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Review article

Soil contact bioassay for rapid determination of acute toxicity with *Eisenia foetida*

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ABSTRACT

A rapid bioassay is presented for determining acute toxicity directly in soil. Modifying the Organisation for Economic Cooperation and Development (OECD) protocol 207, it uses a thin layer of moistened soil laid directly in the bottom of the bioassay jar into which the earthworms are placed and incubated. Examples are presented in comparisons between the soil contact bioassay vs. the filter paper bioassay run on Toxicity Characteristic Leaching Procedure (TCLP) extracts of pesticide contaminated soil and petroleum drilling cuttings. In 2,4-dichlorophenoxy-acetic acid (2,4-D) contaminated soil (300mg/Kg), no mortality was found in soil extracts, but 100% mortality was found when exposed directly to soil. Treatment with the Daremend® product in five anaerobic/aerobic cycles slowly reduced the 24 h mortality (0%) but still showed 100% mortality at 48 h. However, severe sub-lethal effects (expulsion of celomic/bloody fluids) were reduced from 50% to 37%, and further treatment may reduce the toxicity to acceptable levels. The petroleum drilling cuttings treated by chemical oxidation (1.3% H_2O_2, w/w) and bioremediation (simulation of biopiles), showed a similar response, where 0% mortality in soil extracts was found, but 100% mortality with soil contact. Post-treatment with chemical oxidation resulted in a reduction in the soil contact bioassay to 3% and 13% mortality, within the accepted range ($\leq$10%) of the OECD protocol. Observations are presented with respect to moisture control to prevent earthworm desiccation and recommendation for confirmation using the sub-chronic test in the OECD protocol but by testing the contaminated/treated soil itself rather than artificial soil.

1. Introduction

The characterization of contaminated sites and evaluation of remediation techniques usually involves the determination of contaminant concentration in the soil or other contaminated media (sediment, semi-solid waste, etc.). However, for several decades it has become desirable, and in some cases truly necessary, to include bioassays for such characterization also. Basically, this is due to two factors. First, in the biodegradation or chemical transformation of soil contaminants, the contaminating compounds often are not mineralized completely, but other intermediates are produced. Usually, not all of the metabolites are known, and their toxicity is unknown. Second, many weathering processes, as well as bioremediation/chemical treatments, may not greatly reduce the concentration of the parent compound, but may reduce the bioavailability to sufficiently low levels to be only of minimal risk to public health and the environment. Thus, to characterize a site or treated material adequately, bioassays are needed in addition to chemical determinations.

In the following article, we present a novel bioassay for determining acute toxicity with direct contact of the test organism in the soil, based on a modification of the OECD Protocol No. 207. For this purpose, we offer a literature survey of pertinent studies, and of site-specific characterization and remediation. This section develops the theme of the consciousness of this need, based on previous projects in the literature, benefits and limitations of common bioassays, and the specific need for an acute direct-contact test. Subsequently, we present examples of the application of this novel method in three experimental scenarios. In these sections, the experimental procedures are outlined, but special emphasis is put on the results of the direct contact bioassay in comparison to the current filter paper bioassay. The intention is to focus on those aspects of the research related to toxicity in the water soluble fraction vs. the soil itself. The benefits and complementary of the filter paper test and direct soil contact

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test for acute toxicity evaluation are discussed also. In the last of these experimental scenarios, we showed how the moisture content in the soil can be critical to avoid earthworm desiccation and to obtain precise measurements, and we offer suggestions as to how this may be controlled. Finally, a brief conclusion of the complementary use and benefits of this novel method is presented in the context of the current OECD protocol and common modifications.

2. Main text

2.1. Literature survey: use of bioassays for evaluating toxicity in contaminated and remediated soil and soil-like materials

It has been shown that the toxicity of contaminants in soil may vary due to a variety of factors in addition to contaminant concentration. With respect to petroleum hydrocarbons, weathering and bioavailability, as well as soil concentration, are important to toxicity (Matejczyk et al., 2011; Khan et al., 2013). The weathering of petroleum hydrocarbons, either naturally, or in remediation processes, greatly reduces the toxicity and leachability of contaminants in soil and soil-like wastes, often through sequestration into soil organic matter or clayey constituents of soil (Overton et al., 1997; Steliga et al., 2012). For example, in one study, the remediation of petroleum drilling cuttings resulted in a reduction in hydrocarbon concentration of only about 10% but the overall toxicity was reduced more than five times (Adams Schroeder et al., 1999). Likewise, at a field scale demonstration of a chemical stabilization/bioremediation process for treating oily sediments, the toxicity was reduced to background level even though the total hydrocarbon concentration was only reduced 32% and was still above 30,000 mg/kg (Adams et al., 2013). At this site, the results of these tests convinced the national environmental authority (Secretaría de Medio Ambiente y Recursos Naturales – SEMARNAT; Ministry of Environment and Natural Resources) to use the *Vibrio fischeri* bioassay (SECOFI, 1996) as the only remediation criteria – the overall hydrocarbon concentration was not considered relevant (SEMARNAT, 2007). Other authors have also proposed toxicity as the main criterion for soil remediation (Bosma et al., 1997; Alexander, 1999).

In other cases, even though bioremediation of organic compounds tends to reduce toxicity, an incomplete remediation may actually produce conditions that are more toxic. This was observed in the partial bioremediation of crude oil contaminated peaty soil from a wetland. While the hydrocarbon concentration was reduced 60%, the toxicity increased three times above background levels (Adams et al., 2011). It was speculated that bacteria did not only degrade the oil but also part of the peaty soil, thereby liberating oil that was previously absorbed in the peat, and making it more bioavailable and toxic. Also, some intermediates of hydrocarbon biodegradation, such as epoxides, diols, aldehydes etc. are more toxic than the non-oxidized starting contaminants (Manahan, 1992; Brock et al., 1994). Pradhan et al. (1997) also observed more toxic conditions in the partial treatment (by chemical oxidation/bioremediation) of soil contaminated with tarry manufactured-gas plant waste. Thus, in situations such as these, it is very important not to evaluate the success of remediation based on contaminant concentration only, but also toxicity. With respect to more potentially toxic compounds, for example in the degradation of explosives, some pesticides and chlorinated organics in general, the possibility of producing more toxic metabolites or only achieving partial detoxification, further emphasizes the need for evaluation of toxicity of treated soil and soil-like wastes (Rochkind et al., 1986).

An example of this was the demand made by EPA on a composting technology developed by the US Army to treat explosives-contaminated soil (Kaplan and Kaplan, 1982). It was not considered sufficient to show just that the parent compound was degraded, but also that toxicity was reduced to acceptable levels. This was because it was suspected that some of the biodegradation intermediates could be more toxic than the original compound and that the treatment resulted largely in the chemical sequestration of the contaminant or degradation products (Isbister et al., 1984; Williams et al., 1992; Pennington et al., 1995; Kalderis et al., 2011). Synthetic Precipitation Leaching Procedure (SPLP) extracts were tested on *Cerodaphnia dubia*, flathead minnow larvae (*Pimephales promelas*) and by the Ames test. Survival and fecundity was in same range as the control (artificial rain) in the *Cerodaphnia* and flathead minnow test. In the Ames test, *Samonella* strains TA98 and TA100 showed a number of revertants (mutagenicity) in the same range as the control (Griest et al., 1990). Subsequently, these toxicity tests allowed this remediation technique to be used for cleanup of several munitions contaminated sites in the United States.

Two of the most common bioassays used for evaluating toxicity in contaminated and remediated soil and soil-like materials are the Microtox bioassay using *Vibrio fischeri*, a bioluminescent marine bacterium, and the earthworm bioassay using *Eisenia foetida* (Overton et al., 1997; Pradhan et al., 1997; Salanitro et al., 1997; Jarvis et al., 1998; Adams et al., 2006, 2011, 2015; Elgh-Dalgren et al., 2009; Steliga et al., 2012). The Microtox bioassay was first developed for contaminated water and wastewater, but subsequently modified for sediments and soil in a direct contact test (Isenberg, 1993). In this bioassay the test organism was placed in direct contact with the solid medium in a liquid suspension and incubated for a set time. Subsequently, the mixture was filtered and the bioluminescence of the liquid measured. However, the solid phase method met with problems because the sample filtering post-contact only worked for coarse materials (sands, slightly silty sands). With more silty or clayey materials, the filtering was not sufficient to remove the fine particles from solution, which subsequently interfered in the photometric determination (due to turbidity). If one used a finer filter to remove the silty/clayey material, the test organism was also caught in the filter and bioluminescence was not measured accurately in the filtrate as planned (Volpi Ghirardinia et al., 2009). Thus, the kinds of test used, the characteristics of the test organism, and the methods of sample preparation can also be determinant in the successful use of a bioassay.

Ideally, if one is interested in the toxicity of the soil *per se*, the test organism is in direct contact with the remediated (or contaminated) soil to run the test. However, separating the test organism from the soil media to reduce interference and obtain accurate and reproducible results may be problematic. The use of larger species as test organisms has been able to overcome this problem. For example, earthworms, and in particular *Eisenia foetida*, has been used in direct contact tests to evaluate the toxicity of contaminated/remediated soil. The most common method used is the OECD protocol 207 or modifications thereof. Originally, this test was designed to evaluate the intrinsic toxicity of contaminants, not their toxicity in contaminated media (OECD, 1984). It was designed as a two-stage test, a screening test (48 h acute toxicity) and a sub-chronic 14 day artificial soil test, to further investigate potentially hazardous chemicals identified in the screening test. First, the contaminant is diluted in water and the dilutions placed on filter paper, over which the earthworms are placed for 48 h and incubated. Changes in color, form, reaction to a stimulus (a prick), as well as weight loss and mortality are recorded.

For the sub-chronic test, each week the worms are separated from the artificial soil and evaluated for the same factors as the screening test, which is physically possible due to their size relative to soil particles. These determinations are somewhat tedious but uncomplicated, do not depend on sophisticated equipment, and generally can be run almost anywhere in the world. Also, the use of earthworms to test soil is particularly attractive due to the fact that earthworms are a normal part of the biota of many soils, and not an unassociated species (such as the marine bacteria *Vibrio fischeri* or aquatic *Daphnia* species, for example).

Since the OECD protocol 207 was developed, different researchers have made modifications in the way it is run to facilitate their investigations. Generally, these modifications have been used to investigate the properties of contaminated or remediated soil, rather than the intrinsic toxicity of contaminants. With respect to the filter paper test, the main modification has been to test laboratory produced soil leachates

(Jarvis et al., 1998), often using the TCLP or SPLP tests or equivalents (EPA methods 1311 and 1312; USEPA, 1992, 1994). Usually, this is done for two reasons. First, the use of leachate tests lets the researcher investigate the potential of the contaminated soil to leach out toxic substances and pollute a groundwater or a surface water source. Second, the filter paper test is run easily, and gives a result in only two days. Regarding the sub-chronic test, the main modification has been to run the test on the contaminated soil itself, either diluted with the artificial soil, or directly on the contaminated soil (Adams et al. 2006, 2013). Additionally, longer incubations periods have been used, often up to 28 days.

These modifications have provided very useful data for site-specific research as well as for investigating remediation processes. The advantage of the acute test is the short time it takes to get a result (two days). However, it may not be representative of the toxicity of the soil itself. There are many contaminants of low solubility or with high octanol-water partition coefficients (Kow), and which are sequestered in the soil, and are toxic but not very leachable (Travis and Arms, 1988; Adams et al., 2013). For sites where the land use makes it improbable to have direct contact between the contaminated soil and biological receptors (such as commercial or industrial land use), the filter paper tests on TCLP type leachates may be sufficient. However, with sites that have residential, agricultural, livestock raising, forestry or habitat conservation uses, the toxicity of the soil directly to humans or soil organisms (especially plants, mesofauna and microorganisms) is very important. Thus, the filter paper test may be inadequate for low solubility or sequestered contaminants. In these situations, the direct soil, sub-chronic test may be preferred, especially for 28 days exposures. The main inconvenience with this test, however, is the tediousness of the test (removing all of the worms and evaluating them every week), and the time it takes (14–28 days). Recently, in our laboratory, we have developed a modification of the OECD earthworm bioassay which incorporates the advantage of a quick and easy test, with the ability to detect toxicity of low solubility contaminants directly in soil (the Soil Contact Acute Toxicity Bioassay). In the following sections a comparison of this new method with existing methods is made in the context of specific research projects, and recommendations for its use are made.

2.2. Soil contact acute toxicity bioassay for determining remediation effectiveness of 2,4-D contaminated soil

2.2.1. Methods: experimental design

During experiments to test the Daramend® remediation product on soil contaminated with the herbicide 2, 4-D (2, 4-dichlorophenoxy acetic acid, Polaquímca S.A. de C.V., Veracruz, Mexico), a small amount of clayey soil was artificially contaminated with a starting concentration of 300 mg/kg for research purposes. This concentration was based on technical information from the manufacturer and the US EPA which showed a successful treatment of soil that was contaminated with various organochloride pesticides with starting concentrations in the general range of 150–700 mg/Kg (Przepiora et al., 2010; USEPA, 2006). The Daramend® product (PeroxyChem LLC, Philadelphia, USA) was applied at three different dosages ranging from 1 – 3% (w/w, dry) and three replicates were prepared for each treatment. The treatment cycles consisted of anaerobic conditions for two weeks, followed by aerobic conditions for two weeks. Initially, and after the third, fourth and fifth treatment cycles, toxicity was measured. The soil used for these experiments has been described previously and is tentatively classified as a Vertisol in the FAO/World Reference Base soil classification system (Marín-García et al., 2015; Palma López and Triano Sánchez, 2007).

2.2.2. Filter paper bioassay

TCLP type extracts of the soil were prepared (SEMARNAT, 1993), and tested using the OECD filter paper test mentioned previously (OECD, 1984). For these test, nitrocellulose filter paper was used (8μm pore size, 0.25 mm thick, Eaton Dikeman Co., Mt. Holly Springs, Pennsylvania, USA). The jars were wrapped in newsprint paper to reduce light exposure, and the top of the jar was covered with surgical mask material (so the earthworms could breathe), and sealed with a rubber band to prevent the earthworms from escaping. The earthworms added were two to three months old and weighed 350 mg (±50 mg). They were incubated at 20–25 °C under a table with indirect light only. After 24 h and again after 48 h, the worms were observed for mortality, biomass (moist weight), response to stimulus and other indications of toxicity (inflamed clitellus, expulsion of coelomic fluid, and expulsion of bloody fluid). Ten jars were run per sample (on each of the three treatment replicates = 30 jars per treatment), and the results were compared to a control made with deionized water (also with 10 jars).

Even though we noticed a strong, acrid odor in the recently contaminated, untreated clayey soil, no indications of earthworm toxicity or mortality were detected on the TCLP extracts of the soil in the filter paper bioassay. This observation seemed incongruent with the strong odor in the contaminated soil. It was speculated that perhaps, the 2, 4 -D was adsorbed onto the clay surfaces in the soil, or onto the soil organic matter (or the Daramed® product itself, which is organic-based), thus limiting the extraction in the TCLP procedure, and therefore, no mortality was observed.

2.2.3. Soil contact bioassay for acute toxicity

To test this, the following procedure was developed, which combines an acute bioassay, from the paper filter test of the OECD Protocol, with direct contact in the extended part of the Protocol, originally developed for artificial soil (OECD, 1984). Instead of applying a soil extract to the filter paper, a small amount of moist, contaminated (or treated) soil was applied directly to the bottom of the test jars in a thin layer, and the worms were placed on top as per the normal filter paper test, but without the filter paper. Five grams (dry weight basis) of soil was moistened with deionized water while mixing until all the soil surfaces gleamed with moisture and a thin film of water remained on the soil surface. In this case, the soil was moistened to 40% humidity, (equivalent to 115% Field Capacity) and applied to the bottom of a glass jar (7 cm wide x 9 cm tall). The jar was wrapped in newsprint paper to reduce light getting into the jar, and the top of the jar was covered with surgical mask material (so the earthworms could breathe) and sealed with a rubber band to prevent the earthworms from escaping. The earthworms added were two to three months old and weighed 350 mg (±50 mg). They were incubated at 20–25 °C under a table with indirect light only. After 24 h and again after 48 h, the worms were observed for mortality, biomass (moist weight), response to stimulus and other indications of toxicity (inflamed clitellus, expulsion of coelomic fluid, and expulsion of bloody fluid). Ten jars were run per sample (on each of the three treatment replicates = 30 jars per treatment), and the results were compared to a control made from uncontaminated soil (also with 10 jars).

2.2.4. Results and discussion

All of the worms died during the soil contact test on recently contaminated soil, as opposed to a 0% mortality in the filter paper test of TCLP extracts of the same contaminated soil (Table 1). The earthworms also showed other signs of toxicity. In the uncontaminated control, only small effects were observed (weight loss, loss of reaction to a stimulus, and loss of movement), probably just from the effects of going from an organic rich growth medium to soil –slight dehydration and low food supply. However, the contaminated and treated material showed 100% mortality and heavy stress – inflamed clitellus, expulsion of coelomic fluid and expulsion of bloody fluid. None-the-less, the Daramend® treatment did have some success. After the fifth treatment cycle, all the worms survived at least 24 h and the number of worms showing the most severe effects (expulsion of celomic fluid and bloody fluid) decreased from 50% to 37%. It is probable that with a higher dosage of the product and more cycles (8–13) the reduction in toxicity may be completed to acceptable levels (USEPA, 2006).

With this test, one can observe the utility of the soil contact acute bioassay vs. the filter paper acute bioassay. Whereas the filter paper test

Table 1. Comparison of filter paper and soil contact acute toxicity tests with *E. foetida*.

Soil	Filter Paper Test		Soil Contact Test			
	% mortality		% mortality		Observations	
	24 h	48 h	24 h	48 h		
Uncontaminated soil	0	0	0	0	Weight loss %	50
					No reaction to stimulus %	40
					Loss of movement %	10
2,4-D contaminated soil	0	0	100	100	Inflamed clitellus %	100
					Expulsion of coelomic fluid %	30
					Expulsion of bloody fluid %	20
Daramend treated soil – 3 cycles	ND*	ND*	100	100	Inflamed clitellus %	100
					Expulsion of coelomic fluid %	7–13
					Expulsion of bloody fluid %	23–30
Daramend treated soil – 4 cycles	ND*	ND*	100	100	Inflamed clitellus %	100
					Expulsion of coelomic fluid %	10–13
					Expulsion of bloody fluid %	17–27
Daramend treated soil – 5 cycles	ND*	ND*	0	100	Inflamed clitellus %	100
					Expulsion of coelomic fluid %	13–20
					Expulsion of bloody fluid %	3–17

* ND = Not determined. Since there was no mortality in the filter paper tests on contaminated, untreated soil, no mortality was expected on treated soil. Thus, this (filter paper) test was not run on treated samples.

failed to detect the toxicity of the 2,4-D contaminated soil, the soil contact test did. This is presumably due to the adsorption/absorption of the 2,4-D onto soil particles, thus limiting bioavailability in soil extracts. Other researchers have also found reduced toxicity due to sequestering in soil clays and organic material (see Alexander, 1999, for example). In future degradation experiments, once the soil contact acute test demonstrates an adequately low mortality (≤10% according to the OECD protocol), it would be preferable to confirm, using the extended sub-chronic test but applied directly to the treated soil (rather than to an artificial soil).

2.3. Use of soil contact bioassay for determining chemical oxidation/bioremediation effectiveness for treating petroleum drilling cuttings

Another application of the soil contact acute bioassay is for the evaluation of hydrocarbon-contaminated soil-like materials, specifically, petroleum drilling cuttings. During the drilling of oil wells, control fluids are placed in the well to lubricate the bit, provide the hydraulic pressure to activate the bit, seal the well excavation, prevent aquifer contamination, control pressure in the well, and to transport the cuttings to the surface. Common additives used in drilling fluids are water, diesel, bentonite clay, barium and other products such as salts, pH adjusters, emulsifiers and polymers. At the surface, there is some separation of the cuttings and the drilling mud, usually by physical processes, which is done to recover usable materials and reformulate more drilling mud (Rabia, 1986). After the separation, some of the drilling mud still remains in the cuttings. The cuttings in themselves are geological material that is basically non-toxic. However, some residues of the drilling mud are still in the recovered cuttings and usually contain weathered diesel (used as the base oil in the mud), and other additives that may be toxic (especially some polymers, emulsifiers, and thickeners). Prior to land application of the recovered cuttings, they need to be treated to reduce the toxicity. In many parts of the world, it is permitted to just reduce the total hydrocarbon concentration to 3% (for industrial areas) or 1% (for non-industrial sites; LDNR, 1986; República de Venezuela, 1998; Mathews et al., 1997; Indonesian Ministry of Environment, 2003). However, considering all of the other potentially toxic additives from the drilling mud, it may be important to conduct bioassays in addition to just measuring the hydrocarbon content.

2.3.1. Methods: experimental design

The soil contact acute toxicity bioassay was used to evaluate different methods for the treatment of drilling cuttings contaminated with drilling fluids. These consisted of a treatment train of chemical oxidation – bioremediation - chemical oxidation, either with or without a pre-treatment by alkaline desorption (to reduce water repellency and make the chemical and biological treatments more effective; Adams et al., 2016). For the chemical oxidation treatments, 1.3% (w/w) of hydrogen peroxide was added to the cuttings and mixed well with a spatula for 2 min. For the bioremediation treatment, inorganic fertilizer was added to the cuttings as well as 4% (w/w) of sugar cane cachasse as an organic amendment to improve soil aeration and wettability. Subsequently, an unidentified consortium of hydrocarbon degrading microorganisms previously isolated from oil-contaminated soil was added to the cuttings. The cuttings were placed in shallow aluminum containers and mixed three times a week, maintaining the humidity in the range of 50–70% field capacity, for about 4 ½ months at ambient temperature (~28 °C, average).

In these treatment tests, there was interest to see if just chemical oxidation plus bioremediation would be sufficient to treat the drilling cuttings, without a final chemical oxidation (thereby reducing time and costs). The total hydrocarbon concentration in both treatments (with or without the pre-treatment by alkaline desorption) was similar, being reduced from ~7.9% (w/w) to ~5.1% by the first chemical treatment, and to ~1.3% by the subsequent bioremediation treatment. After the bioremediation treatment, but prior to the final chemical oxidation, the treated cuttings were checked for toxicity by different means. To check if this material could be placed in an agricultural setting (for pasture and livestock use), it was tested for germination using seeds of a locally used pasture, Chontalpo grass (a variety of *Brachiaria decumbens*), as well as by the earthworm filter paper acute toxicity test. The filter paper tests were carried out as previously described in Section 2.2.2. Treated material was also tested with the direct contact bioassay as described in Section 2.2.3. To make sure that the treated cuttings had sufficient moisture for the earthworms, 2.5 ml of water was added to 5 g (dry weight) of cuttings for each test, just sufficient to completely moisten the soil and leave a thin film of moisture on the solid particles. Subsequently, earthworms were placed and exposed to this moistened material (already prepared in glass jars), for 48 h. Post bioremediation, these materials were treated for a last time with chemical oxidation as described previously, and the hydrocarbon concentration and toxicity were tested.

2.3.2. Results and discussion

In soil treated by chemical oxidation plus bioremediation, in the germination tests, the results of both treatments (with or without the alkaline desorption pre-treatment) were moderate, having only 55–70% of the germination rate of an uncontaminated clayey soil (Vertisol, previously mentioned). However, when tested with the filter paper acute toxicity earthworm bioassay, there was 0% mortality (see Table 2). These data seemed incongruent, so the treated material was also tested using the soil contact acute toxicity bioassay.

In these direct contact tests, there was 100% mortality in 48 h. Thus it does indeed appear that there still remained toxic (but poorly leachable) contaminants in the treated drilling cuttings. Afterwards, post bioremediation, these materials were treated for a last time with chemical oxidation and the hydrocarbon concentration was reduced to ~0.8%. Also notable after this last treatment, was the loss of the characteristic odor of drilling cuttings, which is probably due to some of the additives in the oil based mud (emulsifiers, polymers and thickeners). The treated cuttings were then re-tested by the soil contact acute toxicity earthworm test and most of the worms were found to survive at least 48 h. There was only a 3% mortality after 48 h of exposure in the treatment without alkaline desorption pre-treatment and only a 13% mortality in the treatment receiving pre-application of alkaline desorption. This level (3%) is within the normal variation in the test according to the OECD protocol (≤10%), thus basically overcoming the acute toxicity, and presenting the possibility of disposal or use as backfill in a pasture and livestock raising area. It is unlikely that this large reduction in toxicity (100 % mortality to 3–13% mortality) is only a result of the modest additional reduction in hydrocarbon concentration (from 1.3% to 0.8%). In similar work on weathered petroleum in clayey soils, this level of reduction hydrocarbon concentration has only resulted in a corresponding reduction in toxicity of about 30% (Adams et al., 2009). It is possible that this last chemical oxidation step also finally transformed some unknown drilling fluid additive into a substantially less toxic compound, greatly reduced its concentration, or reduced its bioavailability to earthworms in contact. It may also be possible that even through the addition decrease in the total hydrocarbon concentration is only moderate, that the degradation of the bioavailable fraction is much greater, thereby reducing the toxicity considerably. This is an ongoing area of research in our laboratory.

2.4. Importance of moisture content in the soil contact acute bioassay: weathering of contaminated soil - experiment

We have come across one factor that may affect the outcome of the proposed soil contact acute toxicity bioassay - the moisture content on the soil or soil-like material being tested. We have found that the earthworms are very sensitive to insufficient moisture as shown in the following experiment.

2.4.1. Methods: experimental design

Toxicity was tested on two alluvial soils that had been contaminated with medium crude oil (30.2 °API) at 2% (w/w) and let to naturally attenuate for 14 months in shallow (20 cm), open-air cells, in a tropical monsoon environment (average temperature ~28 °C, annual precipitation ~1800 mm, Adams Schroeder et al., 2002). The soils used in this study have been tentatively classified as a Fluvisol and a Gleysol in the FAO/World Reference Base system (Palma López and Triano Sánchez, 2007). The Fluvisol has been described previously (Morales-Bautista et al., 2017). The Gleysol was collected from the first 30 cm of surface soil in a floodable pasture at coords. 15Q 2002333N, 406608E (UTM) in the Buena Vista township in the municipality of Cárdenas, Tabasco, Mexico.

After the incubation period, the soils were tested for toxicity with the direct contact bioassay described previously. In the first run of these tests, the soils were moistened to 13.9% H and 16.7% H in the Fluvisol and Gleysol respectively, to try to achieve approximately 80% of the field capacity of the soil. However, this field capacity calculation was based on the initial (contaminated) conditions in the soil. Initially these soils were very water repellent and had relatively low field capacity due to the petroleum contamination. Initial field capacities in these contaminated soils were 17.4 and 20.8% H (Fluvisol and Gleysol, respectively) when usually the field capacities of these kinds of soil in the region are in the 25–40% H range (Zavala-Cruz et al., 2005). Subsequently, the soils were re-tested but increasing the moisture content to 37.5% H. This allowed the soil to be completely moistened and to show a thin layer of moisture on the soil surfaces (~100% field capacity).

2.4.2. Results and discussion

After 14 months, the hydrocarbon concentration in soil was ~0.40% in the Fluvisol and ~0.36% in the Gleysol. Even though the hydrocarbon concentrations were low and the soils had only a slight odor, there was 100% mortality in these tests. After repeating the tests, adjusting for the higher field capacity of the remediated soil, there was 0% mortality and no signs of stress were observed.

In future experiments it is suggested to use soil that has been moistened until all the soil surfaces just glisten with moisture. For clayey soils or drilling cuttings (that have a lot of fine bentonite clay from the drilling fluid), it could be useful to let the soil set for half an hour and re-check the humidity, similar to the preparation of a saturated paste for salinity determinations (Rhoades, 1982; SEMARNAT, 2002). For fine-textured soils, the added moisture may have absorbed into the soil particles and the true humidity may be less than estimated at first preparation, thus not providing adequate moisture for the earthworms.

Table 2. Comparison of hydrocarbon concentration, grass germination and earthworm bioassays to evaluate the treatment of drilling cuttings.

Soil/Drilling cutting	Total Petroleum Hydrocarbons (mg/Kg)	Chontalpo Grass Germination (%)	Earthworm Filter Paper Test % mortality 48 h	Earthworm Soil Contact Test % mortality 48 h
Uncontaminated Clayey soil (Vertisol)	ND	67	ND	0
Drilling Cutting Treatment: Chemical oxidation + bioremediation	12,900	47	0	100
Drilling Cutting Treatment: Chemical oxidation + bioremediation w/pretreatment by alkaline desorption	13,300	37	0	100
Drilling Cutting Treatment: Chemical oxidation + bioremediation + chemical oxidation	7,300	ND	ND*	3
Drilling Cutting Treatment: Chemical oxidation + bioremediation + chemical oxidation w/pretreatment by alkaline desorption	9,400	ND	ND*	13

* ND = Not determined. Since there was no mortality in the filter paper tests on partially treated soil (chemical oxidation plus bioremediation), no mortality was expected on post-treated soil. Thus, this (filter paper) test was not run on these samples.

It should be noted that in addition to careful control of moisture, one of the main limitation of this bioassay with *E. foetida*, will be soils that are naturally saline or have extremes of pH. This species will not survive under these conditions, or only poorly so, and thus toxicity will be indicated for natural conditions that are not contaminated (false positives). For these kinds of conditions, other tests organisms that are large enough to be physically separated from the soil need to be sought out and studied.

3. Conclusions

The soil contact acute toxicity bioassay has the benefits of being able to determine the toxicity of the soil directly in a rapid bioassay. As seen in these examples, there can be a significant difference in toxicity of soil (or soil like material) itself, and the TCLP-like extracts from such to earthworms. For industrial or commercial soil use, simply preventing the leachate of toxic substances to groundwater and surrounding soil may be sufficient for remediation, but for other soil uses where there can be direct contact between living organisms and the soil, a soil contact bioassay is necessary. This bioassay has the advantage of filling this need, being rapid and easy to use and truly evaluating toxicity in a direct contact situation. As noted, it is important to adjust the soil moisture adequately at the beginning of the test to avoid desiccation of the earthworms. For confirmation of no or low toxicity in those samples which pass the acute test ($\leq$10% mortality according to the OECD protocol) a subsequent 14 days (or 28 days) sub-chronic earthworm test is recommended, but directly on the soil or soil-like material itself rather than on artificial soil.

Declarations

Author contribution statement

Verónica I. Domínguez-Rodríguez, Randy Adams: Conceived and designed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Fabián Sánchez-Madrigal, José de los S. Pascual-Chablé: Performed the experiments.

Rodolfo Gómez-Cruz: Analyzed and interpreted the data.

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The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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