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Crude Oil Hydrocarbon Bioremediation and Soil Ecotoxicity Assessment

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In this study, we determined the limits and extent of hydrocarbon biodegradation, earthworm and plant toxicity, and waste leachability of crude oil-containing soils. Three oils (heavy, medium, and light of API gravity 14, 30, and 55, respectively) were mixed into silty loamy soils containing low (0.3%) or high (4.7%) organic carbon at 4000−27 000 mg/kg TPH. Hydrocarbon bioremediation in these artificially weathered oily soils usually followed first-order removal rates in which 50−75% and 10−90% of the total petroleum hydrocarbons (TPH) were degraded in 3−4 months for the low and high organic soils, respectively. Gas chromatographic profiles (simulated boiling point distillation of saturates and aromatic compounds) showed that, after bioremediation, hydrocarbons in oily soils decreased from 70 to 90%, from 40 to 60%, and from 35 to 60% for those carbon number species in the range of C_{11}−C_{22}, C_{23}−C_{32}, and C_{35}−C_{44}, respectively. Most oily soils were initially toxic to earthworms in which few animals survived 14-day bioassays. In a solid phase Microtox test, most oily soils had EC50 values that were ≤50%. Seed germination and plant growth (21-day test, wheat and oat but not corn) were also significantly reduced (0−25% of controls) in untreated soils containing the medium and light crude oils but not the heavy oil. Bioremediated soils were neither toxic to earthworms, inhibitory in the Microtox assay, nor inhibited seed germination after 5 (high organic soil) or 10−12 (low organic soil) months of treatment. Water-soluble hydrocarbons (e.g., O&G and BTEX) could leach from pretreated soils (medium and light crude oily soils) in column or batch extraction experiments. However, after bioremediation, most of the aromatic compounds were no longer leachable from the soils. These data demonstrate that treated oily soils lose their toxicity and potential to leach significant amounts of BTEX. These nontoxic soils contain 1000−8600 mg/kg residual hydrocarbons as TPH. Furthermore, these data suggest that the remaining petroleum compounds may be bound or unavailable in that they are not (a) biodegraded further, (b) toxic to soil-dwelling species (earthworms and plants), and (c) susceptible to leaching and subsequent impact to groundwater. These findings provide a basis for a framework in which petroleum hydrocarbon-containing soils can be evaluated by ecological assessment methods such as biodegradability, ecotoxicity, and leaching potential of regulated substances.

Introduction

Bioremediation is often a cost-effective method to treat oily soils and petroleum wastes containing biodegradable hydrocarbons and indigenous microbes. This soil cleanup technology has been successfully demonstrated in laboratory and field tests for refineries (*1*−*6*), in oil and gas operations in the treatment of oily sludges, and at pipeline sites to remediate accidental crude oil spills (*7*−*9*). The land treatment process requires the management of appropriate levels (e.g., oil hydrocarbons as percentage total petroleum hydrocarbons, TPH) of applied waste to soil, aeration and mixing, nutrient fertilizer addition, pH amendment as required, and moisture control to optimize degradation by soil microorganisms. Guidance on the lab feasibility assessment, field implementation, and soil sampling strategies required to demonstrate land treatment of wastes have been developed by Huesemann (*10*, *11*) and Sims et al. (*12*). A petroleum industry review based on such land treatment practices several years ago indicated that 70−90% of oily sludge hydrocarbons were removed from surface soils having loading rates of 10 000−50 000 mg/kg oil (*1*). Loehr et al. (*13*) studied the treatability of an oily sludge in field plots in a silty loam soil and demonstrated that 60−70% of the initial O&G (20 000−55 000 mg/kg) hydrocarbons were biodegraded within 2−3 years.

There have been numerous studies and reviews in the literature documenting the ready degradability of crude oil hydrocarbons (alkanes, alkenes, aromatics, and polars) in soils, sludges, sediments, and the marine environment by naturally-occurring microbes. Experiments have shown that differences in the extent of soil hydrocarbon biodegradation may depend upon soil and crude oil types, concentration of total applied hydrocarbon, and nutrient growth stimulants (e.g., NH_3 and PO_4^{3-}) based on optimum C:N:P ratios (*14*−*20*). Research by Huesemann and Moore (*21*) on the influence of oil type on bioremediation in a sandy soil showed that a light-medium (API gravity 39 and high saturate fraction) crude oil biodegraded (O_2 uptake and reduction in oil and grease) more extensively than a heavier crude (API gravity 21). In these experiments, optimum rates of O_2 consumption and CO_2 formation were observed in the first 3−4 months. There have been few definitive studies on identifying the fraction and types of petroleum hydrocarbons that are readily degraded or recalcitrant in oily waste soil treatment systems. Recently, Huesemann and Moore (*22*) showed that 93% of the saturate and 79% of the aromatic compounds having carbon numbers in the range of C_{10}−C_{44+} were degraded in a sandy soil containing weathered Michigan (medium API gravity) crude oil with an initial concentration of 30 000 mg/kg TPH. In this same study, however, the polar fraction was resistant to microbial metabolism and did not degrade during the 5.5-month test. Experiments by Huesemann (*23*) on the limits and extent of bioremediating TPH in different oily soils showed that 90% of the alkanes and monocyclic saturates and 50−70% of aromatic compounds ($<C_{44}$) were degraded. The significance of this work is that overall bioremediation effectiveness was dependent upon hydrocarbon types present and was not affected as much by soil type, nutrient fertilizer addition, microbial populations, or treatment conditions (slurry versus static soil conditions). It was also shown that saturate and aromatic compounds with polycyclic structures were most resistant to removal by enhanced soil biotreatment

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methods. The apparent recalcitrance of petroleum hydrocarbon fractions may be due to factors such as lack of bioavailability (inaccessible because of soil sorption and uptake by soil microbes), lack of requisite oxidizing enzymes, and/or steric hindrance for enzyme attack and toxicity to soil microorganisms.

Currently, there are no universal TPH cleanup standards that have been adopted by federal or state regulatory agencies for soils contaminated with fresh or weathered crude oils. State guidelines developed mainly for oil product (e.g., gasoline, diesel, and other middle distillate fuels) spills to surface or subsurface soils have varying remediation end points such as 10−10 000 mg/kg TPH and 0.1−500 mg/kg BTEX, cleanup to background levels, or allow the use of risk-based criteria coupled to environmental fate and effects (*24*). Based on our current understanding of bioremediation of crude oil-impacted soils, it would be difficult to achieve those low cleanup levels at most sites containing varying types of residual, weathered petroleum hydrocarbons. Doyle and Sweet (*25*) have suggested that soil remediation standards should be based upon the BTEX components in crude oil and oil products (fuels)-impacted soils since these are the most mobile (leachable) hydrocarbons that could be transported to groundwater. Ecologically relevant criteria for estimating the impacts of oil hydrocarbons in soils are also important end points for risk assessment. In this respect, ecotoxicity bioassays such as seed germination and plant growth have been used for monitoring treatment effects and restoration of oiled land sites (*26*−*29*). Plant species have been proposed as indicators of soil quality and toxicity of leachable constituents in assessing damage and risk to impacted ecosystems (*30*, *31*). There have been relatively few studies, however, describing the effects of oil hydrocarbons on soil-dwelling invertebrates such as earthworms, nematodes, other polychaetes and microarthropods (*32*−*34*). Earthworms have been used to evaluate the effects of chemicals and contaminated soils on animal survival, growth, and reproduction (*35*−*39*).

Another factor in ecorisk evaluation of oily soils is the potential for dissolution and leaching of water-soluble aromatic hydrocarbons like benzene, toluene, ethylbenzene, and xylenes (BTEX) into the vadose zone and groundwater environments. Laboratory soil microcosm experiments and field investigations in aquifers have been used to study hydrocarbon source migration from oil and fuel spills (*40*−*42*). Fate and transport methodologies have been developed to validate those natural attenuation factors (e.g., inherent biodegradation of hydrocarbons in groundwater, evaporation rates from spills, and soil sorption/desorption rates) governing the dissolution and dispersion of petroleum compounds into the subsurface (*40*, *43*−*45*).

Clearly, the integration of chemical analysis, ecotoxicity, and remediation potential data is required to properly assess ecological risk in the management of crude oil-impacted soils. In the present laboratory study, we compared the biotreatability of three artificially weathered crude oils (heavy, medium, and light) in soils with high or low organic carbon content using traditional land treatment techniques. Soil samples taken before, during, or after bioremediation were evaluated for TPH content, hydrocarbon composition changes, earthworm survival, seed germination and plant growth, Microtox inhibition, and hydrocarbon and metal leaching potential. Our data demonstrate the effectiveness of bioremediation techniques in reducing hydrocarbon levels, eliminating acute soil toxicity, and reducing leaching of water-soluble aromatic compounds (BTEX).

Materials and Methods

Test Soils and Crude Oils. The effects of hydrocarbon bioremediation on soil toxicity was investigated in two soils with high (4.6%, Norwood/Baccto) and low (0.3%, Norwood) organic matter to which were added three different crude oils of API gravity (measured at 60 °C) 14 (heavy), 30 (medium), and 55 (light). The distribution (%) of saturated/aromatic/polar fractions in the heavy, medium, and light oils was 20.3/28.9/44.1, 56.4/23.7/14.7, and 86.7/6.4/0.7, respectively. Total BTEX concentrations were 1735, 15 140, and 36 100 mg/kg, respectively, in the heavy, medium, and light oils. The predominant polyaromatic hydrocarbons (PAH) naphthalene and phenanthrene were present at combined levels of 180, 460, and 960 mg/kg in the heavy, medium, and light oils, respectively. PAH with four or more rings were present at or below the quantitative detection limit (≤20 mg/kg). Metal analysis of the crude oils indicated that Ni, V, and Zn were present in the heavy and light crudes at 99, 130, and 450 mg/kg, respectively (data not shown). Most other metals (e.g., As, B, Cr, Cu, Hg, Mo, Pb, and Se) were <20 mg/kg.

The Norwood soil used in these studies was obtained from the surface (6 in. depth) of a typical agricultural horizon (cotton field) near College Station, TX, and was characterized as a silty loam containing 15% clay and 60% silt, low organic matter (0.3% organic carbon), and a pH of 8.2. The Norwood/Baccto test soil mixture consisted of 75% v/v Norwood soil and 25% Baccto topsoil, had a pH of 7.1, and had an organic carbon content of 4.65%. The Baccto topsoil was a commercially available sandy loam potting soil of low clay (4%) and silt (11%) content, low pH (4.0), and high organic matter (20.3% organic carbon) due to the presence of peat. Soil grain size analysis indicated that 99% (Norwood) and 95% (Norwood/Baccto) of the particles were ≤0.11 mm. Inorganic nitrogen and phosphorus and organic nitrogen were higher in the Norwood/Baccto (469, 473, and 2921 mg/kg, respectively) as compared to the Norwood soil (20, 315, and 517 mg/kg). The initial moisture content of both soils varied from 18 to 28%.

The pH of the six oily soils during the 12-month study did not change appreciably and varied from 6.8 to 7.5. Total heterotrophic bacteria and hydrocarbon degraders were similar and did not vary during biotreatment. Microbial enumeration of soil samples taken during the first 6 months showed that there were 10^8−10^{10} heterotrophs and 10^7−10^9 hydrocarbon degraders/g of soil. Bacteria were estimated by cell growth in MPN dilution methods using Trypticase soy broth (BBL, Becton-Dickinson) medium for heterotrophs and Bushnell-Haas (Difco) minerals containing 1% hexadecane for hydrocarbon degraders.

Oily Soil Mesocosms. Approximately 4.5 kg (5% w/w) of heavy, medium, or light crude oil was added to 95 kg wet wt of Norwood or Norwood/Baccto soils. The sieved soil (1.3 cm screen) was mixed in a cement mixer to maximize hydrocarbon distribution. The oily soil was placed onto plastic sheeting for aeration and artificial "weathering" (2−3 days) and to manually break up clumps of clay and oil. A significant fraction of the volatile hydrocarbons was lost by this procedure. We calculated, for example, that based on the total BTEX hydrocarbons applied to the soil (5% oil addition) and the BTEX level at the start of the bioremediation process, about 40−95% were "volatilized" during the "mixing and weathering" process. Fertilizer solution was added to each 95 kg of oily soil as N (100 g of NH_4NO_3) and P (40 g of K_2HPO_4) at a C:N:P ratio of 100:1:0.2 (assuming a carbon content of 80% for crude oils). Deionized water was added to soils to a moisture content of 50−80% of the field moisture capacity. The fertilizer-amended oily soils were placed (12 in. soil depth) into 128-L capacity stainless steel chambers (45 cm × 45 cm × 30 cm) fitted with plexiglass covers. The mesocosms were continuously swept over the soil surface with humidified air at a flow rate of 250 L/h to minimize moisture loss and to aerate the soil. When mesocosms were sampled for residual TPH and O&G, soil was mixed and aerated and five randomly selected 400-g portions were withdrawn. This 2-kg sample was subsampled and submitted

for O&G, TPH, and BTEX and toxicity tests.

Methods for Hydrocarbon Analyses. (A) Total Petroleum Hydrocarbons. Duplicate samples (40 g wet wt) of oily soils from each treatment were taken monthly for determinations of O&G and TPH. O&G content was measured gravimetrically after evaporation of the Freon 113 solvent used in the Soxhlet extraction according to Method 5520E (*46*). This analysis is similar to EPA Method 413.1 for total O&G. The Freon extract was either (a) treated with silica gel to remove polar compounds and analyzed by an infrared analyzer (Horiba Instrument Co.) according to EPA Method 418.1 as TPH-IR or (b) dried under N_2 and the residue weighed and reported as gravimetric TPH (TPH-Gr) according to Method 5520F (*46*). The calibration standard used in the TPH-IR method was 25% (v/v) *n*-hexadecane, 37.5% (v/v) isooctane, and 37.5% (v/v) chlorobenzene; absorption was measured in the IR spectral range of 3400−3500 cm^{-1}.

(B) Aromatic Hydrocarbons. Polyaromatic compounds (two-, three-, and four-ring PAH) were extracted using sonication and methylene chloride from 2 g of soil according to EPA Method 3550 and analyzed by a direct injection GC/MS determination based on EPA Method 8270 (*47*). Volatile organics such as BTEX were determined using a modification of Method 8240 (*47*) by extracting (vortex mixing) 10 g of soil with 10 mL of high-purity methanol and then analyzed by GC/MS.

(C) TCLP Organics and Metals. The extraction procedures (Method 1331) for organics (Methods 8240 and 8270) and metals (Methods 6010 and 7470) were described in the SW-846 manual (*48*) and performed by Chester Laboratories, Houston, TX. Total fixed metals in soil were determined by Methods 6010 and 7471 as given in SW-846.

(D) Group-Type Separation Analysis. In the analysis of the saturate, aromatic, and polar fractions of the whole oils, TPH extracts were dried and redissolved in cyclopentane and separated on a packed silica gel glass column. The saturates, aromatics, and polar fractions were eluted with pentane, pentane−benzene (60:40), and benzene−2-propanol (80:20), respectively. The dry weight of each fraction was obtained by evaporating the solvent at 60 °C and weighing the residue.

(E) Hydrocarbon Distribution by "Simulated Boiling Point" Gas Chromatography. A gas chromatographic simulated high-temperature distillation of hydrocarbons by carbon number was performed on methylene chloride extracts of the untreated and bioremediated oily soils using a modification of ASTM Method D-2887 (*49*, *50*). Hydrocarbon fractions (saturates and aromatics) from C_{11}−C_{44} were separated, and a standard normal paraffin mixture was used for matching retention time with carbon number in the temperature-programmed column distillation.

Leaching Potential. The ready desorption and dissolution of water-soluble hydrocarbons and metals from each oily soil before and after bioremediation was determined by batch and column extraction methods. In the batch test, 20 g of soil was sequentially extracted five times with 200-mL aliquots of 0.01 M $CaSO_4$−2% sodium azide solution on a rotary platform agitator at 20 rpm for 24−48-h intervals. Sodium azide was added to the $CaSO_4$ solution to prevent microbial growth and biodegradation of the soluble hydrocarbons released from the soil. Soil slurries were centrifuged (2000 rpm, 45 min), and the combined supernatants were analyzed for O&G, TPH, BTEX, and metals (e.g., V, Ni, and Cu). These batch extraction methods were modified from the California Waste Extraction Test Procedures (*51*). In the column leaching studies, 500 g of soil was packed into a 2 in. × 6 in. glass column between 0.5 in. layers of Ottawa sand (Mallinckrodt Chem. Co.; 95% of the particles pass a no. 50 sieve). Columns were operated in an upflow direction using a syringe pulse pump flowing 2 pore vol/day of 0.01 M $CaSO_4$−2% sodium azide solution. These conditions simulated a water leaching flow rate through soil of 1 ft/day. Column leachates were also analyzed for O&G, TPH, BTEX, and metals.

Ecotoxicity Bioassays. (A) Earthworm Survival Test. The common earthworm species, *Eisenia foetida*, was used to determine acute toxicity of the oily soils before, during, and after biotreatment. Animals were obtained from Carolina Biological Supply Company (Burlington, NC) and held in uncontaminated soil until testing. The assay methods were similar to those described in an EPA protocol (*52*). Ten adult animals (five replicates) were placed into 200 g (dry wt) of soil in 1-L wide-mouth jars with loose fitting lids. The LC50 for each oily soil was estimated using five concentrations of bioremediated soil (100, 50, 25, 12.5, 6.5, and 0%) prepared with control (oil-free) Norwood or Norwood/Baccto soil. The soil water content was adjusted to 12−18% for the Norwood and to 30% for the Norwood/Baccto soils. Surviving earthworms were counted after a 14-day incubation at room temperature under constant fluorescent lighting conditions. The LC50 end point was calculated using probit techniques.

(B) Microtox Solid Phase Assay. The Microtox Analyzer M500 and solid-phase test kit (Microbics Corp., Carlsbad, CA) were used to evaluate the response of the luminescent bacteria (*Photobacterium phosphoreum*) to oily soils. The test methods employed were described in the Microbics Manual (*53*). Soil dilutions were prepared (0.3 g/3 mL of Microtox diluent), incubated for 20 min with reconstituted lyophilized bacteria, and then sampled for substrate-induced (Microtox ATP reagents) photoluminescence activity. The EC50 soil dilution that inhibits 50% of the light output relative to the control (hydrocarbon-free soil) was calculated for each soil.

(C) Plant Seed Germination and Growth. The methodology used in these seed germination/plant growth studies was similar to that outlined in the OECD Guideline for Testing of Chemicals (*54*). The effects of untreated and bioremediated oily soils were determined in corn, wheat, and oat species. Corn (*Zea mays*), wheat (*Triticum aestivum*), and oat (*Avena sativa*) were purchased from Carolina Biological Supply Company (Burlington, NC), and seeds were stored at room temperature until used in germination tests.

Oily soils or oil-free (control) soils were dispensed (ca. 80 g/cell) into molded plastic trays (57 cm long × 27 cm wide × 6 cm high) containing 36 cells/tray. Seeds (5 per cell) were placed 1−1.5 cm below the soil surface in each of 20 cells (100 seeds) for each soil treatment. Seed cultures were exposed to 12-h light/dark cycles at a soil surface light intensity of 310−350 lm provided by six 34-W white fluorescent lamps. The room temperature varied from 20 to 23 °C. Soil treatments were kept moist (ca. 30% of the soil mixture holding capacity) by spraying the soil surface with unchlorinated well water.

The percent of seeds germinated before and after (at 8 and 10 months) bioremediation was determined after 21 days. Plant foliar and root dry weights were also measured from all germinated seeds. Plants from a cell were removed as a group, washed to remove soil particles, and then dried at 120 °C for 3 h. The average dry weight/plant was calculated for all plants, and incompletely germinated seeds were not included in the plant dry weight. Plant germination data (where applicable) were compared between treatments using the χ^2 test with a continuity correction or the Irwin−Fisher exact test. A sample size of 100 seeds per treatment can detect a 20% treatment effect when the control group germination rate is ≥60%. The plant dry weight data were analyzed by analysis of variance, followed by the method of least significant difference (LSD) for assessing treatment effects (*55*).

Results and Discussion

Hydrocarbon Biodegradation in Oily Soils. The initial soil concentrations of the applied hydrocarbon varied from 12 000 to 14 000 mg/kg, from 26 000 to 27 000 mg/kg and from 4000

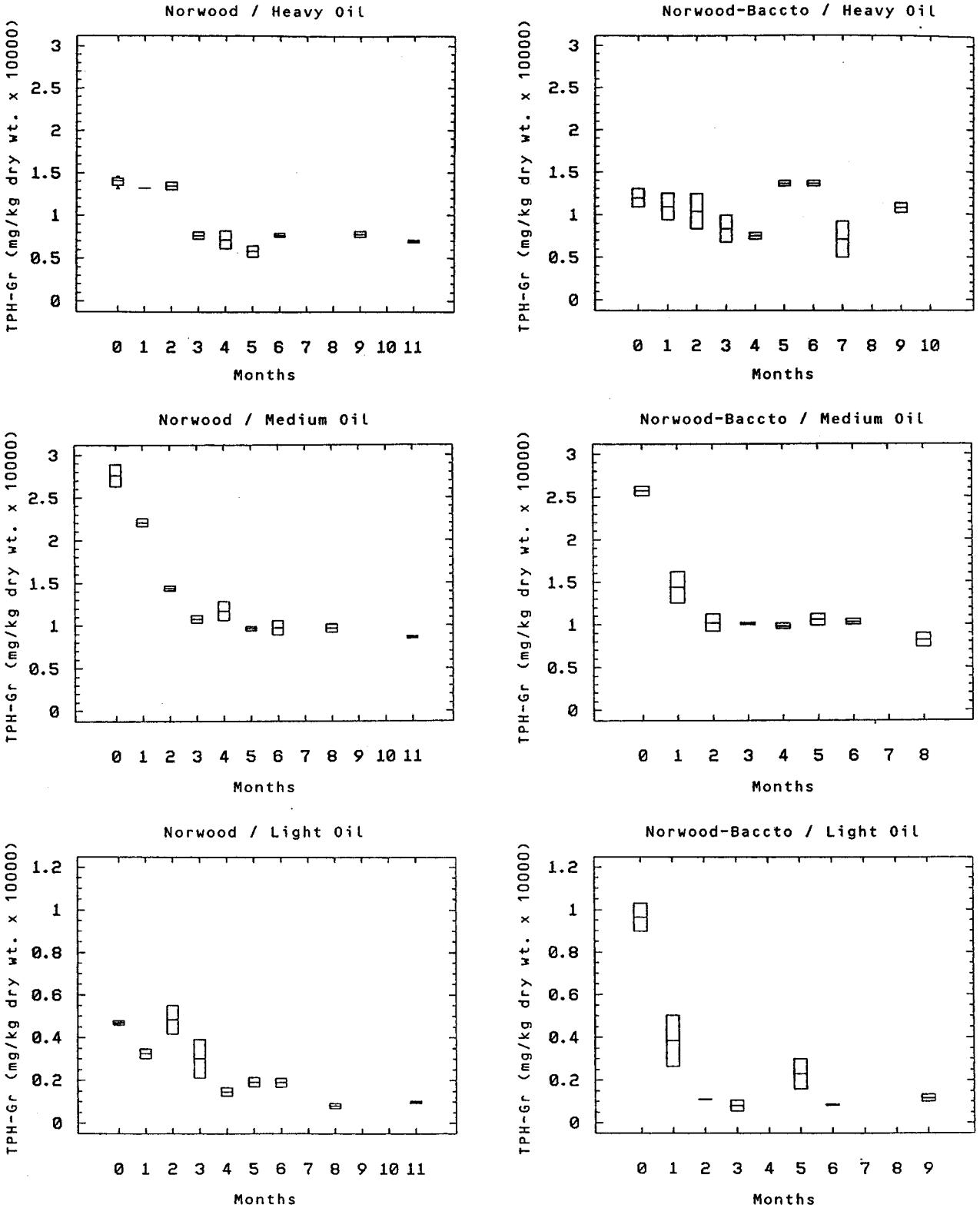

FIGURE 1. TPG-Gr analyses of oily soils during bioremediation. Values given represent the median and actual levels for duplicate soil samples at each time point.

to 9600 mg/kg TPH-Gr for the heavy, medium, and light oils, respectively. Most duplicate soil samples taken at each time point were within 10−20% of each other based on bulk hydrocarbon analysis. Profiles of the decline in oil hydrocarbons during soil bioremediation are shown in Figure 1. Table 1 is a summary of the initial and final (8−11 months) hydrocarbon levels in both soils as analyzed by TPH-Gr, TPH-IR, and O&G methods. The TPH-Gr data show that the heavy, medium, and light oils were significantly degraded in low (Norwood) and high (Norwood/Baccto) organic soils. The overall maximum decline in TPH was similar for the two soils, but different between oil types. For example, the decrease in TPH in soils with heavy, medium, or light oils was 10−50%, 65−70%, and 75−90% of the initial TPH-Gr levels after 8−11 months (see Table 3). Similar net reductions in heavy, medium, and light hydrocarbons were noted for both soils based on O&G (5 and 45%; 55 and 60%; 50 and 80%) and TPH-IR (12 and 73%; 70−80%; 95%) analyses (see Table 1).

TABLE 1. Decline in Bulk Hydrocarbon Levels in Oily Soils after Bioremediation As Measured by TPH, O&G, and TPH-IR

soil	oil type	initial concn (mg/kg)[a]			% degraded based on[b]			degradation rate (%/mo)[b,c]
		TPH	O&G	TPH-IR	TPH	O&G	TPH-IR	
Norwood	heavy	14 000	23 600	35 700	50	44	63	16
	medium	26 600	34 800	81 400	67	57	71	21, 30
	light	4 200	9 760	40 100	76	83	95	26, 48
Norwood/Baccto	heavy	11 900	37 000	64 700	10, 40	6	12, 73	12, 42
	medium	25 700	41 400	122 200	68	62	83	46, 69
	light	9 600	14 000	77 600	88	52	97	108, 126

[a] Based on dry wt of soil. [b] Calculations were the average of duplicate soil samples (±10%−20%, otherwise individual values are given) after 7−9 (Norwood/Baccto) and 8−11 (Norwood) months treatment. [c] Based on a best fit to a first-order decay curve.

TABLE 2. BTEX and Hydrocarbon Number Distribution in Oily Soils[a]

soil type	oil type	treatment	B	TEX (mg/kg)	carbon range (mg/kg)		
					$C_{11}-C_{22}$	$C_{23}-C_{32}$	$C_{33}-C_{44}$
Norwood	heavy	untreated	<0.02	1.64	6010	6234	4308
		bioremediated	<0.02	<0.02	1743 (71)[b]	3615 (42)	2848 (34)
	medium	untreated	3.19	256	7269	6688	3835
		bioremediated	<0.02	2.21 (<0.02)[c]	1887 (74)	3272 (51)	2384 (38)
	light	untreated	63.7	1027	4723	477	157
		bioremediated	<0.02	56.0 (<0.02)[c]	586 (88)	185 (61)	73 (53)
Norwood/Baccto	heavy	untreated	1.77	43.4	5545	6682	5603
		bioremediated	<0.02	<0.02	1100 (80)	2653 (60)	2910 (48)
	medium	untreated	10.0	35.0	5168	4845	3335
		bioremediated	<0.02	<0.02	944 (82)	1880 (61)	1764 (47)
	light	untreated	53.0	1624	13 796	854	218
		bioremediated	0.18 (<0.02)[c]	<0.02	1308 (90)	435 (49)	82 (62)

[a] Soils extracted for BTEX or other hydrocarbons after 2 or 8−11 months bioremediation, respectively. Concentrations are mg/kg dry wt soil. [b] Number in parentheses is the percent reduction of each fraction from the untreated soil. [c] Number in parentheses is the BTEX concentration after 8−11 months.

Calculation of the TPH-Gr rates of degradation (based on best-fit first-order equation during the first 4 months) was highly variable between soils and oils. In general, degradation rates were greater in the high organic Norwood/Baccto soil for the medium (73%/month) and light (81%/month) oil and lower (13−31%/month) for the heavy oil in either soil and for the three oils in the lower organic Norwood soil (Table 1). Lowest hydrocarbon levels in all oily soils were achieved within 4 months, and further biotreatment did not significantly decrease hydrocarbons. TPH-Gr analyses from the soil with heavy crude were the most variable (Figure 1) in which concentrations of TPH-Gr (also TPH-IR, data not shown) in the Norwood/Baccto soil samples varied from 5000 to 13 500 mg/kg during the 9-month treatment. These variations were not observed with the O&G analysis (profile not shown). It is possible that compounds extracted from the high organic soil interfered with removal of polar petroleum hydrocarbons during the silica gel adsorption step for the TPH determination. Indeed, inaccuracies (up to 85% relative error) and biases in the use of silica gel for the determination of TPH in soils containing petroleum products have been discussed by George (*56*). The heavy crude oil contains a larger fraction (44%) of polar material than the medium and light oils.

Analyses of hydrocarbons (mainly saturates and aromatics extracted with CH_2Cl_2) based on a simulated gas chromatographic distillation profile in the range of $C_{11}-C_{44}$ in untreated and bioremediated oily soils are summarized in Table 2. The extent of biodegradation of hydrocarbons was higher (70−90%) for those compounds in the $C_{11}-C_{22}$ range and lower for those in the $C_{23}-C_{33}$ (40−60%) and $C_{34}-C_{44}$ (35−60%) ranges. These degradation values are consistent with the decline in the hydrocarbon concentrations observed in both oily soil types based on TPH-Gr, TPH-IR, and O&G determinations (Table 1). The data also indicate that 8−18% more hydrocarbons were degraded in the higher organic carbon soil (Norwood/Baccto mixture) as compared to the Norwood soil. Residual hydrocarbons ($C_{11}-C_{44}$ fractions) in biotreated soils containing heavy and medium oils was 4500−8200 and 850−1825 mg/kg in the soil with light oil. These hydrocarbon concentrations are also consistent with those TPH residues (8000−10 000 mg/kg for the heavy and medium oily soils and 1000 mg/kg for the light oily soils) that remain after bioremediation (see Figure 1).

Leaching Potential of Oily Soils. It has been recognized that the predominant leachable components from petroleum-containing wastes are the more water-soluble hydrocarbons benzene, toluene, ethylbenzene, and xylenes (BTEX). Table 2 shows data on the residual BTEX components in the six oily soils before and after bioremediation. Solvent-extractable (CH_2Cl_2) B was detected (1.8−64 mg/kg) mainly from the medium and light oils. After 2 months of biotreatment, most soils contained little or no detectable B (<0.02 mg/kg). Initial TEX concentrations (35−1624 mg/kg) in the Norwood/Baccto soils were also reduced to low levels (<0.02 mg/kg) during the same period. Although residual TEX from the medium and light oils (2 and 56 mg/kg) were detected in the Norwood soil after 2 months, these hydrocarbons were below the detection level after 8−11 months. Data on batch and column leaching experiments on the oily soils are summarized in Table 3. Soluble O&G levels in aqueous (neutral pH) batch extractions were only 10−30 mg/L in the bioremediated soil after the first extraction (Table 3, Section A). Subsequent extractions reduced the O&G levels to <5−15 mg/L. No BTEX compounds (<5 μg/L) were detected in the first or subsequent O&G extracts. Soil column leaching tests (Table 3, Section B) also showed that the highest B leachate concentrations (900−10 000 μg/L) were from the soils containing medium and light crude oils and lowest in soils weathered with heavy crude. Biotreated soils had substantially reduced levels of BTEX after 10−30 column pore vol varying from <1 to 50 μg/L leachate from initial high levels of 10 000 μg/L. We also observed that no heavy metals such as V, Ni, or Cu were released (<0.4 mg/L leachate) from any oily soil during the column leaching experiment. These data indicate that

TABLE 3. Leaching Potential of Oily Soils in Batch Extractionand Column Leaching Tests

Section A: Batch Extraction—Hydrocarbon Levels after Bioremediation[a]

		O&G (mg/L) after extraction[b]			
soil	**oil**	**1**	**2**	**3**	**5**
Norwood	heavy	15	11	7	<5
	medium	30	17	12	9
	light	12	<5	<5	<5
Norwood/Baccto	heavy	16	11	9	6
	medium	31	16	14	8
	light	<5	<5	<5	<5

Section B: Soil Column—Hydrocarbon Levels (μg/L Leachate) before and after Bioremediation[a,c]

		B		TEX	
soil	**oil**	**untreated**	**bioremediated**	**untreated**	**bioremediated**
Norwood	heavy	<2	<2	17	2
	medium	630	<2	5260	8
	light	4900	<2	18270	6
Norwood/Baccto	heavy	160	<2	700	<2
	medium	1660	<2	5980	48
	light	7690	<2	16980	5

[a] 8–11 months treatment. [b] TPH and BTEX concentrations in aqueous extractions were <5 mg/L and <5 μg/L, respectively. [c] Values given are the BTEX concentrations in the first 2–3 pore volumes of leachate.

TABLE 4. Earthworm (*Eisenia*) and Microtox Tests on Oily Soils

Section A: Earthworm Survival[a]

		LC50 as % soil after bioremediation month							
soil	**oil**	**0**	**0.5**	**1**	**3**	**5**	**8**	**10**	**12**
Norwood	heavy	22	26	28	100	92	100	100	–[b]
	medium	4	9	4	22	90	100	100	–
	light	1	1	1	6	30	–	66	100
Norwood/Baccto	heavy	34	27	100	100	100	100	–	–
	medium	10	4	79	100	100	100	–	–
	light	1	9	23	100	100	100	–	–

Section B: Microtox[c]

		EC50 in percent soil after bioremediation month						
soil	**oil**	**0**	**0.5**	**1**	**3**	**5**	**8**	**10**
Norwood	heavy	100	–	–	–	–	100	–
	medium	52	88	30	81	100	100	–
	light	36	53	49	67	68	100	100
Norwood/Baccto	heavy	7	7	100	100	100	–	–
	medium	33	92	44	100	100	–	–
	light	41	63	42	100	100	–	–

[a] Percent survival after 2 weeks incubation in dilutions of oily soil. [b] (–) not done. [c] Solid phase modification.

bioremediated oily soils will contain very low levels of leachable aromatic hydrocarbons. Oily soils of similar composition that are undergoing land treatment remediation would present a very low risk from BTEX infiltration to the subsoil and groundwater environment since it is well known also that BTEX concentrations of 10–5000 μg/L are rapidly biodegraded by naturally-occurring soil microbes (*57*). There have been reports demonstrating the low leachability of oily waste components from soil. Huddleston and Myers (*3*) showed that heavy metals and water-soluble organics leaching was <0.01 to <1% of the total metal and organic content of refinery oily waste during rainfall simulation experiments. Bioremediation studies by Huesemann and Moore (*7*) on a weathered Michigan crude oily soil also showed that no BTEX (<1 μg/L leachate) was detected in batch extractions (pH 7) of the soil. Laboratory lysimeter experiments by Dibble and Bartha (*58*) on land treatment of refinery oily sludges (5% w/w) in an acidic (pH 3.7) sandy loam showed that little or no ether-extractable (O&G determination) material was detected in column leachates of bioremediated waste. More recently, Gould and Pardus (*44*) presented a simple one-dimensional model to describe the potential for migration of organic compounds to groundwater by estimating leaching potential using soil/waste characteristics, contaminant concentrations, rainfall rates, soil hydraulic conductivity, groundwater gradients, and distance to receptor wells. These types of models would be helpful in assessing the mobility of residual hydrocarbons (e.g., BTEX) in treated and untreated oily soils.

Earthworm Survival and Microtox Assays. In estimating the environmental toxicology and efficacy of the bioremediation process on oily soils, we chose tests utilizing representative soil-dwelling species such as earthworms and plants. In the earthworm bioassay, survival of adult *Eisenia* was determined after a 2-week exposure to soil. These results shown in Table 4, Section A, indicate that all oily soils were acutely toxic to *Eisenia* in the first 2–4 weeks of the bioremediation experiment. The Norwood soils with heavy, medium, and light oils were toxic to earthworms for at least 8 months. In contrast, all animals survived in the three

TABLE 5. Effects of Oily Soil Bioremediation on Seed Germination[a]

				% germination in soil					
		TPH-Gr (mg/kg in soil)		untreated			bioremediated		
soil	oil	untreated	bioremediated	corn	wheat	oat	corn	wheat	oat
Norwood	none	ND[c]	523	90, 77[b]	90, 92[b]	89, 87[b]			
	heavy	14 000	7 000	81	89	68	87	87	95
	medium	26 600	8 600	100	81	95	85	82	95
	light	4 200	1 000	74	51[d]	19[d]	82	77	90
Norwood/Baccto	none	ND	523	93, 83[b]	92, 86[b]	70, 92[b]			
	heavy	11 900	10 800	93	86	88	73	72	88
	medium	25 700	8 200	97	25[d]	71	84	89	96
	light	9 600	1 200	4[d]	0[d]	0[d]	89	88	83

[a] Determined after 10 (Norwood) or 8 (Norwood/Baccto) months treatment. [b] Different values represent the variation in seed germination of control (no oil and untreated) soil initially and after 8−10 months. [c] Not done. [d] Values are significantly ($p < 0.01$) less than the control soil with no oil.

TABLE 6. Effects of Oily Soil Bioremediation on Plant Growth[a]

		plant growth (mg dry wt/plant in soil)					
		untreated			bioremediated		
soil	oil	corn	wheat	oat	corn	wheat	oat
Norwood	none	82.1, 68.4[b]	15.2, 18.3[b]	16.6, 14.3[b]			
	heavy	119[c]	16.7	8.2[c]	88.8	16.9	14.3
	medium	123[c]	12.1[c]	8.3[c]	91.3[c]	15.6	16.9
	light	83.7	8.3[c]	5.1[c]	68.9	10.3[c]	8.2[c]
Norwood/Baccto	none	73.4, 58.9[b]	16.3, 18.4[b]	12.5, 13.9[b]			
	heavy	135[c]	19.0	17	40.3[c]	14.5	9.5[c]
	medium	113[c]	9.4[c]	8.2[c]	98.9[c]	18.4	16.5
	light	46[c]	0[c]	0[c]	60.5	18.7	11.7

[a] Determined after 10 (Norwood) or 8 (Norwood/Baccto) months treatment. For corresponding TPH concentrations before and after bioremediation, see Figure 8. [b] Different values represent the variation in plant weight of control (no oil and untreated) soil initially and after 8−10 months. [c] Values are significantly ($p < 0.05$) less than or greater than the control soil with no oil.

Norwood/Baccto oily soils after only 3−5 months treatment. We previously showed (Figure 1) that the maximum reduction in oil hydrocarbons (TPH) was usually after 3−5 months for both soil types. In general, loss in earthworm toxicity appeared to correlate with optimum hydrocarbon biodegradation with the exception of the low organic Norwood soil. It is not known why toxicity persisted in the Norwood soils; however, it is possible that residual or uncharacterized petroleum compounds (undegraded or incompletely metabolized) contributed to the acute effects on *Eisenia* survival. In contrast, hydrocarbons may have degraded more rapidly or were sequestered (not bioavailable) in the higher organic Norwood/Baccto soil.

Results of the solid phase Microtox assay utilizing sensitivity to the luminescent microbe, *Photobacterium*, to dilutions of oily soils are shown in Table 4, Section B. The Microtox test appears to be less sensitive and more variable than the earthworm bioassay. Also, bioremediated soils lose most of their Microtox inhibiting activity after 3 months.

Seed Germination and Plant Growth. Data on the effects of heavy, medium, and light oily soils on the 21-day seed germination and plant growth bioassays before and after bioremediation are summarized in Tables 5 and 6. In the untreated soils, seed germination for corn, wheat, and oat species was inhibited (50−100%) by the presence of 25 000−26 000 and 4200−9600 mg/kg TPH, respectively, of medium and light crude oils. In contrast, seed germination in the bioremediated soils was not significantly different from control soils that contained no crude oil (Table 5). It is interesting to note that the residual TPH in which germination was not affected in both bioremediated soils varied from 7000 to 10 000, from 8200 to 8600, and from 1000 to 1200 mg/kg for the heavy, medium, and light oily soils, respectively.

Results of the effects of oily soils on plant growth (Table 6) show that, in the untreated material, heavy and medium crude oils significantly enhanced growth (mg/plant dry wt) of the corn plant by 40−70% over control plants grown in oil-free soil. The growth stimulating effect was still apparent in the bioremediated soils. This enhanced effect of crude oil hydrocarbons on plant growth has been reported in the literature. Over 75 years ago, Carr (*59*) observed that soybean yields increased at least 50% in field plots of a sandy peat soil with 7500 mg/kg oil from an accidental pipeline release. Concentrations of crude oil in soil ≥25 000 mg/kg, however, affected nodule formation and growth. Also, Baker (*60*) cited (a) studies on increased yields of saltmarsh grass exposed to soils containing a heavy crude fraction (high boiling cut) of Kuwati oil and (b) experiments by Russian workers on increased crop yields associated with a heavy polar oil fraction containing naphthenic acids. In our studies, growth yields of germinated wheat and oat seeds were significantly reduced (20−70% less) in both untreated soils containing medium and light oils. After 8−11 months bioremediation, wheat and oat growth yields were significantly improved and similar to control plants grown in oil-free soil. However, some plant growth inhibition was still apparent in both soil types with the heavy, medium, and light oils. This reduction in growth between plant species (corn, wheat, and oat) varied from 0 to 40% from the control (no oil) soils. These results indicate that undegraded petroleum compounds (other than BTEX) or metabolites may be affecting plant growth. The phytotoxicity of petroleum hydrocarbons has not been studied sufficiently in recent years. Work by Baker (*60*) and Currier and Peoples (*61*) several years ago indicated that high concentrations of light hydrocarbons (octane, decane), aromatics (BTEX), and naphtha(cyclohexanes) and phenolic-like compounds reduced respiration, transpiration, and photosynthesis in grasses (barley, mustard) and crop plants (carrot, citrus). More current experiments by Wang and Bartha (*62*) showed that soybean and rye germination and

dry wt yield in soil lysimeters contaminated with a jet fuel, heating oil, or diesel fuel (55 000−75 000 mg/kg) were significantly improved after 2−5 months biotreatment. In field plot studies of land treating heavy crude oils, Raymond et al. (*26*) observed that although 30−50% of the initial O&G levels (25 000−35 000 mg/kg) were degraded in 6 months, germination and growth of radish, beans, and turnip plants were restricted, indicating that residual hydrocarbons or metabolites were phytotoxic. Huddleston and Myers (*3*) applied a mixed oily waste (15% w/w) to field plots and showed that soils which contained 17 000−22 000 mg/kg residual hydrocarbons had no adverse effects on wheat and bermuda grass germination and growth. These latter studies suggest that hydrocarbon phytotoxicity cannot be predicted and varies widely with oil and soil type, concentration and plant species tested.

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