UNIVERSITAT POLITÈCNICA DE CATALUNYA BARCELONATECH

UPCommons

Portal del coneixement obert de la UPC

http://upcommons.upc.edu/e-prints

Aquesta és una còpia de la versió *author's final draft* d'un article publicat a la revista Environmental science and pollution research.

URL d'aquest document a UPCommons E-prints:
http://hdl.handle.net/2117/90589

Article publicat / *Published paper*:

Bori, J., Vallès, B., Ortega, L. et al. (2016) Bioassays with terrestrial and aquatic species as monitoring tools of hydrocarbon degradation. Environmental science and pollution research, 23, 18, pp 18694–18703.
doi:10.1007/s11356-016-7097-z

Bioassays with terrestrial and aquatic species as monitoring tools of hydrocarbon degradation

Jaume Bori[1*], Bettina Vallès[1], Lina Ortega[2], Maria Carme Riva[1]

[1] *Center for Research and Innovation in Toxicology (CRIT-Innotex Center), Technical University of Catalonia (UPC). Ctra. Nac. 150 Km 15, 08227 Terrassa (Barcelona) Spain.*
[2] *Geotecnia 2000 (Grupo ATISAE), Tres Cantos, Madrid, Spain.*
** Corresponding author. +34 937398396 / jaume.bori@crit.upc.edu*

Keywords: Hydrocarbon degradation, Remediation, Ecotoxicology, Soil contamination, Aqueous extracts,

Abstract In this study chemical analyses and ecotoxicity tests were applied for the assessment of a heavily hydrocarbon-contaminated soil prior and after the application of a remediation procedure that consisted in the stimulation of soil autochthonous populations of hydrocarbon degraders in static-ventilated biopiles. Terrestrial bioassays were applied in mixtures of test soils and artificial control soil and studied the survival and reproduction of *Eisenia fetida* and the avoidance response of *E. fetida* and *Folsomia candida*. Effects on aquatic organisms were studied by means of acute tests with *Vibrio fischeri*, *Raphidocelis subcapitata* and *Daphnia magna* performed on aqueous elutriates from test soils. The bioremediation procedure led to a significant reduction in the concentration of hydrocarbons (from 34264 mg kg^{-1} to 3074 mg kg^{-1} i.e. 91% decrease) and toxicity although bioassays were not able to report a percentage decrease of toxicity as high as the percentage reduction. Sublethal tests proved the most sensitive terrestrial bioassays and avoidance tests with earthworms and springtails showed potential as monitoring tools of hydrocarbon remediation due to their high sensitivity and short duration. The concentrations of hydrocarbons in water extracts from test soils were 130 μg L^{-1} and 100 μg L^{-1} before and after remediation, respectively. Similarly to terrestrial tests, most aquatic bioassays detected a significant reduction in toxicity, which was almost negligible at the end of the treatment. *D. magna* survival was the most affected by soil elutriates although toxicity to the crustacean was associated to the salinity of the samples rather than to the concentration of hydrocarbons. Ecotoxicity tests with aqueous soil elutriates proved less relevant in the assessment of hydrocarbon-contaminated soils due to the low hydrosolubility of hydrocarbons and the influence of the physicochemical parameters of the aquatic medium.

1. Introduction

Soils are considered major sinks of hazardous environmental pollutants. Among them, total petroleum hydrocarbons (TPHs) have become a worldwide cause of concern due to their environmental persistence, bioconcentration and bioaccumulation (McElroy et al. 1989), their potential toxicity, mutagenicity and carcinogenicity (Brown et al. 1999; White and Claxton 2004). The environmental release of TPHs into soils is known to occur through several ways: pipeline blow-outs, waste dumping, disposal after drilling oil and gas wells, road accidents, leakage in underground storage tanks, or uncontrolled landfill activities among others (Chaineau et al. 2003). Once in soils and depending on the solubility and hydrophobicity of hydrocarbon fractions, TPHs can reach the water compartment through leaching (Stroo et al. 2000). Furthermore, highly-mobile TPHs might reach ground waters and become more toxic to soil organisms (Cvancarova et al. 2013).

Hydrocarbon-contaminated sites require the application of proper management and remediation procedures to render their soils environmentally acceptable. To achieve this goal, autochthonous populations of hydrocarbon degraders can be stimulated under certain environmental conditions (temperature, soil moisture, nutrients, etc) and their success in reducing hydrocarbons contents can be evaluated through chemical analyses. However, those analyses have proven insufficient for a proper characterization of the overall soil quality because they are unable to identify all compounds in soil (Fernandez et al. 2005). Moreover, they cannot detect toxic intermediary metabolites that increase soil toxicity (Haeseler et al. 2001; Loibner et al. 2003) nor provide information on bioavailability, synergic, and antagonistic phenomena (Juvonen et al. 2000). On the other hand, ecotoxicological tests do integrate all soil-occurring phenomena and are therefore recommended for the ecological risk assessment of polluted soils (Bori and Riva 2015; Bori et al. 2015; Bori et al. 2016) and as monitoring tools of hydrocarbon remediation (Salanitro et al. 1997; Saterbak et al. 1999; Mendonça and Picado 2002; Lors et

al. 2009; Megharaj et al. 2011). In order to obtain useful information on potential ecological risks, such tests are usually applied in batteries that include species from different taxonomical groups and routes of exposure (Békaert et al. 1999; Bispo et al. 1999; Rila and Eisentraeger 2003; Fernandez et al. 2005).

In the evaluation of the environmental risk posed by contaminated soils, most efforts have focused in the study of the effects to soil-dependent organisms (Keddy et al. 1995; Walker et al. 2006). Within this group, soil invertebrates (earthworms and springtails) are most frequently used for the assessment of lethal and sublethal responses. Among the available endpoints, chronic studies have the advantage of being more sensitive than acute tests and providing information on potential effects on the soil habitat function (DECHEMA 1995). However, their higher costs and time consumption make them unsuitable for the assessment of polluted soils or as monitoring tools. On the other hand, sublethal tests that evaluate the tendency of earthworms and springtails to avoid contaminated soils proved quick and sensitive tools for soil quality assessment. Due to their relatively recent standardization (ISO 2008; ISO 2011), the application of such tests for the evaluation of remediation procedures is scarcer. At the same time, aquatic ecotoxicity tests traditionally used for the assessment of water contamination (Riva 1991; Riva et al. 1993; Riva and Lopez 2001; Riva et al. 2007) can be used as indicators of soil quality through their application on aqueous elutriates from polluted soils. However, those tests are considered less relevant from an ecological point of view (Van Gestel et al. 2001).

The aims of this study were: (i) to apply chemical analyses in combination with ecotoxicity tests for the evaluation of a hydrocarbon-contaminated soil prior and after applying a bioremediation procedure, and (ii) to compare the sensitivity of bioassays carried out directly on soils and on their water extracts in order to determine the most suitable battery of tests to evaluate the habitat and retention function of a hydrocarbon-contaminated soil and to monitor its remediation.

2. Materials and methods

2.1 Soil samples collection and analyses

The soil of study consisted in a heavily hydrocarbon-contaminated soil from an industrialized area in Getafe (South of Madrid, Center of Spain) with a long history of oil spills from storage and distribution tanks. Biotreatability assays were applied to the soil in order to ensure that its physicochemical properties enabled the biodegradation of hydrocarbons. Once confirmed, the test soil was homogenized, mechanically aerated and distributed into 3 biopiles (37 m length, 28 m width and 2 m high) that contained a total amount of 1800 m^3 of soil. Autochthonous populations of hydrocarbon degraders were stimulated through nutrient supply (mainly urea, $Ca(H_2PO_4)$, and K_2SO_4) prior to the beginning of the procedure and through constant aeration during remediation. Contents of CO_2, O_2 and VOCs were checked weekly whereas the concentration of hydrocarbons was assessed on a monthly basis. The remediation procedure lasted 120 days.

Test soils were collected prior (untreated sample; UTR) and after (treated sample; TR) applying the bioremediation process. Composite samples of test soils (4 samplings per composite sample) were collected, homogenized, sieved through a 2 mm mesh and kept refrigerated (4ºC) until requested. The following physicochemical parameters were evaluated (N=3): pH (KCl, 1 mol L^{-1}), Electrical Conductivity (EC) (1:5, soil:water suspension), Soil Organic Matter (SOM) (by loss on ignition at 550 ºC for 2 hours), texture (Pipette method) and Water Holding Capacity (WHC) (ISO 2011). TPHs (C10-C40) in soils were analyzed by Geotecnia 2000 (Madrid, Spain) in accordance with a method accredited by the Spanish National Accreditation Body (ENAC). Briefly, hydrocarbons were extracted with hexane, purified with Florisil (reagent grade, Sigma Aldrich) and quantified through Gas Chromatography using a Flame Ionization Detector (GC-FID). Cd, Cr, Cu, Hg, Ni, Pb and Zn contents were analyzed through Atomic Absorption Spectroscopy (AAS) by Analiza Calidad (Barcelona, Spain). Reference materials were used for quality control.

2.2 Water samples collection and analyses

Water extracts from test soils were obtained according to the British Standard EN 12457-2 (2002). Suspensions were prepared into 2-L glass vessels by mixing soils and deionized water at a ratio of 1:10 (w/v). Soil mixtures were thoroughly agitated on an orbital shaker Unimax 2010 (Heidolph, Germany) during 24 hours at a temperature of 20±2 ºC. After a settling period of 15 minutes, samples were centrifuged (2000 g, 10 minutes) and filtered through a 1 µm pore size membrane filter. Supernatants were kept refrigerated until use. Values of pH, Electrical Conductivity and Total Organic Carbon (TOC) were determined with a Microph 2001 ph-meter (Crison, Spain), a Ecoscan Con 5 conductivity meter (Eutech Instruments, UK) and a TOC-VCSH analyzer (SHIMADZU, Japan), respectively. A subsample

of each water extract was sent to Analiza Calidad (Barcelona, Spain) for the quantification of metals and total hydrocarbons through AAS and Fourier Transform Infrared Spectroscopy (FTIR), respectively.

2.3 Terrestrial ecotoxicity tests

Direct toxicity bioassays were performed using whole soils. When dilution was needed, test soils were mixed with an artificial soil (69% quartz sand, 20% caolinite clay, 10% finely ground sphagnum peat, 1% calcium carbonate and pH adjusted to 6.0 ± 0.5) (ISO 2011) that acted as control (0%). In order to obtain different percentages of effect that allowed the calculation of effective and lethal median concentrations (EC_{50} and LC_{50} respectively), test concentrations ranged from 0 to 100% of sampled soils mixed with artificial soil. All soil bioassays were carried out at 40-60% of their water holding capacity. EC_{50}s and LC_{50}s were expressed as the percentage of sampled soil mixed with artificial soil (w/w) that reduced by 50% the measured endpoint.

Earthworms from the species *Eisenia fetida* and springtails from the species *Folsomia candida* were obtained from synchronized cultures maintained at the Centre for Research and Innovation in Toxicology of the Technical University of Catalonia in Terrassa. Earthworms were cultured in 30-L breeding boxes and a 1:1 mixture of horse manure and peat. Only clitellate adults between 300 and 600 mg of body weight were selected for the performance of the tests. Earthworms were acclimated in control soil during 24 to 48 hours prior to the beginning of the tests. Springtails were cultured in vessels filled with a substrate of plaster of Paris and charcoal (8:1, w/w) at 20±2 ºC. Individuals were fed twice a week with granulated dry yeast added in small amounts to avoid spoilage by fungi. Organisms between 10 and 20 days old were used for toxicity testing.

2.3.1 E. fetida acute toxicity test

Acute toxicity tests with earthworms were adapted from the OECD 207 (1984) guideline. Ten organisms were placed in plastic containers (140x140x80 mm) containing 500 g dry weight (dw) of test soil. Test containers were kept under constant light (400-800 lux) at a temperature of 20±2 ºC. Survival was determined after 7 and 14 days of exposure. Each test ran with 5 concentrations (10-18-31-54-100% for UTR and 41-51-64-80-100% for TR) and 5 replicates per treatment. Artificial soil was used in control (0%) tests.

2.3.2 E. fetida reproduction test

Effects on the reproduction of earthworms were studied by means of the OECD 222 (2004) guideline. Ten earthworms were placed in 1-L plastic containers filled with 500 g of soil (dw). Test vessels were incubated in a controlled chamber at 20±2 ºC and a 16:8 h light:dark cycle. Animals were fed weekly with 2 g of moistened bread during 4 weeks. Surviving earthworms were sorted by hand after 28 days. Juvenile production was recorded after 56 days of exposure to test soils. Tests ran with 5 concentrations (0.25-0.5-1.0-2.0-4.0% for UTR and 3.13-6.25-12.5-25-50% for TR) and three replicates per treatment. Six replicates with artificial soil were used as control (0%).

2.3.3 Avoidance tests with E. fetida and F. candida

Avoidance tests with *E. fetida* and *F. candida* were adapted from ISO 17512 (2008) and ISO 17512 (2011) standards, respectively. Rectangular plastic containers (220x140x50 mm) were used in tests with earthworms while cylindrical vessels (diameter 8 cm; depth 8 cm) were selected for tests with springtails. Test containers were divided into two equal sections by a vertically introduced plastic card. Each section (control and test) of the test containers was filled with 250 g dw (test with earthworms) or 30 g (wet weight) (test with springtails) of the corresponding soil. Ten adult earthworms or twenty adult collembolans were carefully placed on the line separating both soils. After removing the divider, test containers were covered with a transparent plastic lid and incubated for 48 hours in an environmental chamber at 20±2 ºC and under a 16:8h light:dark photoperiod. At the end of the test period the plastic card was reinserted and the number of individuals at each section was counted. In tests with springtails, the soil from each section was carefully emptied into two different vessels and flooded with water. After gentle stirring, the animals floating on the water surface were counted. Avoidance tests ran with 5 concentrations plus a control (0%) and five replicates per treatment. Assays with earthworms were performed at 0.16-0.31-0.63-1.25-2.5% (UTR) and 1.25-2.5-5.0-10-20% (TR) of test soil mixed with artificial soil whereas the tested concentrations in assays with springtails were 2.5-5.0-10-20-40% (UTR) and 5.0-10-20-40-80% (TR). Results were expressed as the percentage of avoidance at the end of the test.

2.4 Aquatic ecotoxicity tests

Soil elutriates from test soils were tested through indirect toxicity bioassays. Test concentrations were prepared by mixing water samples with the corresponding test medium. Toxicity results were expressed as the percentage of water sample in the test medium (v/v) reducing by 50% the endpoint measured (EC_{50} or LC_{50}). Organisms from the species *R. subcapitata* and *D. magna* were cultured in the Centre for Research and Innovation in Toxicology.

2.4.1 Bacteria luminescence inhibition test

Acute toxicity to the bioluminescent bacteria *V. fischeri* was assessed in accordance with the ISO 11348 (2007) standard. Organisms were exposed to 4 concentrations of aqueous elutriates (5.63-11.25-22.5-45%) plus a control (0%) and the luminescence emitted was measured after 15 minutes with a Microtox® 500 system (Microbics©). Three replicates per treatment were analyzed.

2.4.2 Algal growth inhibition test

Effects on the growth of microalgae were assessed according to the OECD 201 (2011) guideline. Cultures of *R. subcapitata* were kept under constant illumination (4000-5000 lux) at a temperature of 20±2 ºC. Only populations in the exponential phase were used in tests. Assays were carried out in tubes containing 9 mL of test solution and 1 mL of algal inoculums that were placed in a controlled room at 20±2 ºC and under constant illumination (4000-5000 lux) and agitation. After 72 hours of incubation, the absorbance of each replicate was measured at 665 nm with a CECIL CE9200 spectrophotometer. Tests ran with 6 concentrations (10-17-29-49-84-90%) plus a control consisting in OECD TG 201 algae culture medium (OECD 2011). Three replicates were assessed per treatment. In order to avoid interferences in the spectrometric measure of the aqueous soil elutriates at the end of the test due to the presence of suspended particles, one extra tube containing 9 mL of extract, 1 mL of culture medium (OECD 2011) and no algae was measured. Results were expressed as percentage of algal growth inhibition.

2.4.3 Daphnia magna acute immobilization test

The acute toxicity test with *D. magna* was carried out according to the OECD 202 (2004) guideline. Bulk cultures of 15 daphnids were kept in 2.5 L of ASTM hard synthetic water (ASTM 1988). Culture medium was changed three times per week and supplemented by organic extract and a concentrate of *Chlorella vulgaris* as food. Cultures were maintained at 20±2 ºC in a 16:8h light:dark cycle. Neonates were removed daily but, only those less than 24 hours old and obtained from the third brood, were used for toxicity testing. Assays were performed in glass tubes containing 10 mL of test medium and 5 daphnids. Test vessels were kept in an incubator at 21±2 ºC and in the dark. Immobilization was visually recorded after 24 and 48 hours of exposure. Daphnids were exposed to 10 dilutions of water-extracts (0.1-0.22-0.48-1.0-2.2-4.8-10-22-48-100%) plus a control in four replicates per treatment. Mortality at the end of the test was expressed as a percentage.

2.5 Statistical analysis

Statistical analysis was performed using SPSS software (SPSS 15.0 for Windows; SPSS Inc., Chicago, IL, USA). Data were checked for their homogeneity of variances and normality. Differences between means were tested with one-way ANOVA. Whenever significant differences were found ($p< 0.05$), Tukey post-hoc test was applied to further elucidate differences. Non-normal data were transformed and, when the assumption of normality was not reached, non-parametric Kruskall-Wallis tests alongside with Mann-Whitney post hoc tests were performed. Data from avoidance tests were analyzed using the Fisher Exact test (Zar 1998), which compares the distribution of organisms between test sections with an expected distribution with no avoidance. A two-tailed test was used to check the homogeneous distribution of the organisms in dual-control tests whereas a one-tailed test was used in avoidance assays with the sampled soils. The percentage of avoidance was calculated in each replicate by the equation x = [(nc - nt) / N] x 100, where x = percent avoidance, nc = number of individuals in the control soil, nt = number of individuals in the test soil, and N = total number of individuals. Median effective, lethal and inhibitory concentrations (EC_{50}, LC_{50} and IC_{50} respectively) and their 95% confidence intervals were calculated by Probit regression. A normal or logistic distribution was assumed depending on results from normality tests. Estimated values were compared using the Confidence Interval Ratio Test recommended by Wheeler et al. (2006).

3. Results and discussion

3.1 Physicochemical analyses of soil samples

As expected, physicochemical properties were very similar between soils (Table 1). Both samples presented slightly acidic pH (6.54 in UTR and 6.79 in TR) and high salinity (1355 µS cm^{-1} in UTR and 1249 µS cm^{-1} in TR). Both soils presented low organic matter contents (1.99% in UTR and 1.08% in TR) and a silt loam texture. The water holding capacity was markedly higher in the TR soil (40.56%) than in the UTR one (13.13%). Both sites were heavily contaminated by petroleum hydrocarbons although their concentration before treatment (34264 mg kg^{-1} dw) was one order of magnitude higher than afterwards (3074 mg kg^{-1} dw). The application of the bioremediation procedure reduced by 91% the total concentration of hydrocarbons. Similar microbial degradation rates were reported in previous studies (Bossert and Bartha 1984; Morgan and Watkinson 1989; Atlas and Bartha 1992; Salanitro et al. 1997; Suguira et al. 1997). Aliphatic compounds predominated over aromatic ones and represented approximately 73% (UTR) and 89% (TR) of the quantified hydrocarbons. Between fractions, the average degradation of aliphatic compounds (87.52%) was slightly lower than that of aromatic ones (95.6%). At the same time, both fractions (aliphatic and aromatic) were almost exclusively composed by C16-C21 and C21-C35 compounds. The lower presence of lighter hydrocarbons was associated to volatilization. On the other hand, metal concentrations in both soils were very low and similar to the local geochemical background (BOCM 2006). Furthermore, metal contents were at least one order of magnitude lower than the intervention values for soil remediation established by Dutch regulations (VROM 2000) and were not considered to pose a risk to soil organisms.

Table 1. Physicochemical properties (mean±SD; N=3), contents of hydrocarbons and contents of metals in soils before and after treatment. EC: Electrical Conductivity; SOM: Soil Organic Matter; WHC: Water Holding Capacity.

	Untreated soil (UTR)	Treated soil (TR)
Physicochemical parameters		
pH	6.54±0.12	6.79±0.15
EC (µS cm^{-1})	1355±57	1249±39
SOM (%)	1.99±0.09	1.08±0.21
Texture	Silt Loam	Silt Loam
WHC (%)	13.13±0.18	40.56±2.14
Hydrocarbons (mg kg^{-1} dw)		
Total	34264	3074
Aliphatic fraction		
C10-C12	< 50	< 50
C12-C16	64	< 50
C16-C21	3100	450
C21-C35	22000	2300
Aromatic Fraction		
C10-C12	< 50	< 50
C12-C16	< 50	< 50
C16-C21	1000	54
C21-C35	8100	270
Heavy metals (mg kg^{-1} dw)		
Cd	0.03	0.09
Cr	0.84	0.21
Cu	8.01	9.39
Hg	<0.05	<0.05
Pb	17.56	12.85
Zn	5.88	9.63
Ni	0.43	0.30

3.2 Physicochemical analyses of water samples

The physicochemical characteristics of the water extracts are shown in Table 2. The sample from UTR was slightly acidic (pH of 6.45) whereas the extract from TR presented slightly alkaline pH (7.91). Both extracts presented high salinity (2263 µS cm^{-1} and 2410 µS cm^{-1}, respectively) and organic carbon content (18.37 mg L^{-1} to 21.97mg L^{-1}). The standard water extraction procedure gave low extraction yields (expressed as the ratio of pollutant concentration in water extract to concentration in soil: [µg/L]/[µg/kg]). Ratios of extraction were in the range of 10^{-8} and 10^{-9} for hydrocarbons and 10^{-2} and 10^{-3} for metals, which were in accordance with the hydrosolubility of each type of substances. Total contents of hydrocarbons reached 130 µg L^{-1} and 100 µg L^{-1} in UTR and TR, respectively and did not correlate with soil contents, where a difference of one order of magnitude was detected between samples. Such

difference was explained by the fact that UTR presented a markedly higher concentration of heavier petroleum hydrocarbons (C16-C21 and C21-C35), which are less soluble in water than lighter ones (Brassington et al. 2007). On the other hand, the concentration of light petroleum hydrocarbons was very similar between sites (Table 1). Among the analyzed metals, only Cd, Cr and Cu could be quantified in the extracts and their concentrations (from 1.27 to 18.76 µg L^{-1}) were considered too low to represent a threat to aquatic organisms (USEPA 2016).

Table 2. Physicochemical properties (mean±SD; N=3), contents of hydrocarbons and contents of metals in water extracts from test soils. EC: Electrical Conductivity; TOC: Total Organic Carbon.

	Untreated soil (UTR)	Treated soil (TR)
pH	6.45±0.08	7.91±0.11
EC (µS cm^{-1})	2263±91	2410±65
TOC (mg L^{-1})	18.37±0.03	21.97±0.02
Hydrocarbons (µg L^{-1})	130	100
Cd (µg L^{-1})	1.27	<0.5
Cr (µg L^{-1})	4.46	4.20
Cu (µg L^{-1})	18.76	16.26
Hg (µg L^{-1})	<1	<1
Pb (µg L^{-1})	<1	<1
Zn (µg L^{-1})	<100	<100
Ni (µg L^{-1})	<2.5	<2.5

3.3 Toxicity to terrestrial organisms

Test soils proved moderately to extremely toxic to soil invertebrates (Table 3). Even so, all terrestrial bioassays detected higher toxicity in UTR than in TR, thus confirming that toxicity to terrestrial organisms was related with hydrocarbons content.

Table 3. LC_{50} and EC_{50} values (95% confidence limits) of terrestrial tests in percentage (%) of test soil in test substrate (w/w). *Significantly different from the untreated soil ($p<0.05$; Confidence Interval Ratio Test).

	Eisenia fetida			*Folsomia candida*
	Survival (LC_{50})	Reproduction (EC_{50})	Avoidance (EC_{50})	Avoidance (EC_{50})
Untreated soil (UTR)	56.16% (29.62-73.42)	0.83% (0.69-0.99)	1.25% (0.85-1.83)	10.33% (7.05-15.11)
Treated soil (TR)	71.07% (51.25-85.78)	2.45%* (1.36-3.27)	6.53%* (4.85-8.79)	51.74%* (33.21-80.61)

In acute tests, *Eisenia* survival rate decreased throughout time. LC_{50}s of 81.90% and 56.16% were estimated after 7 days and 14 days of exposure to UTR. Similar values (83.13% and 71.07% respectively) were estimated for TR. Earthworms body mass decreased in controls, which was associated with the lack of food supply during tests. Likewise, body mass loss increased with increasing test concentrations and reached 100% (i.e. 100% mortality) at the highest test concentrations. Despite being recommended for the assessment of hydrocarbon-contaminated soils (Saterbak et al. 1999; Son et al. 2003; Van Gestel and Weeks 2004; Eom et al. 2007; Lors et al. 2009), mortality of earthworms was the least sensitive terrestrial endpoint and the only direct test that was not able to distinguish between soils according to their toxiciy.

Earthworms' survival in reproduction tests was only affected in the exposure to the highest concentration (4%) of UTR soil (10% mortality). However, a marked decrease in body weight was appreciated (Figure 1A). After 28 days of exposure, the lowest concentration of UTR soil (0.25%) caused 18.5% decrease in earthworms' weight, which further decreased to 36.70% in the highest test concentration (4%) (Figure 1A). In the TR soil, the slight increase in biomass (14.6% to 17.6%) observed at low test concentrations (≤12.5%) was followed by an abrupt decrease at higher ones (≥25%; Figure 1B). After 56 days of exposure, both soils caused a significant decrease in the average number of juveniles per treatment. When compared with controls, the inhibition of juvenile production was statistically significant in concentrations higher than 1% of UTR soil (Figure 1A) and in all tested concentrations with TR soil (Figure 1B). EC_{50}s for the inhibition of juvenile production in UTR soil and TR soil were estimated at 0.83% and 2.45% of test soil in test substrate, respectively and confirmed the higher sensitivity of sub-lethal endpoints suggested by other authors (Hund-Rinke et al. 2002; Davies et al. 2003).

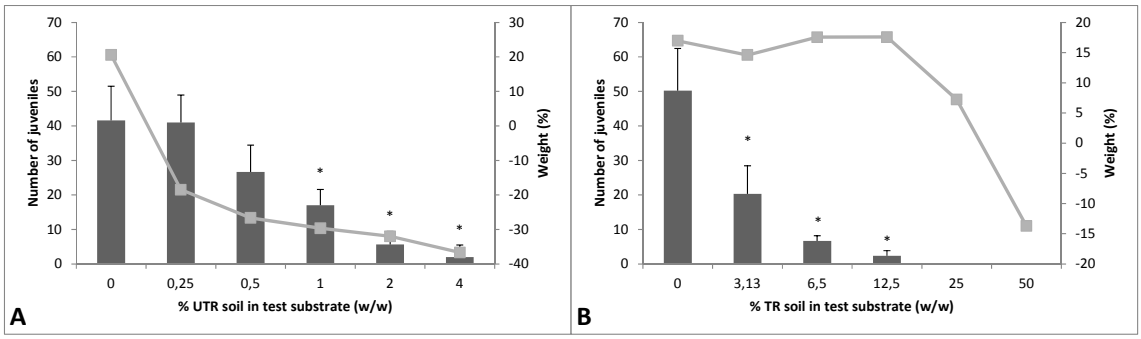

Figure 1. Number of juveniles (bar; left *Y*-axis) and weight variation (curve; right *Y*-axis) of *E.fetida* exposed to the UTR (A) and TR (B) soils in reproduction tests. Mean values ± standard deviations of 3 replicates. '*': Significantly different from control ($p<$ 0.05; Mann Whitney *U* Test).

Dual-control avoidance tests with *E. fetida* and *F. candida* showed an equal distribution of individuals between sections of the test containers. Mortality was not detected in tests with earthworms and a clear preference for the artificial soil was observed. Statistically significant avoidance responses (Fisher Exact test; $p<0.05$) were detected at concentrations of UTR soil higher than 0.31%, with avoidance responses ranging from 32% to 80% (Figure 2A). Statistically significant avoidance responses were also observed in exposures to concentrations of 5% to 20% of TR soil (Figure 2B; 40% to 80% of avoidance). EC_{50} for UTR soil was estimated at 1.25% whereas that for the TR soil was slightly higher (6.53% i.e. less toxicity detected). Despite the reduction of soil toxicity due to the remediation treatment, both tested soils were considered to present a limited habitat function because avoidance responses reached values higher than 60% (Hund-Rinke and Wiechering 2001).

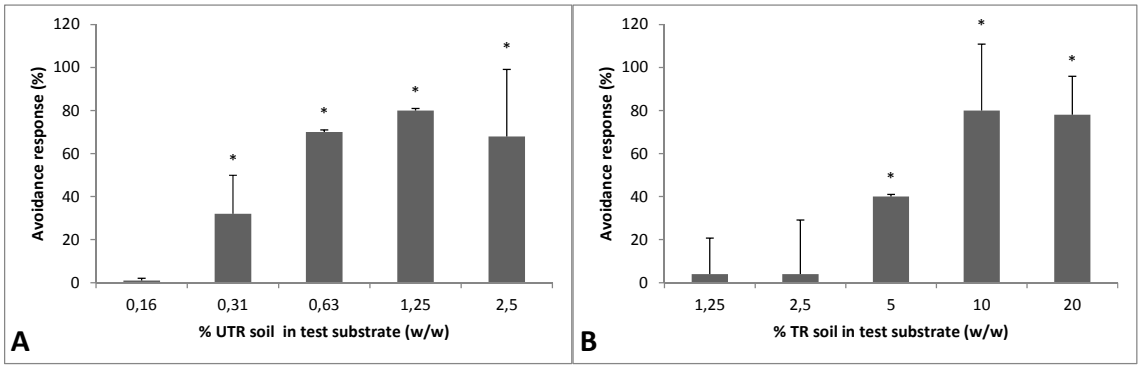

Figure 2. Percentage of soil avoidance by earthworms *E. fetida* exposed to the UTR (A) and TR (B) soils. Mean values ± standard deviations of 5 replicates per treatment. '*': Statistically significant avoidance response ($p<$ 0.05; Fisher Exact Test).

The number of dead or missing springtails in avoidance tests never reached values higher than 20% per treatment, thus accomplishing with the requirements of the ISO standard. The results were in agreement with those from tests with *E fetida* although, significant responses were detected at higher hydrocarbon concentrations (Figure 3). Statistically significant avoidance responses (Fisher Exact test; $p<0.05$) of *F. candida* were detected at concentrations higher than 5% of UTR soil (Figure 3A) and 10% of TR soil (Figure 3B). EC_{50}s were estimated at 10.33% for UTR soil and 51.74% for TR soil. Despite the high sensitivity of *F. candida* to hydrocarbon-contaminated soils (Bori and Riva 2015), our results were in accordance with those from Natal-Da-Luz et al. (2008) and Hentati et al. (2013) and confirmed the higher sensitivity of *E. fetida* to contamination by hydrocarbons.

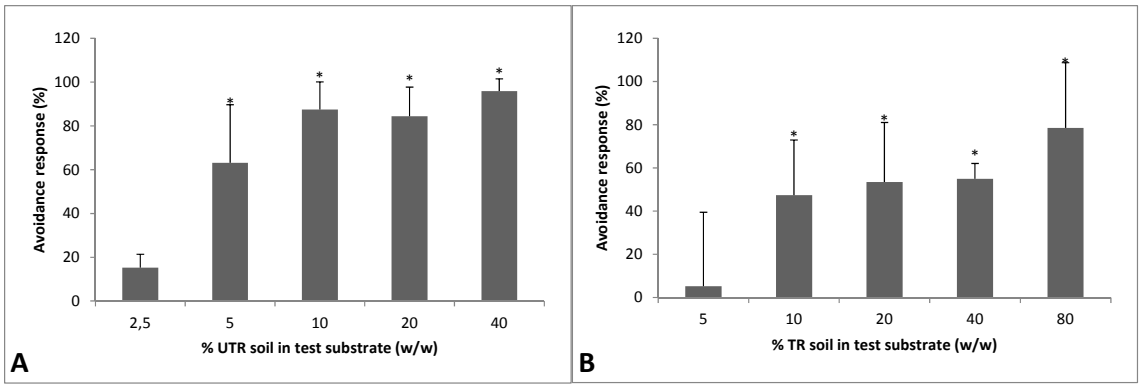

Figure 3. Percentage of soil avoidance by springtails *F. candida* exposed to the UTR (A) and TR (B) soils. Mean values ± standard deviation of 5 replicates per treatment. '*': Statistically significant avoidance response ($p < 0.05$; Fisher Exact Test).

In this study, several soil bioassays recommended for the assessment of contaminated soils (Cortet et al. 1999) were successfully performed. The sensitivity ranking according to the detected toxicity was as follows (in decreasing order): Earthworm reproduction > Earthworm avoidance > Collembola avoidance > Earthworm survival. In an attempt to evaluate the suitability of soil ecotoxicity tests with invertebrates as complementary tools for the monitoring of soil remediation procedures, the percentage decrease in the concentration of hydrocarbons throughout the treatment was calculated and compared with the percentage decrease in toxicity (i.e. the increase in LC_{50} or EC_{50} values). Calculations were performed as follows: % decrease = 100 - [(X_A / X_B) x 100], where X_A = value after remediation, and X_B = value before remediation. Following this equation, none of the terrestrial ecotoxicity tests reported a toxicity decrease as high as the percentage reduction in the contents of hydrocarbons although avoidance tests were close (80.86% and 80.06% for earthworms and springtails respectively). Earthworms' survival was the least sensitive to changes in hydrocarbon contents (21% toxicity decrease) whereas earthworms' reproduction reported 66.08% decrease of toxicity despite presenting the highest sensitivity to hydrocarbons. Discrepancies between contamination and toxicity reduction after remediation of hydrocarbon-contaminated soils were previously reported by Hubálek et al. (2007) and Al-Mutairi et al. (2008). Such discrepancies were attributed to the presence of intermediate metabolites and to their synergic or antagonistic behavior, which are difficult to detect through chemical methodologies.

3.4 Toxicity to aquatic organisms

Results of bioassays carried out with elutriates from test soils are summarized in Table 4. All aquatic bioassays estimated significantly lower EC_{50}s (i.e. higher toxicity detected) for the elutriate from the UTR soil. Even more, the elutriate obtained after remediation (TR soil) proved innocuous to most test organisms.

Table 4. LC_{50} and EC_{50} (95% confidence limits) of aquatic bioassays performed with water extracts from test soils. '*'Significantly different from the untreated soil ($p<0.05$; Confidence Interval Ratio Test).

	V. fischeri Luminescence Inhibition (IC_{50})	*R. subcapitata* Growth Inhibition (IC_{50})	*D. magna* Acute Immobilization (LC_{50})
UTR soil	47.84% (39.51-56.18)	49% (44-56)	2.30% (1.0-4.7)
TR soil	>100%*	>100%*	91%* (70-139)

The elutriate from the untreated soil was moderately toxic to aquatic microorganisms *V. fischeri* and *R. subcapitata*. The concentration of water extract reducing bacterial luminescence by 50% after 15 min was 47.84%, and that decreasing algal growth after 72 hours was 49%. *D. magna* survival was more severely affected by water-extracted pollutants and 50% of decrease in viability was estimated at a concentration of 11.9% after 24 hours and of 2.3% after 48 hours. The water extract from the treated soil was toxic to *D. magna* after 48 hours of exposure (EC_{50} at 91%) but not after 24 hours (EC_{50}>100%).

Despite all the studied endpoints were focused on acute responses, aquatic bioassays showed marked differences in sensitivity to the aqueous extracts (in decreasing order): *D. magna* immobilization > *R.*

subcapitata growth inhibition ≈ *V. fischeri* luminescence inhibition. These results were not in agreement with previous studies that reported the markedly higher sensitivity of *R. subcapitata* and *V. fischeri* in comparison with *D magna* towards elutriates from hydrocarbon-contaminated soils (Rojíčková-Padrtová et al. 1998; Bispo et al. 1999; Mendonça and Picado 2002; Eom et al. 2007). The higher toxicity to *D. magna* was associated with the salinity of the elutriates, which was already reported by Thavamani et al. (2015) after assessing the toxicity of a leachate from a hydrocarbon-contaminated soil to *Daphnia carinata*. Aquatic bioassays were successfully applied to aqueous elutriates from test soils and were able to detect a decrease in toxicity. However, their performance was markedly influenced by the physicochemical parameters of the soil elutriates and by the limited hydrosolubility of hydrocarbons. Consequently, they were considered less relevant than direct tests for the assessment of hydrocarbon-contaminated soils (Van Gestel et al. 2001).

4. Conclusions

The bioremediation procedure applied to a heavily hydrocarbon-contaminated soil led to a significant reduction in the content of hydrocarbons as well as in toxicity. Even so, the treated soil still presented toxic contents of hydrocarbons. Our study confirmed the higher sensitivity of sublethal endpoints in comparison with lethal ones. Reproduction tests with earthworms showed highest sensitivity to hydrocarbon-contaminated soils and were followed by avoidance tests with earthworms and springtails. Due to their short duration and high sensitivity, avoidance tests represent a promising tool for routine assessment of hydrocarbon-contaminated soils. Despite their sensitivity, none of the bioassays showed a reduction in toxicity as remarkable as the reduction in the contents of hydrocarbons, thus demonstrating the need to complement chemical analysis with ecotoxicological tools in the evaluation of contaminated soils.

The concentration of hydrocarbons in water extracts and their toxicity to aquatic organisms also decreased after bioremediation. Most aquatic bioassays detected a significant reduction in toxicity, which was almost negligible at the end of the treatment. However, the low hydrosolubility of hydrocarbons and the influence of water physicochemical parameters to some aquatic test organisms limited the performance of aquatic bioassays for the assessment of hydrocarbon-contaminated soils and their remediation.

Acknowledgments

Authors want to thank Geotecnia 2000 for supplying the soils. This research was funded by Universitat Politècnica de Catalunya (UPC) and R&D Gestió i Serveis Ambientals S.L. (Spain) through a doctoral grant to Jaume Bori (Beca UPC Recerca 2012-2016) and by the Spanish Ministry of Economy and Competitiveness through the project CTM2010-18167.

Compliance with ethical standards

The authors declare that they have no conflict of interest.

5. References

Al-Mutairi, N., Bufarsan, A., Al-Rukaibi, F. (2008). Ecorisk evaluation and treatability potential of soils contaminated with petroleum hydrocarbon-based fuels. Chemosphere, 74, 142-148.

ASTM (1988). Standard Guide for Conducting Acute Toxicity Tests with Fishes, Macroinvertebrates, and Amphibians. E729-88a. American Society for Testing and Materials, Philadelphia, PA. 20 pp.

Atlas, R.M. & Bartha, R. (1992). Hydrocarbon biodegradation and oil spill bioremediation, Advances in Microbial Ecology, 12, 287–338.

Békaert, C., Rast, C., Ferrier, V., Bispo, A., Jourdain, M.J., Vasseur, P. (1999). Use of in vitro (Ames and Mutatox tests) and in vivo (Amphibian Micronucleus test) assays to assess the genotoxicity of leachates from a contaminated soil. Organic Geochemistry, 30, 953–962.

Bispo, A., Jourdain, M.J., Jauzein, M. (1999). Toxicity and genotoxicity of industrial soils polluted by polycyclic aromatic hydrocarbons (PAHs). Organic Geochemistry, 30, 947–952.

Bossert, I. & Bartha, R. (1984). The Fate of Petroleum in Soil Ecosystem. In R. M. Atlas (ed.), Petroleum Microbiology, Macmillan Co., New York, 435–476.

Brassington, K.J., Hough, R.L., Paton, G.I., Semple, K.T., Risdon, G.C., Crossley, J., et al. (2007). Weathered hydrocarbon wastes: a risk management primer. Critical Reviews in Environmental Science and Technology 37: 199-232.

British Standard EN 12457-2 (2002). Characterization of waste. Leaching. Compliance test for leaching of granular waste materials and sludges. One stage batch test at a liquid to solid ratio of 10 l/kg for materials with particle size below 4 mm (without or with size reduction). British Standards Institutions.

Bori, J., & Riva, M.C. (2015). An Alternative Approach to Assess the Habitat Selection of Folsomia candida in Contaminated Soils. Bulletin of Environmental Contamination and Toxicology, 95(5), 670-674.

Bori, J., Ribalta, C., Domene, X., Riva, M.C., Ribó, J.M. (2015). Environmental effects of an imidacloprid-containing formulation: from soils to waters. Afinidad, 571(72), 169-176.
Bori, J., Vallès, B., Navarro, A., Riva, M.C. (2016). Geochemistry and environmental threats of soils surrounding an abandoned mercury mine. Environmental Science and Pollution Research International. In revision.
Brown, D.G., Knightes, C.D., Peters, C.A. (1999). Risk assessment for polycyclic aromatic hydrocarbon NAPLs using component fractions. Environmental Science and Technology, 33, 4357–4363.
Chaineau, C.H., Yepremian, C., Vidalie, J. F., Ducreux, J., Ballerini, D. (2003). Bioremediation of a crude oil-polluted soil: biodegradation, Leaching and Toxicity Assessments. Water, Air, and Soil Pollution, 144, 419–440.
Cortet, J., Gomot-De Vaufleury, A., Poinsot-Balaguer, N., Gomot, L., Texier, C., Cluzeau, D. (1999). The use of invertebrate soil fauna in monitoring pollutant effects. European Journal of Soil Biology, 35(3), 115–134.
Cvancarova, M., Kresinova, Z., Cajthaml, T., 2013. Influence of the bioaccessible fraction of polycyclic aromatic hydrocarbons on the ecotoxicity of historically contaminated soils. Journal of Hazardous Materials 254–255, 116–124.
Davies, N.A., Hodson, M.E., Black, S. (2003). Is the OECD acute worm toxicity test environmentally relevant? The effect of mineral form on calculated lead toxicity. Environmental Pollution, 121, 49–54.
Deutsche Gesellschäft für Chemisches Apparatewesen, Chemische Technik und Biotechnologie (1995). Biologische Testmethoden für Böden. Adhoc-Arbeitsgruppe Methoden zur Toxikologischen/Ökotoxikologischen Bewertung von Böden, DECHEMA, Frankfurt am Main, Germany.
Eom, I.C., Rast, C., Veber, A.M., Vasseur, P. (2007). Ecotoxicity of a polycyclic aromatic hydrocarbon (PAH)-contaminated soil. Ecotoxicology and Environmental Safety, 67, 190-205.
Fernandez, M.D., Cagigal, E., Vega, M.M., Urzelai, A., Babin, M., Pro, J., Tarazona, J.V. (2005). Ecological risk assessment of contaminated soils through direct toxicity assessment. Ecotoxicology and Environmental Safety, 62, 174–184.
Haeseler, F., Blanchet, D., Werner, P., Vandecasteele, J.P. (2001). Ecotoxicological characterization of metabolites produced during PAH biodegradation in contaminated soils, In: Bioremediation of Energetics, Phenolics, and Polycyclic Aromatic Hydrocarbons, 6(3), 227-234, Magar, V.S., Johnson, G., Ong, S.K., Leeson A. (Eds), Batelle press, San Diego, USA, 313 pp.
Hentati, O., Lachhab, R., Ayadi, M., Ksibi, M. (2013). Toxicity assessment for petroleum-contaminated soil using terrestrial invertebrate and plant bioassays. Environmental Monitoring and Assessment, 185, 2989-2998.
Hubálek, T., Vosáhlová, S., Matějů, V., Kováčová, N., Novotný, C. (2007). Ecotoxicity Monitoring of Hydrocarbon-Contaminated Soil During Bioremediation: A Case Study. Archives of Environmental Contamination and Toxicology, 52, 1-7.
Hund-Rinke, K. &Wiechering, H. (2001). Earthworm avoidance test for soil assessment: An alternative for acute and reproduction tests. Journal of Soils and Sediments,1, 15-20.
Hund-Rinke, K.., Koerdel, W., Hennecke, D., Achazi, R., Warnecke, D., Wilke, B.M., Winkel, B., Heiden, S. (2002). Bioassays for the ecotoxicological and genotoxicological assessment of contaminated soils (results of a round-robin test): part II—assessment of the habitat function of soils-tests with soil microflora and fauna. Journal of Soils and Sediments, 2 (2), 83–90.
ISO 11348-3 (2007). Water quality: Determination of the inhibitory effect of water samples on the light emission of Vibrio fischeri (Luminescent bacteria test) - Part 3: Method using freeze-dried bacteria. International Organization for Standardization, Geneva, Switzerland.
ISO 17512-1 (2008). Soil quality: Avoidance test for determining the quality of soils and effects of chemicals on behaviour - Part 1: Test with earthworms (Eisenia fetida and Eisenia andrei). International Organization for Standardization, Geneva, Switzerland.
ISO 17512-2 (2011). Soil quality: Avoidance test for determining the quality of soils and effects of chemicals on behaviour - Part 2: Test with collembolans (Folsomia candida). International Organization for Standardization, Geneva, Switzerland.
Juvonen, R., Martikainen, E., Schultz, E., Joutti, A., Ahtiainen, J., Lehtokari, M. (2000). A battery of toxicity tests as indicators of decontamination in composting oily waste. Ecotoxicology and Environmental Safety, 47, 156–166.
Keddy, C.J., Greene, J.C., Bonnell, M.A. (1995). Review of whole-organism bioassays: soil, freshwater sediment, and freshwater assessment in Canada. Ecotoxicology and Environmental Safety, 30, 221–251.
Loibner, A., Szolar, O., Braun, R., Hirmann, D. (2003). Ecological assessment and toxicity screening in contaminated land analysis. In: Thompson, K.C., Nathanail, C.P. (Eds.), Chemical Analysis of Contaminated Land. Blackwell, Oxford, UK, 29–267.
Lors, C., Perie, F., Grand, C., Damidot, D. (2009). Benefits of ecotoxicological bioassays in the evaluation of a field biotreatment of PAHs polluted soil. Global NEST Journal, 11 (3), 251-259.
McElroy, A. E., Farrington, J. W., & Teal, J. M. (1989). Bioavailability of polycyclic aromatic hydrocarbons in the aquatic environment. In U. Varanasi (Ed.), Metabolism of polycyclic aromatic hydrocarbons in the aquatic environment (pp. 1–39). Boca Raton: CRC Press.
Megharaj, M., Ramakrishnan, B., Venkateswarlu, K., Sethunathan, N., Naidu, R., 2011. Bioremediation approaches for organic pollutants: A critical perspective. Environment International, 37, 1362–1375.
Mendonça, E. & Picado, A. (2002). Ecotoxicological Monitoring of Remediation in a Coke Oven Soil. Environmental Toxicology, 17(1), 74-79.
Morgan, P. & Watkinson, R.J. (1989). Hydrocarbon degradation in soils and methods for soil biotreatment. Critical Reviews in Biotechnology, 4, 305–333.
Natal-Da-Luz, T., Römbke, J., Sousa, J.P. (2008). Avoidance tests in site-specific risk assessment - Influence of soil properties on the avoidance response of Collembola and earthworms. Environmetal Toxicology and Chemistry, 27 (5), 1112-1117.
OECD 201 (2011). Freshwater Alga and Cyanobacteria, Growth Inhibition Test. *Guideline for testing of chemicals*. Organization for Economic Cooperation and Development.
OECD 202 (2004). Daphnia sp. Acute Immobilization Test. *Guideline for testing of chemicals*. Organization for Economic Cooperation and Development.
OECD 203 (1992). Fish, Acute Toxicity Test. *Guideline for testing of chemicals*. Organization for Economic Cooperation and Development.
OECD 207 (1984). Earthworms acute toxicity tests. *Guideline for testing of chemicals*. Organization for Economic Cooperation and Development.
OECD 222 (2004). Earthworm Reproduction Test (Eisenia fetida/Eisenia andrei)**.** *Guideline for testing of chemicals*. Organization for Economic Cooperation and Development.
Official Bulletin of the Community of Madrid (BOCM) (2006). Order 2770/2006 , of August 11th, laying proceeds to establishing generic reference levels of heavy metals and other trace elements in contaminated soils from Madrid.

Rila, J.P., Eisentraeger, A. (2003). Application of bioassays for risk characterization and remediation control of soils polluted with nitroaromatics and PAHs. Water, Air and Soil Pollution, 148, 223–242.
Riva, M.C. (1991). Nociones y planteamientos en la preservación del medio ambiente acuático. Boletin INTEXTER (UPC), 99, 63-83.
Riva, M.C., Cegarra, J., Crespi, M. (1993). Effluent ecotoxicology in the wool-scouring process. The Science of the Total Environment, 134 (2), 1143-1150.
Riva, M.C. and Lopez, D. (2001). Impacto ambiental de los efluentes del proceso de blanqueo de algodón: parámetros químicos y biológicos. Boletin INTEXTER (UPC) 119, 51-57.
Riva, M.C., Ribó, J., Gibert, C., Alañón, P. (2007). Acute toxicity of leather processing effluents on Vibrio fisheri and Brachydanio rerio. Afinidad, 528, 182-188.
Rojíčková-Padrtová, R., Maršálek, B., Holoubek, I. (1998). Evaluation of alternative and standard toxicity assays for screening of environmental samples: Selection of an optimal test battery. Chemosphere, 37(3), 495-507.
Salanitro, J.P., Dorn, P.B., Hueseman, H., Moore, K. O., Rhodes, I. A., Rice Jackson, L. M., Vipond, T. E., Western, M., Misniewski, H.L. (1997). Crude oil hydrocarbon bioremediation and soil ecotoxicity assessment. Environmental Science and Technology, 31, 1769–1776.
Saterbak, A., Toy, R.J., Wong, D.C.L., Mcmain, B.J., Williams, M.P., Dorn, P.B., Brzuzy, L.P., Chai, E.Y., Salanitro, P.S. (1999). Ecotoxicological and analytical assessment of hydrocarbon-contaminated soils and application to ecological risk assessment. Environmental Toxicology and Chemistry, 18 (7), 1591–1607.
Son, A.J., Shin, K.H., Lee, J.U., Kim, K.W. (2003). Chemical and ecotoxicity assessment of PAH-contaminated soils remediated by enhanced soil flushing. Environmental Engineering Science, 20 (3).
Stroo, H.F., Jensen, R., Loehr, R.C., Nakles, D.V., Fairbrother, A., Liban, C.B., 2000. Environmentally acceptable endpoints for PAHs at a manufactured gas plant site. Environ Sci Technol 34, 3831–3836.
Suguira, K., Ishihara, M., Shimauchi, T., Harayama, S. (1997). Physicochemical properties and biodegradability of crude oil. Environmental Science and Technology, 31, 45–51.
Thavamani, P., Smith, E., Kavitha, R., Mathieson, G., Megharaj, M., Srivastava, P., Naidu, R. (2015). Risk based land management requires focus beyond the target contaminants—A case study involving weathered hydrocarbon contaminated soils. Environmental Toxicology and Innovation 4: 98-109.
US Environmental Protection Agency (EPA)(2016). Water Quality Criteria. http://www.epa.gov/wqc/national-recommended-water-quality-criteria-aquatic-life-criteria-table (accessed May 2016).
Van Gestel, C.A.M., Van der Waarde, J.J., Derksen, J.G.M.A., Van der Hoek, E.E., Veul, M.F.X.W., Bouwens, S., Rusch, B., Kronenbur, R., Stokman, G.N.M. (2001). The use of acute and chronic bioassays to determine the ecological risk and bioremediation efficiency of oil-polluted soils. Environmental Toxicology and Chemistry, 20(7), 1438-1449.
Van Gestel, C.A.M., & Weeks, J.M. (2004). Recommendation of the 3rd international workshop on earthworm ecotoxicology, Aarhus, Denmark, august 2001. Ecotoxicological Environment and Safety, 57, 100–105.
Ministry of Housing, Spatial Planning and Environment (VROM). Circular on target values and intervention values for soil remediation. The Hague, Netherlands, 2000.
Walker, C.H., Hopkin, S.P., Sibly, R.M., Peakall, D.R. (2006). Principles of Ecotoxicology, third ed. Taylor and Francis, CRC Press, Boca Raton.
Wheeler, M.W., Park, R.M., Bailer, A.J. (2006). Comparing median lethal concentration values using confidence interval overlap or ratio tests. Environmental Toxicology and Chemistry, 25(5), 1441-1444.
White P.A. and Claxton L.D. (2004). Mutagens in contaminated soil: a review. Mutation Research, 567, 227-345.
Zar, J.H. (1998). Biostatistical analysis, 5th Edition. Prentice-Hall, Upper Saddle River, NJ, USA, pp 561-569.