and Microtox® adapted Organic Solvent Sample Solubilization Test (c). CI: confidence interval, 95 %, ND: not detectable.

Sample	Soil (a)			Soil elutriate (b)			Soil organic extract (c)		
	$EC_{50,soil}$ (g_{soil}/L_{slurry})	CI	$C_{COCs,soil}$ (mg/kg_{soil})	$EC_{50,elutriate}$ (g_{soil}/L)	CI	$C_{COCs,elutriate}$ (mg/L_{water})	$EC_{50,organic}$ (g_{soil}/L_{MeOH})	CI	$C_{COCs,soil}$ (mg/kg_{soil})
TS L0	2.22	0.12	1	N.D.	–	0.07	N.D.	–	1
TS L1	1.95	0.32	42	N.D.	–	0.05	N.D.	–	42
TS L2	1.29	0.25	409	306.5	18.95	4.74	16.66	3.56	409
TS L3	1.03	0.21	802	312	29.05	4.68	19.23	4.63	802
TS L4	0.94	0.26	1147	125	26.55	10.92	8.49	2.49	1147
SS-C L0	1.62	0.12	20	N.D.	–	0.06	27.80	3.94	20
SS-C L1	0.70	0.01	518	56.5	9.6	31.30	3.79	0.21	518
SS-C L2	0.66	0.07	3682	29	0.35	56.90	0.55	0.04	3682
SS-C L3	0.65	0.02	6372	31	3.15	41.00	0.44	0.02	6372
SS-F L0	3.30	0.04	33	N.D.	–	0.07	29.53	0.98	33
SS-F L1	1.34	0.18	1042	67	9.5	33.1	1.31	0.06	1042
SS-F L2	1.10	0.13	3024	47.5	12.25	36.5	0.48	0.01	3024
SS-F L3	0.96	0.06	9528	29.5	4.6	48.6	0.38	0.08	9528

$C_{COCs,soil}$ (mg/kgsoil) of soil (a) and soil organic extract (c) corresponds to the same samples and measurement procedure.

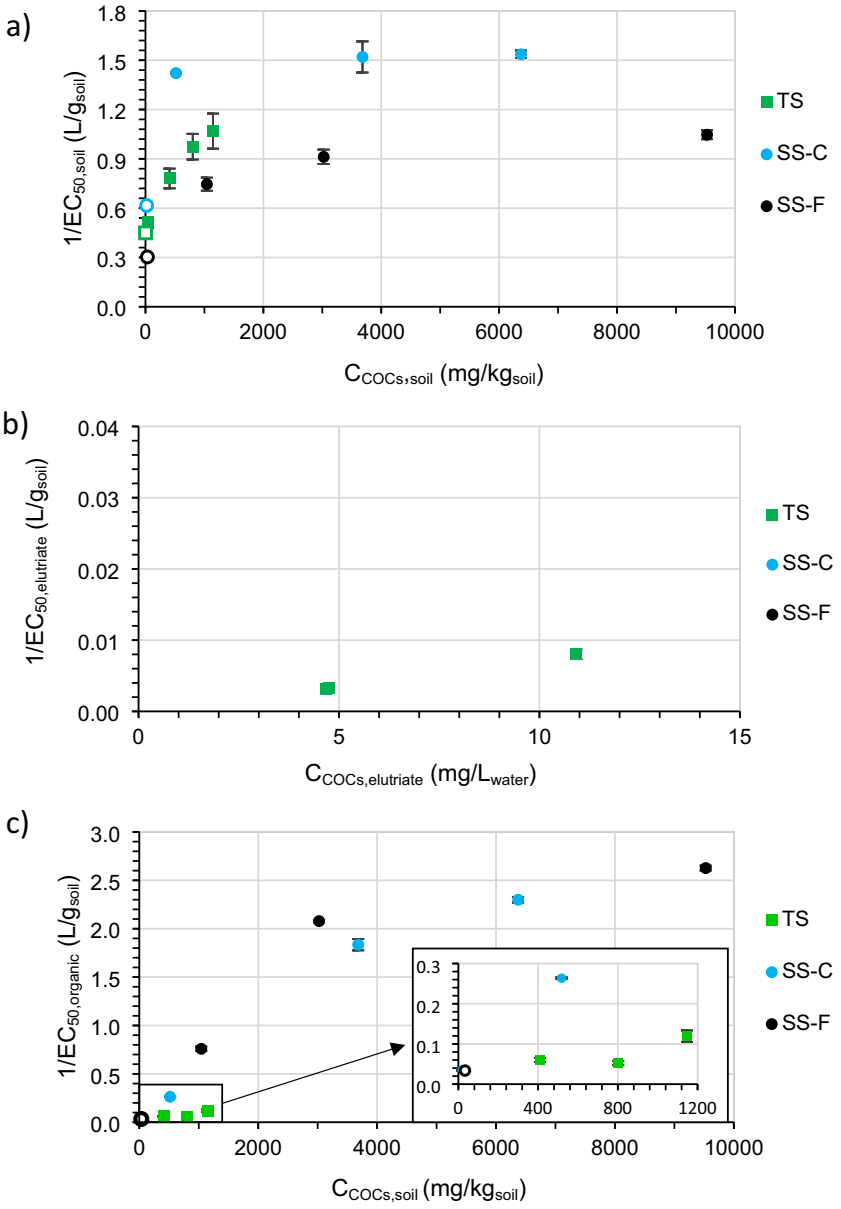

Fig. 6. a) Soil toxicity ($1/EC_{50,soil}$, Microtox® mBSPT), b) Elutriate toxicity ($1/EC_{50,elutriate}$, Microtox® Basic Test) and c) Soil organic extract toxicity (1/EC50,organic, Microtox® adapted Organic Solvent Sample Solubilization Test) vs. COCs concentration. The unfilled markers denote the reference soil samples (L0).

It is also noticed that at similar values of COCs concentration soil toxicity differed depending on its origin: SS-C > TS > SS-F (Fig. 6a). The fact that fraction fine (F) toxicity was lower than fraction coarse (C) for the same soil type (SS) could be justified based on the contaminant bioavailability concept. Contaminants are more strongly adsorbed to the soil in the fraction F and are not as accessible to bacteria, resulting in lower toxicity. Secondly, the greater toxicity of SS compared to TS for the same particle size (C) and COCs concentration could be associated with the contaminants type (DNAPL and HCH particulate matter), as will be further discussed.

3.3. Toxicity of soil elutriates

Elutriate toxicity results ($EC_{50,elutriate}$, Microtox® *Basic Test*), the confidence interval, and COCs concentration are given in Table 2. The calculation of toxicity values that would require dilutions below 45 % (maximum concentration measured in this protocol) has been obtained by extrapolation. The toxicity evaluation has been carried out according to the classification proposed by Libralato et al. (2010): EC_{50} > 100 g/L low toxicity sample, 10 g/L ≤ EC50 ≤ 100 g/L moderately toxic sample, EC_{50} < 10 g/L very toxic sample. As seen in Table 2, topsoil elutriates (TS) presented low toxicity, which could indicate that the possible leachates from the surface soil of the landfills are not an environmental issue in terms of acute toxicity. However, subsoil fraction elutriates, C-SS and F-SS, exhibited moderate toxicity from pollution level L1 (reference levels toxicity was not detectable), meaning that groundwater in contact with contaminated subsoil (C_{COCs} > 500 mg/kg) would imply a moderate but significant environmental risk in terms of acute toxicity, fact that highlights the need to remediate these sites in the short term.

Elutriate toxicity is expected to be related to solubilized compounds from soil: COCs, other organic compounds (if any) and inorganic species, such as metals, present in the soil (Čvančarová et al., 2013). Since elutriate reference samples (L0) did not exhibit toxicity (Table 2) and metals concentration does not vary with the contamination level, it can be inferred that these species don't contribute to the elutriate toxicity. Elutriate toxicity results (expressed as $1/EC_{50,elutriate}$ in L/g_{soil}) for each soil type (TS, SS-C, and SS-F) and pollution level have been plotted vs. COCs concentration in these aqueous phases (Fig. 6b) (COCs concentration has been considered as a single compound to simplify). Regardless of soil type, an approximately linear trend can be observed.

The effective nominal concentration (Eq. (2)) of HCH particulates and DNAPL (as a single compound) and the most representative COCs (commercially available) of these contaminants have been determined (Microtox® *Basic Test*, Fig. 7a). The concentration of these pollutants in saturated aqueous solution is shown in Table S.M. 4.

HCH particulates exhibited an increase in bioluminescence at the minimum concentration of the measured sample (Fig. S.M. 3a), meaning that the inhibition percentage reached a negative value. This behavior is due to hormesis, a phenomenon of response to potentially toxic agents characterized by stimulation at low doses, and inhibition at higher doses, following a hyperbolic trend (Kwan and Dutka (1990)).

α-HCH and β-HCH did not present acute toxicity toward the bacterium *Vibrio fischeri*, as occurred with HCH particulate matter (the maximum bioluminescence inhibition of the aqueous phase saturated in these pollutants did not reach 20 %). The fact that these compounds represent around 90 % of the HCH particulate matter (Fig. 3a) would justify that no toxicity response had been detected in the aqueous phase for this source of contamination (the concentration of COCs in HCH particulate matter was 7.75 mg/kg, Table S.M. 4). Contrarily, the aqueous phases of γ- and δ-HCH, and especially chlorobenzenes (1,2,3-TCB, 1,2,4-TCB and 1,2,3,4-TetraCB), which practically represent 50 % of the DNAPL composition, exhibited considerable acute toxicity, which would justify the high acute toxicity of DNAPL ($EC_{50,DNAPL}$ = 4.45 mg/L, the concentration of COCs in this aqueous solution was 41.8 mg/kg, Table S.M. 4).

The results here obtained are in agreement with those found in the literature. Svenson et al. (1996) analyzed the acute toxicity of sediments contaminated with chlorinated pesticides, including α-HCH. According to their results, this compound was not potentially toxic to the bacteria

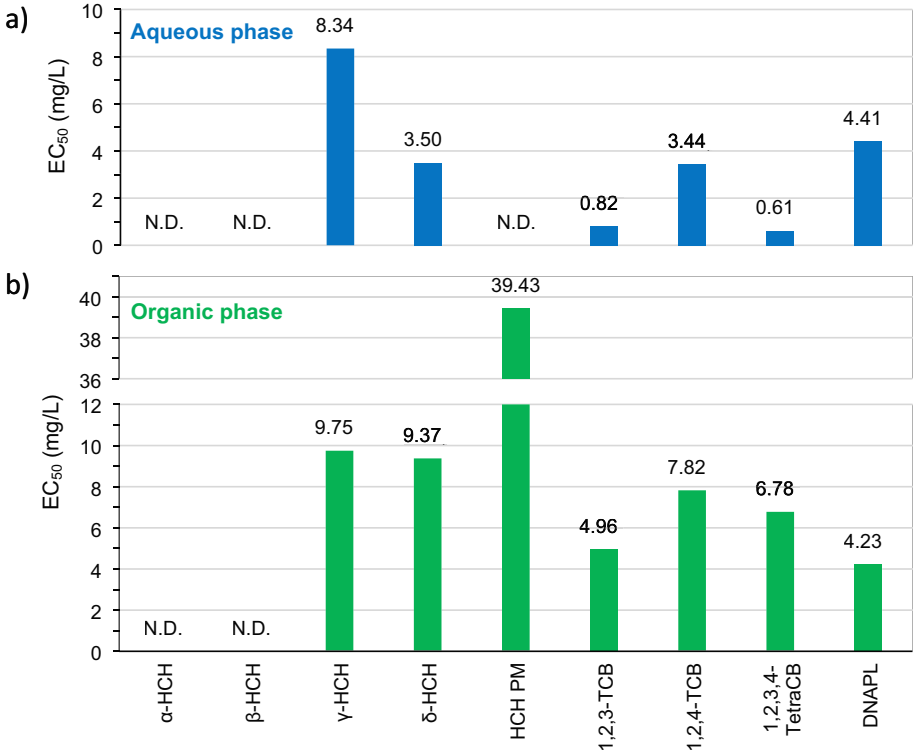

Fig. 7. EC_{50} of HCH particulate matter (HCH PM), DNAPL and pure commercial contaminants a) in aqueous phase (Microtox® Basic Test) and b) organic phase (Microtox® adapted Organic Solvent Sample Solubilization Test). ND: toxicity not detected.

tested. Garg et al. (2016) studied the toxicity of β-HCH and δ-HCH in saturated aqueous solutions. A bioluminescence reduction was not detected for these compounds, but in the case of δ-HCH, negative inhibition (hormesis) was observed, indicating that this isomer exhibits acute toxicity at higher concentrations. Finally, Kaiser and Palabrica (1991) collected about 1350 acute toxicity data of individual organic compounds for the *Vibrio fischeri* bacterium. The EC_{50} ranges (or exact values) for γ-HCH, 1,2,4-TCB, 1,2,3-TCB, and 1,2,3,4-TetraCB were 5.67–11.1, 3.01–3.70, 2.50–2.62, and 1.88 mg/L, respectively. The acute toxicity reported for γ-HCH and 1,2,4-TCB agrees with that obtained in the present study (8.34 and 3.44 mg/L, respectively (Fig. 7a)), while in the case of 1,2,3-TCB and 1,2,3,4-TetraCB, the EC_{50} values found in the literature were slightly higher than those obtained in the present study (0.82 and 0.61 mg/L, respectively (Fig. 7a)). The low water solubility of these compounds implies that their concentrations are very low, which can be associated with a significant error in their quantification. Thus, determining the toxicity of poorly water-soluble compounds in an organic matrix is recommended to minimize this issue (Microbics Corporation (1995), Ruiz et al. (1997)). Nevertheless, it should be highlighted that only acute toxicity in the aqueous phase had been studied to date for the pollutants under study.

3.4. Toxicity of soil organic extracts

Determining the toxicity of soil organic extracts ensures the total bioavailability of the pollutants (Čvančarová et al., 2013). These results ($EC_{50,organic}$, Microtox® *adapted Organic Solvent Sample Solubilization Test*), the confidence interval, and the corresponding COCs concentration are given in Table 2. The calculation of toxicity values that would require concentrations of soil organic extract above 3.6 % (maximum study concentration in this protocol) has been obtained by extrapolation. To the best of our knowledge, there is no toxicity classification in the literature regarding the organic extract samples.

Toxicity results of soil organic extracts ($1/EC_{50,organic}$, expressed in L/g_{soil}) for each soil type (TS, SS-C, and SS-F) and pollution level have been plotted vs. COCs concentration (Fig. 6c, COCs concentration has been considered as a single compound to simplify). An inset of the plot on the range of low COCs concentrations was zoomed in to facilitate the interpretation of the results.

The higher the soil COCs concentration, the higher the acute toxicity of the organic extract (Fig. 6c). Values obtained for F and C subsoil samples suggest a logarithmic trend between toxicity and contaminants concentration, which corresponds to the logarithmic relationship between bacteria inhibition and the concentration of the sample studied. The toxicity of similar COCs concentrations values differs depending on the soil origin (TS or SS): SS-C ≈ SS-F > TS. The similar COCs composition of C-SS and F-SS explains the similar toxicity values with COCs concentrations obtained for these soils. The fact that the TS organic extracts are considerably less toxic than the two SS fractions (SS-C and SS-F) can be explained again attending to the different contaminant types (HCH particles in TS and DNAPL in SS).

The effective nominal concentration (Eq. (2)) of HCH particulates and DNAPL (as a single compound) and the most representative COCs (commercially available) of these contaminants solved in methanol have been determined (Microtox® *adapted Organic Solvent Sample Solubilization Test*, Fig. 7b). Since COCs solubility in methanol is significantly higher than in water, the analysis of the organic phase allows for determining their acute toxicity at higher concentrations. As previously stated, COCs concentration in methanol for pure compounds ranged from 400 to 4000 mg/kg, depending on their acute toxicity. The exact pollutants concentration in each sample can be found in Table S.M. 4. In the case of HCH and DNAPL, the concentrations were 752 and 296 mg/kg, respectively (solutions with lower HCH particles concentrations were analyzed but toxicity was below the detection level, data not shown).

In the organic phase (methanol) it was possible to dissolve a much higher concentration of HCH particulate matter than in the aqueous phase (752 mg/kg vs. 7.75 mg/kg), which made it possible to obtain a toxicity value, EC_{50} = 39.43 mg/L, by extrapolation. As can be seen, the DNAPL concentration that induces 50 % of bioluminescence inhibition was significantly lower (4.23 mg/L), highlighting the higher acute toxicity of this pollutant type. This finding explains the higher toxicity values obtained for the organic extracts in the case of SS (contaminated by DNAPL) than TS (contaminated by HCH particulates) (Fig. 6c).

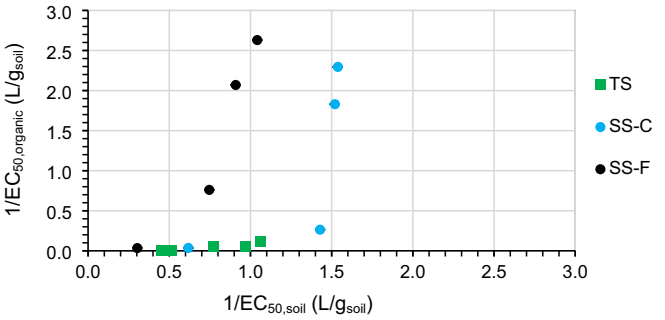

Fig. 8. Soil organic extract toxicity ($1/EC_{50,organic}$, Microtox® adapted Organic Solvent Sample Solubilization Test) vs. soil toxicity ($1/EC_{50,soil}$, Microtox® mBSPT).

The low toxicity of HCH particulate matter in this test can be explained again attending to its composition. The average EC_{50} values (mg/L) obtained in the Microtox® test for the organic phase showed that α-HCH and β-HCH (≈ 90 % of HCH particulates, Fig. 3a) did not present acute toxicity toward the bacterium *Vibrio fischeri* either, despite being in much higher concentration than in the aqueous phase (Table S.M. 4).

Soil organometallic compounds soluble in methanol pass into organic extracts (Roig et al., 2011) and considering that the concentration of these species is not affected by the pollutant level and is similar for the three soil types (Table S.M. 1), results corresponding to reference samples (L0) with negligible pollutants concentration would indicate if these organometallic compounds participated in the toxicity response. Since TS L0, with a COCs concentration below 1 mg/kg, does not exhibit toxicity, soil metals effect can be ruled out. Hence, the toxicity values obtained for samples SS-C L0 and SS-F L0 (EC_{50} = 27.80 and 29.53 mg/L_{MeOH}) are attributable to their concentration of COCs (20 and 33 mg_{COCs}/kg_{soil}, respectively).

Finally, soil organic extracts toxicity values have been plotted vs. those corresponding to soils (Fig. 8). The results obtained for low COCs concentrations indicate that soils exhibited more significant toxicity than their organic extracts, which was especially notable for topsoil samples. As can be seen, this behavior was reversed as the concentration of COCs increased. Soil organic extracts toxicity increased exponentially with soil toxicity for subsoil samples. This phenomenon would confirm that: i) contaminant-free soil has non-negligible intrinsic acute toxicity and, ii) the mBSPT test considers the bioavailability of contaminants, although the solubility equilibrium limits it. Moreover, it should be highlighted that the *Microtox® adapted Organic Solvent Sample Solubilization Test* resulted in the most reliable test for evaluating the toxicity of these types of soils, considering the lower standard deviation of $1/EC_{50}$ values obtained.

3.5. Statistical comparisons of total concentration of COCs and acute toxicity

Pearson and Spearman correlation coefficients obtained between soil toxicity (EC_{50} values) for TS, SS-C and SS-F and COCs concentration are summarized in Table S.M. 5. The evaluation has been applied for the three types of toxicity analysis performed: soils, soil elutriates and soil organic extracts. The Pearson coefficient obtained for TS soils and elutriates was higher than those corresponding to SS-C and SS-F. Since topsoil presents lower COCs concentration than subsoil, the solubility limit of the contaminants was not reached and, therefore, the points were in the range of linear dependence, which is not the case for subsoil. Spearman coefficient values were close to +1.00 in all cases, confirming a positive association between the toxicity of the samples and the pollutants concentration.

Thus, considering the statistical analysis, the discussion of results conducted in the previous subsections can be corroborated.

Therefore, it can be concluded that the three methods used reveal interesting and complementary information. The measurement of soil toxicity provides information on the contaminants present in the soil and on the intrinsic toxicity of the soil, so it should be carried out in each case study (different soil characteristics). The measurement of soil elutriates toxicity is necessary to know the toxicity risk associated with the waters in contact with the polluted soils (leachates or groundwaters), and it is of special relevance when there are superficial waters in the vicinity of the contaminated site. Finally, measuring the toxicity of the organic extracts is essential in the case of having hydrophobic compounds as soil pollutants, such as HCHs. Therefore, all three measures are necessary to have a complete overview of the acute toxicity of contaminated soils. In the specific case of soils contaminated with lindane wastes, the most conclusive and reproducible method is the *adapted Organic Solvent Sample Solubilization Test* (which also presents better statistical parameters with respect to COCs concentration).

4. Conclusions

This paper studies and compares the acute toxicity of real soils contaminated with lindane wastes by Microtox® bioassays. It is the first study that applies three different Microtox® tests (soil, soil elutriates and soil organic extracts), with the scope of a more complete and comprehensive evaluation of the acute toxicity of these soils. The three tests revealed interesting and complementary information. COCs concentration, type and distribution in top and subsoils at different pollution levels were found to be related to the contamination source: HCH particulate matter (α-HCH and β-HCH) and DNAPL (a mixture of 28 COCs, HCHs, HeptaCHs, TCBs, TetraCBs, DCBs, CB, PCXs and HCXs), respectively. Soil samples presented high toxicity in the *Basic Test* ($EC_{50,\ soil} \leq 5$ g/L). The higher the COCs concentration in soil, the higher the toxicity. However, an asymptote in EC_{50} was obtained as the COCs concentration in soil increased due to the low solubility of COCS in the aqueous phase. This was also noticed when aqueous elutriates were analyzed. EC_{50} of soil elutriates was lower than EC_{50} of soils, indicating that soil was more toxic than the COCs solubilized to the aqueous phase. The toxicity of soil elutriates was well correlated with dissolved COCs. Soil organic extracts represent more plainly the acute toxicity of the soil without this asymptotic trend. Moreover, EC_{50} of HOCs with low acute toxicity (high EC_{50} values) could be determined by this method. Contamination from DNAPL produces a greater inhibition in bacteria light emission than contamination by HCHs, and therefore, subsoils showed higher toxicity than topsoils. Regarding their bioavailability, contaminants in the finest subsoil fraction are more strongly adsorbed and are less accessible to *Vibrio fischeri* (and, consequently, less toxic). Topsoil and subsoil elutriates showed low ($EC_{50} > 100$ g/L) and moderate (10 g/L ≤ EC50 ≤ 100 g/L) toxicity, respectively, indicating that the possible leachates from the landfills' surface soil are not an environmental issue, but the toxicity of groundwater in contact with contaminated subsoil has to be considered, and remediation treatments should be applied. This analysis, essential for assessing the toxicity of soils with HOCs, resulted to be the most representative of the current study. It revealed that acute toxicity increased with the concentration of COCs and highlighted the need for remediation of these sites. In future works, it would be interesting to evaluate the toxicity of these soils considering other aquatic organisms, such as *Daphnia magna*. In addition, evaluating the chronic toxicity of soils contaminated with lindane wastes is needed.

Abbreviations

C	coarse (soil particles)
CB	chlorobenzene
CI	confidence interval
COCs	chlorinated organic compounds
DCB	dichlorobenzene
DNAPL	dense-non aqueous phase liquid
EC_{50}	effective nominal concentration of toxicant that reduces the intensity of light emission by 50 %
ECD	electron capture detector
F	fine (soil particles)
FID	flame ionization detector
GC	gas chromatography
HCH	hexachlorocyclohexane
HCX	hexachlorocyclohexene
HeptaCX	heptachlorocyclohexane
HOCs	hydrophobic organic compounds
I	light emitted by the bioluminescent bacteria
IC	inorganic carbon
IC_{50}	sample dilution ratio that yields a 50 % reduction of bacteria light emission
ISTD	internal standard
L	pollution level
LOD	limit of detection
LOQ	limit of quantification
MAC	maximum allowable concentration
mBSPT	modified Basic Solid-Phase Test
MS	mass spectrometry detector
ND	not detectable
OAS	osmotic adjustment solution
PCX	pentachlorocyclohexene
PM	particulate matter
PS	persulfate
PTFE	polytetrafluoroethylene, Teflon®
S.M.	Supplementary Material
SARGA	Sociedad Aragonesa de Gestión Agroambiental
SE	soil elutriates
SOE	soil organic extract
SS	subsoil
T	temperature
TCB	trichlorobenzene
TetraCB	tetrachlorobenzene
TOC	total organic carbon
TS	topsoil

CRediT authorship contribution statement

Carmen M. Dominguez: Conceptualization, Methodology, Writing-Original draft preparation, Writing - review & editing. **Paula Ventura**: Investigation, Writing-Original draft preparation **Alicia Checa-Fernandez**: Methodology, Investigation. **Aurora Santos**: Funding acquisition, Resources, Conceptualization, Supervision, Project administration.

Data availability

No data was used for the research described in the article.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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