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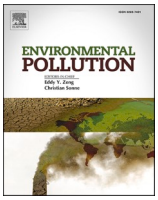

Comparative toxicological assessment of three soils polluted with different levels of hydrocarbons and heavy metals using *in vitro* and *in vivo* approaches

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ABSTRACT

The biological effects induced by the pollutants present in soils, together with the chemical and physical characterizations, are good indicators to provide a general overview of their quality. However, the existence of studies where the toxicity associated to soils contaminated with mixtures of pollutants applying both *in vitro* and *in vivo* models are scarce. In this work, three soils (namely, Soil 001, Soil 002 and Soil 013) polluted with different concentrations of hydrocarbons and heavy metals were evaluated using different organisms representative of human (HepG2 human cell line) and environmental exposure (the yeast *Saccharomyces cerevisiae*, the Gram-negative bacterium *Pseudomonas putida* and, for the *in vivo* evaluation, the annelid *Enchytraeus crypticus*). *In vitro* assays showed that the soluble fraction of the Soil 001, which presented the highest levels of heavy metals, represented a great impact in the viability of the HepG2 cells and *S. cerevisiae*, while organic extracts from Soils 002 and 013 caused a slight decrease in the viability of HepG2 cells. In addition, *in vivo* experiments showed that Soils 001 and 013 affected the survival and the reproduction of *E. crypticus*. Altogether, these results provide a general overview of the potential hazards associated to three specific contaminated sites in a variety of organisms, showing how different concentrations of similar pollutants affect them, and highlights the relevance of testing both organic and soluble extracts when *in vitro* safety assays of soils are performed.

1. Introduction

Soils are essential for the global ecosystems functioning since they are involved in several crucial processes including climate control, nutrient dynamics or establishing the habitat for a wide variety of organisms (Vogel et al., 2019). Thus, and closely connected to these implications, these systems have had a determining role in the establishment and development of civilizations, constituting a highly valuable natural resource for human beings providing food and other relevant products such as fibre (Mueller et al., 2010).

Soil quality, also referred to as soil health, is defined as "the capacity of a living soil to function, within natural or managed ecosystem boundaries, to sustain plant and animal productivity, maintain or enhance water and air quality, and promote plant and animal health" (Doran, 2002; Doran et al., 1996). Together with air and water qualities, these three elements define the environmental quality (Bünemann et al., 2018). Among others, the pollution level is one of the main factors that critically affects soil quality, representing a direct impact for health and the environment (Arellano et al., 2015; Guo et al., 2012; Okereafor et al., 2020; Poggio et al., 2009). For this reason, and considering that soils are a non-renewable source, their protection has become one of the main priorities for the international community and several legal approaches were approved by the European Union in this regard (Pérez and Eugenio, 2018).

Different chemicals with potential toxic effects for the organisms and the ecosystem can be detected in polluted soils. Specifically, organic

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contaminants such as mineral oil or polycyclic aromatic hydrocarbons (PAHs), and heavy metals are of particular interest for the environmental field and related areas, since according to a review prepared by Panagos et al., both are the main pollutants in soils and groundwater of the European Union (Panagos et al., 2013). These substances can reach soils through natural and anthropogenic means, being anthropogenic activities, including accidental and deliberate processes, the primary cause of contamination to the soils. Thus, sectors such as mining and petrochemical industries, as well as the improper use of wastewaters and organic wastes in agricultural activities, are important sources of heavy metal pollution in soils (Wuana and Okieimen, 2011), while PAHs showed to have a pyrogenic origin in urban soils, suggesting the important role of vehicle emissions (Morillo et al., 2007).

In addition to chemical and physical analysis, the use of biological assays is a valuable tool to evaluate the quality of a soil. These analyses are based on the application of representative *in vitro* and *in vivo* models that provide information about the potential hazards that a contaminated site may represent for the human health and for the environment. Therefore, the toxicity associated to different real polluted soils was reported in previous works using several model organisms such as earthworms (Li et al., 2020; Oliveira Resende et al., 2018), plants (Loureiro et al., 2006; Massa et al., 2018), or cell lines, being the latter exposed to contaminant extracts directly obtained from the soils (Baderna et al., 2014, 2013; Husejnovic et al., 2018). Besides in contaminated soils, these assays also showed to be an appropriate approach to study the toxicity of landfill soils (Alimba et al., 2016; Swati et al., 2017), as well as in other polluted substrates such as sediments (Pinto et al., 2014a, 2014b).

The aim of the present study was to evaluate and compare the quality of three contaminated soils presenting different levels of hydrocarbons and heavy metals from a toxicological perspective. The first soil under study (named as Soil 001 in this work) was taken from an industrial area located in Toledo, Spain, which is 3 km from an urban nucleus. This emplacement has been used for decades for machinery repairing and maintenance activities, and the soil sample evaluated has been selected from excavation and removal of soil affected by accidental spills, so it is polluted with the presence of different types of hydrocarbons, mineral oils, and heavy metals from fuel and engine oil leaks. The second soil analysed (named as Soil 002 in this work) was taken from a food manufacturing site located in the outskirts of Carlow. This factory was operative from 1920 to 2006. Since its closure, this site has been undergoing a Closure, Restoration and Residues Management plan. During the site investigation a number of small pockets of hydrocarbon contaminated soil were identified, originating from a leaking heavy fuel oil tank used to run the generators on site during its operation. Finally, the third soil selected (named as Soil 013 in this work) was obtained from one of the largest oil fields of China: Shengli Oil Field. Specifically, this soil was taken from a site located in Gudao, and it is polluted with crude oil. The toxicity associated to these three soils was analysed through the application of a battery of assays combining *in vitro* and *in vivo* models. To perform the *in vitro* assays, soluble and organic extracts were obtained from the soils, and their effect on the viability of different organisms was studied separately. The HepG2 cell line, a model of human liver, together with the yeast *Saccharomyces cerevisiae* and the bacteria *Pseudomonas putida*, both representative of environmental organisms, were applied in these assays. Additionally, the terrestrial annelid *Enchytraeus crypticus* was used to carry out the *in vivo* assays, studying the effects on the survival and the reproduction of this organism after being directly exposed to different concentrations of the polluted soils. The obtained results provide information about the biological quality of the three soils under study, showing how the different concentrations of the contaminants influence this parameter.

2. Materials and methods

2.1. Selected sites and soil samples preparation

Three soils polluted with different levels of hydrocarbons and heavy metals, and intended for being treated with diverse bioremediation technologies, were selected to be toxicologically analysed in this study. The location and contamination source of these sites are summarised in supplementary material (Table S1). Soils 001 and 013 were excavated and homogenized, and appropriate amounts from them were prepared for both chemical and toxicological analyses. In the case of Soil 002, the samples analysed were taken from an ecopile constructed with an excavated soil that was treated with N:P fertiliser and inoculated with 1 L m^{-3} of a Total Petroleum Hydrocarbons (TPH) degrading inoculum. As with the other soils studied in this work, appropriate amounts of it were prepared for chemical and toxicological analysis.

Before their preparation for the different analysis, all the soil samples were air dried and sieved at 2 mm.

2.2. Chemical characterization

2.2.1. TPH quantification

For the TPH extraction, 1 g of each soil previously dried at 30 °C was weighted, and 20 mL of an acetone:hexane mixture (1:1 v/v) was added in a microwave-assisted extraction equipment (Ethos X, Milestone, Sorisole, Italy) for 20 min at 150 °C. After this time, samples were cooled down and centrifuged at 2500×*g* for 30 min. The supernatant obtained was filtered (pore size 0.22 μm) and evaporated to a volume of 1 mL in a speed-vac evaporator (SAVANT SPD111V, Thermo).

Fractionation of Extracted Petroleum Hydrocarbons (EPHs) between linear and aromatic hydrocarbons was carried out using the solid phase extraction (SPE) method according with Jiménez et al. (2014). For this purpose, Isolute EPH fractionation cartridges (25 mL/5 g, Biotage, Uppsala, Sweden) were used in a SPE-24G column processor (JT Baker). The cartridges were first activated by adding 30 mL of hexane and, before it dried, the loading step was started, dissolving the samples in 1 mL of hexane. Then, the elution stage was carried out without applying pressure at a speed of 2–3 mL min^{-1} adding 12 mL of hexane to collect the aliphatic fraction, and 20 mL of dichloromethane to collect the aromatic fraction. Both fractions were concentrated in a speed-vac evaporator to 1 mL. Quantification of EPHs and PAHs was performed in a Varian 3900 gas chromatography instrument equipped with a FID device and a Varian CP8907 capillary column (25 m, 0.25 mm inner diameter, nominal film thickness 0.25 mm).

2.2.2. Trace elements quantification

To perform the extraction, 0.2 g of each soil were weighed, and 10 mL of nitric acid were added to Teflon tubes. Samples were digested at 180 °C during 20 min in a microwave-assisted digestion system (Ethos One, Milestone, Sorisole, Italy). The digested samples were then filtered using a double filter paper into 50 mL volumetric flasks and made up with ultrapure water. All the materials used were previously cleaned in diluted HNO_3.

Those metals that were expected to be present at high concentrations (Al and Fe) were analysed by inductively coupled plasma - optical emission spectrometry (ICP-OES) using a SPECTRO GENESIS spectrometer. In the case of those elements that were expected to be present at very low concentrations (As, Cd, Cr, Co, Cu, Mn, Mo, Ni, Pb, Ti and Zn), the quantification was performed by inductively coupled plasma mass spectrometry (ICP-MS) using an Agilent 8900 ICP-QQQ instrument in the University of Burgos. A 21-multielement standard solution (100 mg L^{-1}, VWR Chemicals, Leuven, Belgium) was used for the calibration-verification procedure.

2.3. In vitro assays

2.3.1. Sample preparation

2.3.1.1. Organic fraction extraction. The pollutant extraction was performed following a protocol adapted from Pinto et al. (2014a). Firstly, samples were dried at 40 °C in the dark, pulverized and homogenized with a mortar before being extracted with a mixture of dichloromethane (DCM):methanol (2:1 v/v). To carry out this step, 3.3 mL of the solvent mixture were added to every 2 g of dry pulverized soil sample, and the extraction was done mechanically by reciprocal shaking during 15 min (60 rpm). Samples were then centrifuged (2000×*g*, 20 min) and the supernatant was recovered and evaporated using a rotary evaporator, adding the supernatant little by little. To prepare the organic extract stocks 2 mL of dimethyl sulfoxide (DMSO) were added to each tube, and the samples were submitted to gentle shaken (300 rpm) combined with several 1-h sonication steps in an ultrasonic bath during 1 week with the aim to solve as much as possible the obtained precipitate. The final soil: extract proportion was 1 g soil dry weight per mL of extract. Finally, samples were filtered with a 0.22 μm nylon filter. Three independent extractions per each soil were performed. A blank sample was created following the same extraction process without soil.

2.3.1.2. Soluble fraction extraction. Prior to the extraction, soil samples were completely rehydrated. The extraction was performed adding water at room temperature in in a ratio 1:4 (w/v) (4 mL per gram of dried weight soil). Then, samples were vortexed and sonicated in an ultrasonic bath with ice cold water for 1 h. After this time, samples were vortexed again, centrifuged at 4000×*g* for 10 min, and the supernatants were collected and evaporated until a concentration equivalent to 1 g soil dry weight per mL of water. Finally, samples were filtered with a 0.22 μm polyethersulfone filter.

2.3.2. Organisms and culture conditions

HepG2 cell line was cultured in commercial Eagle's Minimum Essential Medium (EMEM) supplemented with 10% (v/v) fetal bovine serum (FBS), 1% non-essential amino acids, 1% sodium pyruvate and 100 U mL^{-1} penicillin and 100 mg L^{-1} streptomycin. This cell line was incubated under standard conditions in 37 °C humidified 5% CO_2 atmosphere.

The BY4741 strain of *S. cerevisiae* was maintained in standard Yeast extract Peptone-Dextrose (YPD) medium (1% yeast extract, 1% yeast bacto-peptone, 2% glucose). Liquid cell cultures were done on a rotary shaker at 185 rpm at 30 °C.

P. putida CECT 4064 (DSMZ 548) was maintained at 30 °C in Mueller-Hinton broth or agar.

2.3.3. Viability analysis of HepG2 cells

The neutral red uptake assay was carried out to determine the viability of HepG2 cells after being exposed to the different contaminant fractions. Briefly, cells were seeded in 96 well plates (4×10^4 cells per well) and, 24 h after seeding, cells were incubated for 24 h in presence of the contaminants diluted in fresh culture medium. For the organic fraction, which was resuspended in DMSO, the concentration tested was 10 mg of soil equivalent per mL (s.e. mL^{-1}). For the experiments analysing the soluble fraction, culture medium 10 × was previously prepared and directly diluted to 1 × in the samples, being the concentration tested 900 mg s.e. mL^{-1}. From this point on, the assay was performed following the protocol previously explained in other works developed by our group (Rumbo et al., 2021, 2020).

2.3.4. Viability analysis of S. cerevisiae

S. cerevisiae cells were grown on YPD medium in an orbital shaker (185 rpm) at 30 °C until reach an $O.D._{600\ nm} = 1$. Cells were then exposed for 2 and 24 h to 10 mg s.e. $mL^{-1}$of the organic fraction and 900 mg s.e. mL^{-1} of the soluble fraction in 24-well plates (final volume of 1 mL). Yeast colony forming units were determined inoculating cells on solid YPD medium (6% agar) and incubated at 30 °C. Results were expressed as percentage of control (CFUs in absence of contaminants).

2.3.5. Experiments using P. putida: growth curves analysis

The influence of both contaminant fractions in microbial growth was monitored using a Synergy HT microplate reader (BioTek Instruments, Inc.). Cultures of *P. putida* were first grown in MHB overnight at 30 °C with shaking. Cultures were then diluted 1:100 in medium alone (as control) and in medium supplemented with 10 and 900 mg s.e. mL^{-1} of the organic extracts and the soluble fractions respectively. The growth rate was monitored every hour recording the optical density at 600 nm in 24-well plates for 20 h.

2.4. In vivo assays using Enchytraeus crypticus

Toxicity test with *E. crypticus* was carried out according to OECD 220 (OECD, 2004). Loamy sand soil (LUFA-Speyer 2.2, Sp 2121, Germany, 2009) with a pH-$CaCl_2$ of 5.5, a total organic carbon content of 2.09%, a cation exchange capacity (CEC) of 10 meq/100 g and a water-holding capacity (WHC) of 46.5% was used. After mixtures (contaminated soil + LUFA 2.2) preparation, additional Milli-Q was added to achieve a soil moisture content of 50% of the maximum WHC.

The organisms were cultured in dishes containing Bacto agar, kept at 20 ± 1 °C with a 12/12 h photoperiod, and fed with boiled oatmeal. Animals with clearly visible clitellum (sexually mature) and the same size were used in the experiment. Selected organisms were transferred from the culture to a Petri dish with water and then introduced in the test containers filled with 30 g of moist soil. For each concentration, four replicate test containers were used. Test containers were closed with perforated aluminium foil, and 3 mg crushed oatmeal was added as food. Then, containers were placed in a climate room at 22 °C, with 75% of relative humidity and 12/12 h light/dark cycle. Twice a week, containers were checked for water loss and compensated for by weighing, and additional food was added if needed. After 21 days, enchytraeids were fixed adding a solution of 10 mL ethanol to each test container, and thoroughly stirring the container. After 1 min, the suspension was transferred to a plastic jar and 100 mL of distilled water was added. The enchytraeids were stained by adding 300 μL of a 1% Bengal rose solution. The samples were shaken again vigorously and incubated for 24 h in the refrigerator at approx. 4 °C to achieve an optimal dying effect. Then the bright pink coloured enchytraeids were isolated by sieving over 160 μm and counted in 80 × 50 photo trays using a magnifying glass. The number of surviving adults and juveniles produced were determined in each test container.

2.5. Statistical analysis

Data are presented as means ± SD. Statistical analysis was performed by the one-way analysis of variance (ANOVA), followed by Tukey *post hoc* test for multiple comparisons. Statistical tests were carried out using Prism 8.0.2 (GraphPad Prism, GraphPad Software, Inc.), considering the differences significant at $P \leq 0.05$.

3. Results

3.1. Chemical characterization of the soils

The three soils under study were analysed to characterize their main physico-chemical parameters (Supplementary Material, Table S2) and their contaminant levels (Table 1). In these soils, the Total Petroleum Hydrocarbons (TPHs; TPHs = EPHs + VPH) fraction was equivalent to extractable fraction (EPHs), since the volatile fraction (VPH) was almost completely depleted, therefore only remaining the medium and high molecular weight, in both linear aliphatic and aromatic hydrocarbons.

Table 1
Contamination levels (per contaminant class) of the three soils.

		Soil Sample		
		Soil 001	Soil 002	Soil 013
Contaminant				
Organic (mg kg^{-1})[a]				
EPHs		3723	1355.2	13,162
LAHs	>C10 – C12	2	0.2	26
	>C13 – C16	117	6	897
	>C17 – C21	112	41	1750
	>C22 – C35	2661	462	5857
	>C35	208	134	1143
	∑LAHs	3100	643.2	9673
Aromatics	>EC10 - EC12	15	3	37
	>EC13 - EC16	11	2	99
	>EC17 - EC21	111	152	1690
	>EC22 - EC35	340	400	1639
	>EC35	146	155	24
	∑ Aromatics	623	712	3489
Inorganic (mg kg^{-1})[b]				
	Al	11,000	8200	17,000
	Fe	32,000	15,000	24,000
	Mn	4200	530	295
	As	77.28	3.20	4.26
	Cd	7.78	3.26	0.06
	Cr	14.9	14.2	16.7
	Co	5.8	4.4	5.3
	Cu	8.5	21.4	15.2
	Mo	1.71	0.71	<d.l.
	Ni	9.9	15.1	14.6
	Pb	339	41	10
	Ti	188	85	398
	Zn	681	70	44.5

<d.l., below detection limit.
[a] Values obtained considering the percentage of the measurement uncertainty associated to the analytical method (5%).
[b] Values obtained considering the percentage of the measurement uncertainty associated to the analytical method (10% for Al and Fe; 4% for Mn, As, Cd, Cr, Co, Cu, Mo, Ni, Pb, Ti, Zn).

All the soils displayed metallic and organic contaminants, being the concentrations for the EPHs and some particular trace elements above their correspondent threshold levels, according to legislation. Clear differences in the levels of the different contaminants between the soils were observed. Thus, the levels of EPHs in Soil 013 were ≈ 3- and 9-fold higher than in Soils 001 and 002 respectively. Linear aliphatic hydrocarbons (LAHs) were the most abundant hydrocarbons in this soil, specifically those of >C22 – C35 chains. Regarding the aromatics fraction, those of >EC17 - EC35 n-alkane equivalent C chains were predominant in Soil 013. Soil 001 represented the intermediate sample in terms of EPHs pollution. LAHs >C22 – C35 corresponded to the major fraction, as well as in the aromatics fraction, where this equivalent C-chain range was also predominant. Finally, Soil 002 showed the lower EPHs content, being similar the levels of LAHs and aromatics and, as observed in Soil 001, >C22 – C35 n-alkane chain hydrocarbons were the most representative in both fractions.

Regarding metals and metalloids content, Al and Fe were the most abundant elements in these three soils. In Soil 001, the levels of Mn were particularly high, being the concentration of this element ≈ 8- and 14-fold higher than in Soils 002 and 013 respectively. In addition, the concentrations of As, Cd, Pb and Zn stand out over the levels found in the other soils. In Soil 002, Mn, together with Al and Fe, constituted the most abundant metals, while in Soil 013, Ti ranged the 3rd position, just above the Mn.

3.2. Effect of polluted soil extracts in human HepG2 cell line

The effect of both organic and soluble extracts obtained from the three contaminated soils in the viability of the human hepatoma cell line HepG2 was evaluated using the neutral red uptake assay. Fig. 1 shows the results obtained after exposing HepG2 cells to 10 mg s.e. mL^{-1} of the different organic extracts for 24 h. Due to the difficulty to redissolve the organic condensate obtained after the evaporation, and the impossibility to achieve its complete dissolution, three independent extractions from each soil were performed, and the redissolved extracts were tested separately to ensure that the procedure was homogeneous across the process, and to confirm that the results obtained were similar. Extracts from Soil 001 showed a slight decrease in the percentage of viable cells (5–10%) (Fig. 1A). In the case of the extracts from Soils 002 and 013, both caused a decrease of ≈10% in the viability of HepG2 cells, being these values statistically significant when compared to control in the three samples obtained from Soil 002.

Moreover, cells were exposed to a concentration of 900 mg s.e. mL^{-1} of the soluble extracts from the three soils for 24 h. Results showed that the extracts from Soil 001 presented a critical impact in the viability of HepG2 cells, where the percentage of dead cells was almost 100% (Fig. 1B). On the other hand, soluble extracts from Soil 002 caused a slight decrease in the viability of these cells (≈10%), while those exposed to soluble extracts from Soil 013 presented a percentage of viable cells similar to that of the control (Fig. 1B).

3.3. Effect of polluted soil extracts in S. cerevisiae

To determine the toxicological potential of both organic and soluble soil extracts, the fungal model *S. cerevisiae* was included in the assays. Thus, the viability of this organism was evaluated at two exposure times (2 and 24 h). As displayed in Fig. 2A, any of the organic extracts caused a negative effect on the viability of *S. cerevisiae* when exposed to a

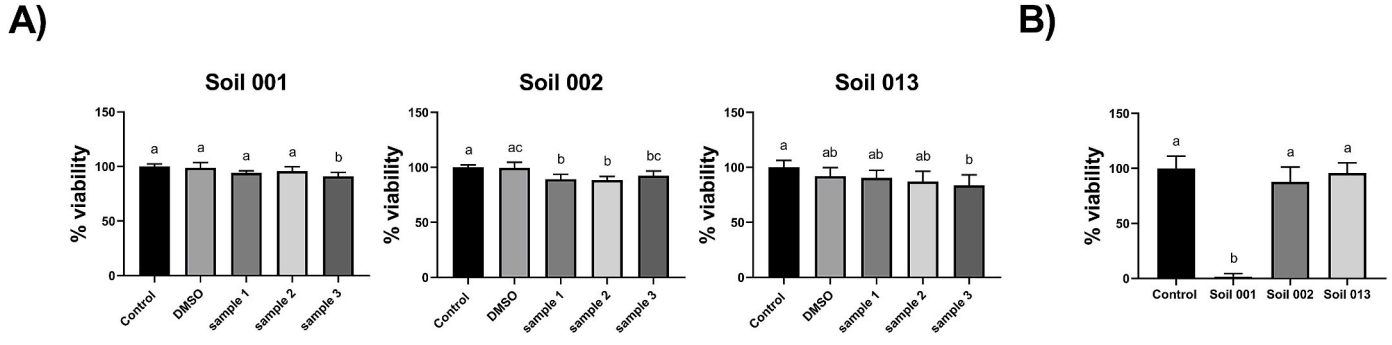

Fig. 1. HepG2 cells viability after being exposed to 10 mg s.e. mL^{-1} of the organic extracts (A) and to 900 mg s.e. mL^{-1} of the soluble extracts (B) for 24 h. Results are expressed as % of control (untreated cells). Data represent the mean of at least 5 biological replicates (± standard deviation, SD) obtained in two independent experiments (A) or the mean of at least 6 biological replicates (± standard deviation, SD) obtained in three independent experiments (B). Differences were established using a One-way ANOVA followed by multiple comparisons test (Tukey test) and considered significant at $P \leq 0.05$. Different letters indicate statistically significant differences between treatments.

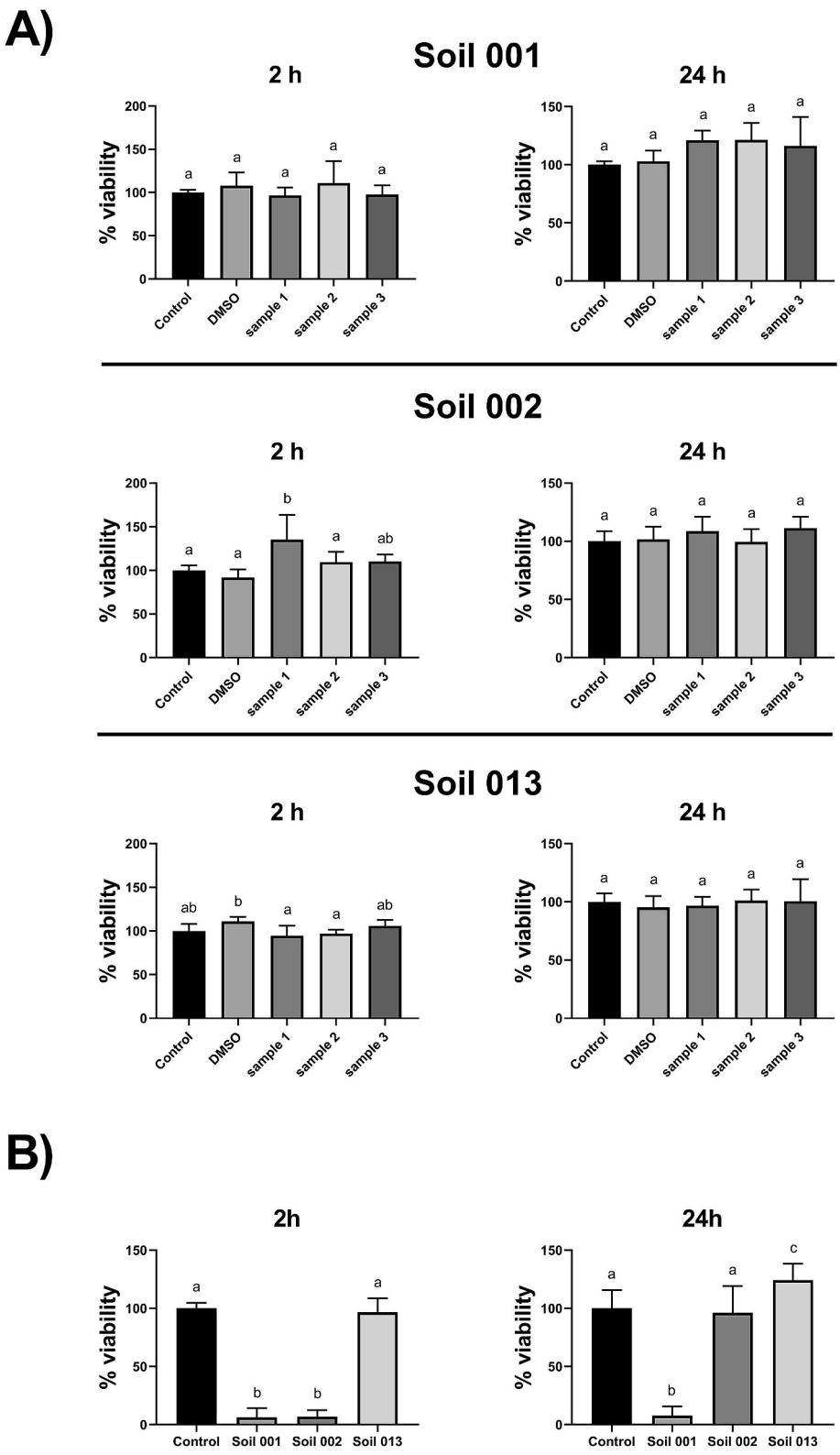

Fig. 2. Colony forming units (CFUs) of *S. cerevisiae* cells exposed to 10 mg s.e. mL^{-1} of the organic extracts (A) and to 900 mg s.e. mL^{-1} of the soluble extracts (B), at two exposure times (2 and 24 h). Results are expressed as the percentage (%) of CFUs determined for each exposure condition using as reference value the non-exposed cells condition, which was assigned a value of 100%. Data represent the mean of at least 5 biological replicates (± standard deviation, SD) obtained in two independent experiments (A) or 6 biological replicates (± standard deviation, SD) obtained in three independent experiments (B). Differences were established using a One-way ANOVA followed by multiple comparisons test (Tukey test) and considered significant at $P \leq 0.05$. Different letters indicate statistically significant differences between treatments.

concentration of 10 mg s.e. mL^{-1}. On the other hand, and as it was observed in HepG2 cells, soluble extracts from Soil 001 at 900 mg s.e. mL^{-1} showed to have a critical impact on this organism. Fig. 2B shows that even at 2 h of incubation, the viability of these cells was decreased ≈95%, being this percentage similar at 24 h. Interestingly, soluble extracts from Soil 002 drastically reduced the viability of *S. cerevisiae* at 2 h of incubation but presenting this organism a similar percentage of viable cells than in the control after a longer exposure time (24 h). Finally, the soluble extracts from Soil 013 did not cause any negative effect on the viability of *S. cerevisiae* at any of the time points selected, being even significantly higher than control at 24 h.

3.4. Effect of polluted soil extracts in P. putida

Pseudomonas putida, a representative bacterium found in water and soil, was used to evaluate the toxicity of the different soil extracts. The toxic effect of both organic and soluble extracts on bacterial growth was evaluated first performing MICs, showing that any of the concentrations tested inhibited the growth of this bacterium (Supplementary Material; S1, Table S3). Moreover, to analyse if the highest concentrations of the soil extracts could represent any perturbation in bacterial growth in spite of not inhibiting it, growth curves were carried out. Fig. 3 shows that neither organic nor soluble extracts caused any negative effect on bacterial growth, presenting all conditions similar curves than the control condition.

3.5. Effect of polluted soil in E. crypticus survival and reproduction

Control performance of the test animals was evaluated using the quality criteria described in test guidelines. For the enchytraeid, *E. crypticus* survival should be $\geq$ 80% with $\geq$25 juveniles per test container and a coefficient of variation $\leq$50% (OECD, 2004). In our experiment, the enchytraeids produced large numbers of juveniles (average = 321 juveniles) and the coefficient of variation was less than the 50% limit. Soils 001 and 013 showed a statistically significant toxic effect on enchytraeid survival and reproduction. However, no toxic effect was observed when organisms were exposed to Soil 002 (Fig. 4).

Moreover, for contaminated Soils 001 and 013, the EC_{50} values for survival and reproduction were also evaluated exposing the organisms to mixtures of the contaminated soil with control soil LUFA 2.2 at different concentrations. Dose response curves were performed showing that, for Soil 001, the EC_{50} values were 80% [67–109] for survival, and 57% [36–81] for reproduction, while in Soil 013, these values were 57% [53–77] and 46% [36–49] for survival and reproduction respectively, being thus highlighted the greater toxicity of the latter (Supplementary Material, Fig. S1).

4. Discussion

Hydrocarbons and heavy metals are among the main pollutants in the soils of the European Union, representing around 60% of soil contamination according to estimations published in previous works (Panagos et al., 2013). In addition, in the specific case of As, Cd, Cr, Cu, Pb, Zn, Sb, Co and Ni, it was described that in the 28.3% of the total surface area of the European Union, one or more of these metals and metalloids exceed the applied threshold concentration (Tóth et al., 2016). Considering all of this, together with the pernicious effects that both hydrocarbons and heavy metals pose for human health and the environment, the availability of data concerning the potential toxicity of soils presenting these pollutants is critically important to determine their associated hazards.

Physicochemical characterization methods are traditionally applied to analyse the quality of a soil. However, this methodology does not entirely reflect the global effects that xenobiotic mixtures may induce in living organisms. For this reason, the employment of toxicological assays using different model organisms are essential tools to elucidate the potential impact of a soil. In the present study, three polluted soils presenting different concentrations of hydrocarbons and heavy metals have been evaluated from a biological perspective.

The chemical characterization analysis revealed that EPH levels in the three soils were above of 1000 mg kg^{-1}, thereby suggesting notable hydrocarbon pollution. Specifically, Soil 013, which corresponds to an oil extraction field, showed to harbour by far the highest concentration of hydrocarbons, followed by Soil 001 and Soil 002. The presence of trace elements was also studied. In comparison with the others, especially significant were the amounts of Mn in Soil 001, as well as those of As, Cd, Pb and Zn, being the appearance of the latter two elements compatible with the contamination by fuel and engine oil leaks since both metals are present in used engine oils (Kashif et al., 2018; Stout et al., 2018).

The use of organisms belonging to different biology kingdoms provides a more accurate overview about the real implications that a polluted soil may represent for the whole environment since, due to their particularities, they will respond differently to stress and, therefore, they will not be equally affected when exposed to a specific pollutant at particular concentrations. Bearing this in mind, a broad portfolio of model organisms including human cells, fungus, bacteria and worms were applied, combining *in vitro* and *in vivo* methodologies to evaluate the potential hazardous effects associated to these three real polluted soils. In addition, the fact that all the soils were polluted with a mixture of heavy metals and hydrocarbons, provided valuable information extracted from real sites about the implications of different levels of similar contaminants and mixtures in the viability of different organisms.

To carry out the *in vitro* assays, the HepG2 cell line, the yeast *S. cerevisiae* and the Gram-negative bacterium *P. putida* were used in the experiments. HepG2 cell line is considered a suitable organism to be applied in toxicological studies since the liver is one of the main targets of xenobiotics and, in addition, the regulation of xenobiotic genes in this cell line is similar than in primary hepatocytes (Baderna et al., 2013). Therefore, HepG2 cells have been applied in previous works to analyse the cytotoxicity of extracts obtained from environmental contaminated samples (Baderna et al., 2014; Pinto et al., 2014b). Regarding *S. cerevisiae*, this microorganism was included in this work since yeasts are present in soils, involved in several processes such as nutrient transformations or the maintenance of its structure (Yurkov, 2018). In addition, this unicellular fungus is among the most widely applied model organisms in studies focused on a broad range of biological parameters such as in regulation of gene expression or metabolic analyses (Karathia et al., 2011). Moreover, it was also proposed as a suitable

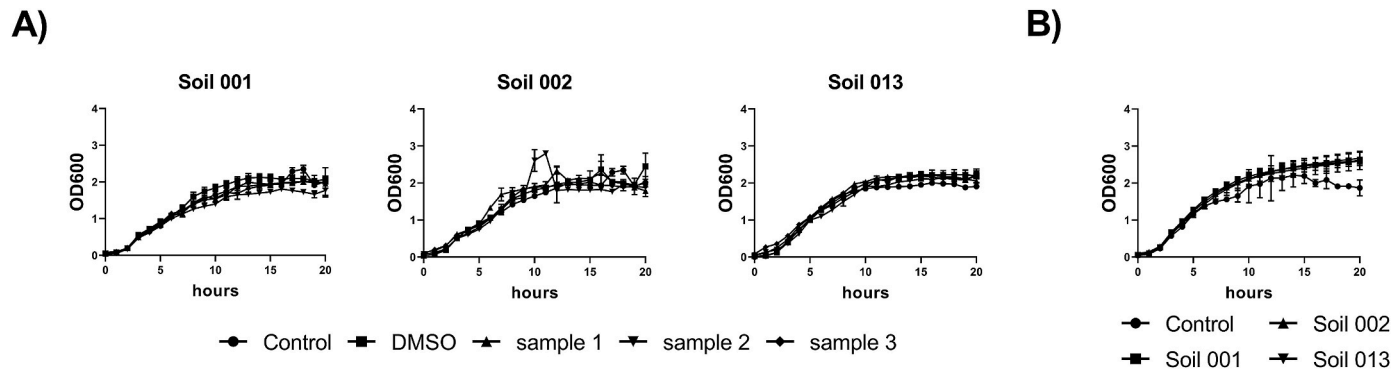

Fig. 3. Growth curves in the presence of 10 mg s.e. mL^{-1} of the organic extracts (A) and 900 mg s.e. mL^{-1} of the soluble extracts (B). Data represent the mean of 3 biological replicates (± standard deviation, SD).

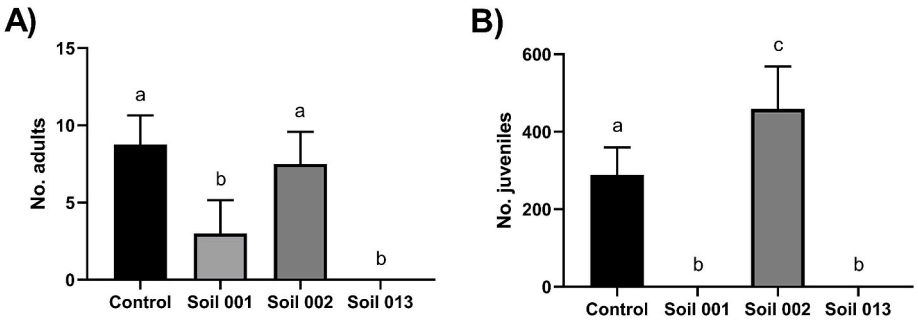

Fig. 4. Effect of contaminated soils (Soil 001, Soil 002 and Soil 013) on survival (A) and reproduction (B) of *E. crypticus* after 28 days of exposure. LUFA 2.2 was used as control soil. Data represent the mean of 4 replicates (± standard deviation, SD). Differences were established using a One-way ANOVA followed by multiple comparisons test (Tukey test) and considered significant at $P \leq 0.05$. Different letters indicate statistically significant differences between treatments.

model to study the toxicity of soils contaminated with pesticides (Gil et al., 2015). Likewise, the Gram-negative bacterium *P. putida* can be found in soils, particularly in rhizosphere, where it interacts with plants stabilising commensal relationships, and being involved in several important functions such as plant growth promotion (Molina et al., 2020). This microorganism was also used in previous works to study the toxicity of contaminated and bioremediated soil extracts (Ahtiainen et al., 2002; Hund and Traunspurger, 1994). To perform *in vivo* assays, the annelid *E. cypticus* was employed as model organism. Soil-dwelling annelids of the genus Enchytraeus are ecologically relevant species for ecotoxicological testing since these organisms play an important role in organic matter decomposition and soil bioturbation. *E. cypticus* has a large tolerance range to soil properties such as pH (4.4–8.2), clay (1–29%) and organic matter content (1.2–42%) (Kuperman et al., 2006; Van Gestel et al., 2011), making it a useful tool to assess the ecotoxicity of soils with different properties.

To perform the *in vitro* toxicological evaluation, soluble and organic extracts were obtained from the soils. The organic extracts were obtained through a DCM:methanol extraction. This methodology was selected since, according to a previous work where the toxicity of sediments containing heavy metals, PAHs, pesticides and polychlorinated biphenyls was evaluated, it has the ability to trap a complex mixture of toxicants, reflecting the original contamination pattern of the samples (Pinto et al., 2014b). Our results showed that a concentration of 10 mg s.e. mL^{-1} caused a slight decrease in the viability of HepG2 cells, mainly in the case of those from Soils 002 and 013, both presenting the higher levels of TPHs. On the other hand, no effect was observed in *S. cerevisiae* and *P. putida*. The evaluation of extracts from soils to analyse their biological impact was previously applied in other works. Baderna et al. evaluated the risk of soils with low levels of pollutants located in two semi-rural areas in the North of Italy using soil organic extracts in their analysis (Baderna et al., 2014, 2013). These authors found that only high doses of polluted soil equivalents resulted in cytotoxic effects. Moreover, Pinto et al. also used this cell line to study the potential genotoxicity and mutagenicity of different extracts of impacted estuarine sediments (Pinto et al., 2014a, 2014b), observing that sediments obtained from industrial areas were significantly more genotoxic and mutagenic than those from rural areas. In our case, despite the large variations observed in the levels of pollutants between the three soils, the experiments analysing the organic extracts did not show notable differences at the used concentration (10 mg s.e. mL^{-1}) for any of the three model organisms. This value was selected as the highest allowable concentration that does not exceed the 1% of solvent (DMSO), which could negatively affect the organisms *per se*.

The extraction of contaminants in metal polluted soils applying water was previously applied in other works (Husejnovic et al., 2018; Vidic et al., 2009), following similar methodologies than that described here. In our study, the most accessible contaminants from the soils were extracted applying sonication and, given that the used solvent is water, a higher concentration of soil equivalent (900 mg s.e. mL^{-1}) could be tested in the different organisms to stablish comparisons. In this case, remarkable differences were observed between the three soils in terms of their effects as well as in their metals and metalloids content (Supplementary Material, Table S4). Thus, the Soil 001 extracts critically affected the viability of HepG2 cells and *S. cerevisiae*. In the case of the extracts from Soil 002, they showed to interfere in the viability of *S. cerevisiae* only at low incubation times, while extracts from Soil 013 did not show any negative effect. Living organisms are exposed in the environment to different metals that, in combination, may pose a risk and cause adverse effects even when their individual concentrations are below toxic levels (Wu et al., 2016). Our results show that, in general, the specific mixtures of metals and metalloids found in the extracts from Soils 002 and 013 are safe in terms of viability effects, while those from Soil 001 result in a critical impact in HepG2 cells and *S. cerevisiae*. Several factors are involved in the toxicity of the heavy metals, including dose, route and time of exposure. In the case of individual metals, their acute and chronic effects have been described for some of them (Balali-Mood et al., 2021; Jaishankar et al., 2014). However, regarding the impact of mixtures of metals and metalloids, the number of existent works in the current literature is scarce, since most of them are focused on their individual effect. For this reason, little is known about the combined toxicity of heavy metals, which can present additive, antagonistic or synergistic effects. The huge differences in their whole content between Soil 001 extracts and the others are the most likely cause of the adverse biological impact of this soil. A Principal Component Analysis (PCA) of the chemical properties of the soluble extracts and their associated toxicity on HepG2 cells and *S. cerevisiae* was also performed to identify those elements that are more likely to be associated with the toxic effects observed (Supplementary Material, S2). This analysis showed that the high concentration of some trace elements such as Zn, Mn, As, Cr or Co could be related with the toxicity against both human cells and the yeast (Supplementary Material, Tables S5–S6; Fig. S2). Particularly, the high levels of Mn detected in this sample probably represent a determinant factor in the observed hazard effects. Excessive levels of Mn have been reported to induce cell death through apoptosis (Alaimo et al., 2014; Hirata, 2002) or neurodegenerative damage in mammals (Peres et al., 2016). In hepatoma cell lines, including HepG2 cells, it has been reported that their viability can be affected when exposed to $MnCl_2$ at concentrations in the millimolar and micromolar range (Chen et al., 2022; Tillman, 2018). Moreover, in *S. cerevisiae*, 0.5 mM or higher concentrations of Mn showed to inhibit the growth of this organism (Blackwell et al., 1998). Therefore, the levels of Mn, acting synergistically with the other elements, could cause the critical decrease observed in the viability of both HepG2 cells and *S. cerevisiae*. In the case of *P. putida*, and as it was observed in the evaluation of the organic extracts, this microorganism was not affected by any of the soil samples. In general terms, the members of this species have the ability to grow and develop in adverse environments, including polluted sites (Ramos et al., 2015). Thus, our results show that the levels of contaminants present in these soils are under the tolerance limits of *P. putida*.

Regarding the *in vivo* toxicological evaluation of the three soils, the differences in EPH levels in Soil 013 compared to the others could explain the high effect of this soil on survival and reproduction of *E. crypticus*. Effects of crude oil contamination on enchytraeids survival were observed at concentrations of 10,000 mg TPH kg^{-1} and 0.4 mg PAH kg^{-1}. In addition, survival has been reported as an index of heavy crude oil contamination of soil because correlated linearly with TPH and PAH (Filimonova and Pokarzhevskii, 2000). The effect of Soil 001 could be partly explained by the high concentrations of As and Zn, which were closed to and up to bibliographic values for LC50 and EC50, survival and reproduction, respectively (LC50: 72.8 mg As kg^{-1} (Li et al., 2021); EC50: 212 mg Zn kg^{-1} (Weltje et al., 1995); and 229 mg As kg^{-1} (Lock and Janssen, 2001)). A PCA performed to prospect for the possible influence of soil physical and chemical properties on the observed toxicity in worms (Supplementary Material, Tables S7–S8; Fig. S3) showed that the load of organic soil pollutants and some trace metals such as Al, Ti and Cr, as well as As, Mn, Pb and Zn, could be associated with harmful effects over the viability and the reproduction of *E. crypticus*. Moreover, other properties such as the electrical conductivity and the percentage of sand seems to affect both parameters.

5. Conclusions

The biological impact of three soils polluted with different levels of hydrocarbons and heavy metals was evaluated in this work. The selected analysis included *in vitro* assays using organic and soluble extracts from the soils, and *in vivo* assays directly using the soil samples. Different model organisms representative of human (cell line HepG2) and environmental (*S. cerevisiae, P. putida* and *E. crypticus*) exposures were employed in the toxicological evaluation.

The obtained results showed that the soluble contaminants of the Soil 001, which presented the highest levels of metals and metalloids, provoked a critical decrease in the viability of the HepG2 cells and *S. cerevisiae*. Moreover, *in vivo* experiments exhibited that this soil, together with Soil 013, which presented the highest levels of hydrocarbons, display a significant effect over the viability and the reproduction of *E. crypticus*.

In summary, these results provide a general overview of the impact of three real polluted soils in the human health and the environment. In addition, this work brought out the importance of evaluating different extracts of the soils when performing *in vitro* assays, and the relevance of using different representative organisms combining *in vitro* and *in vivo* assays to obtain accurate information about the potential toxicity that a polluted site may represent.

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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.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envpol.2022.120472.

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