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Biodegradation and phytotoxicity of crude oil hydrocarbons in an agricultural soil

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ABSTRACT

Land treatment of crude oil is used by the oil industry, since it has been recognized that hydrocarbons (HC) can be metabolized by the indigenous microbial community of soil. The crude oil biodegradation in agricultural soil was studied for 12-mo to determine the HC biodegradation and leaching, the effect of HC on barley productivity and soil properties, and the potential for HC uptake in the plant. Concentration and composition of HC in the soil were periodically determined at a depth of 0 to 75 cm. The HC concentration decreased over time due mainly to the microbial degradation. At the end, 12% of the primary crude oil amount remained constant in the soil. A vertical migration, leaching and metabolic products of HC into subsoil occurred. The HC have changed soil fertility. Barley has been successfully cultivated in soil but the HC reduced some plant growth parameters. However, HC were not detected in plant seeds. Many of oil-utilizing bacteria and fungi were isolated from soil. The HC biodegradation potential of oil polluted soil (6% to 66%) were higher than of unpolluted one (4% to 53%). The bacteria (22% to 66%) were more active than fungi (4% to 46%) in HC biodegradation. The study demonstrated that agricultural lands with low rates of oil contamination allows the growth of plants. They ensure high efficiency of HC biodegradation. Vertical infiltration plays an important role in HC removing from soil. Alkanes were completely assimilated by microorganisms and polar compounds were more resistant to microbial attack.

Key words: Agricultural soil, bacteria, biodegradation, crude oil, fungi.

INTRODUCTION

Environmental pollution due to accidental oil spillage, leakage and disruption of oil pipelines is very common and has become a major concern for governments, ecologists and communities (Ikuesan, 2017). The release of oil into the environment is causing considerable damage to the ecosystem (Han et al., 2016). The toxicity of petroleum hydrocarbons (HC) to organisms is well established (Eze et al., 2014).

Soil pollution with oil is the major global concern today. It may lead to delayed plant growth, soil infertility, and changes in soil physicochemical and microbiological properties (Ikuesan, 2017). It might cause organically contaminated groundwater and poses a significant risk to human health (Chukwura et al., 2016). The oil impacts may lead to lower agricultural productivity and thus adversely affect the socioeconomic of people life (Chorom et al., 2010). The negative impact of oil contamination has necessitated the exploration of many strategies to return contaminated sites to a non-polluted state. The physical and chemical techniques for oil treatment currently used are expensive and not environmentally friendly (Jain et al., 2011). Recently, much effort has been made to treat the oil pollution using natural processes that include bioremediation and phytoremediation (Jahangeer and Kumar, 2013).

Bioremediation is a natural process in which microorganisms transform environmental pollutants into harmless final products to obtain C and energy sources. It is simple way and easy to maintain, applicable to large areas, cost-effective, and conducive to complete destruction of pollutants (Omotayo et al., 2012). Many factors such as nutrients, temperature, oxygen, etc., affect biodegradation of HC (Jahangeer and Kumar, 2013). Basically, there are two different approaches to

bioremediation techniques. The first involves the activation of indigenous microorganisms in the contaminated area by adding nutrients and forming the best environmental conditions (biostimulation). The second is bioaugmentation, which involves adding oil-utilizing or genetically modified microorganisms (Ikuesan, 2017).

A variety of microbes with the ability to metabolize HC can be easily isolated from soil (Eze et al., 2014). The soil with high HC content contains more HC-utilizers than soil with low HC content (Olajire and Essien, 2014). While most HC biodegradation studies focus on bacteria, fewer investigations deal with fungi. Biodegradation of HC in marine environments has been well studied. However, there are lesser studies in soil ecosystems in general and in agricultural soils in particular. Therefore, the objective of this study was to determine the potential HC degradation of bacteria and fungi isolated from contaminated and non-polluting agricultural soils. In addition, HC leaching into the soil was determined and the effects of crude oil on the barley and soil properties were considered. The uptake of HC by plant was also evaluated.

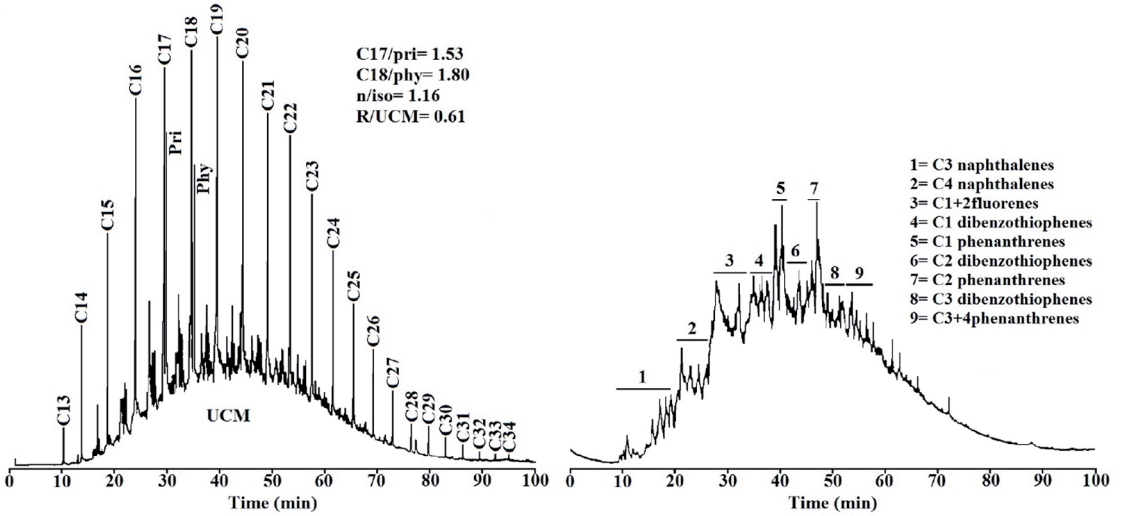
MATERIAL AND METHODS

The study site was located in Abu-Al-Khasib (rural area) (30°27'00" N, 47°59'27" E), Basrah city, southern of Iraq, where the HC contamination is possible from oil spill accidents and oil wastes discharged from various agricultural machines and residents in the region. A tow experimental fields (200 m^2) were designed in an agricultural soil during November 2016 to October 2017. The soil characteristics are shown in (Table 1). One of the fields (F1) was spread with 12 g m^{-2} of Nahran-Omer crude oil with an agricultural spreader. The gas chromatographic analysis of the initial crude oil composition is demonstrated in Figure 1. The crude oil composed of 50% aliphatic HC, 40% aromatics, and 10% polar compounds. The other field (F2) was left unpolluted (control). A drainage system was designed and buried at a distance

Table 1. Characteristics of the agricultural soil at 0 to 75 cm depth.

	0-15 cm	15-30 cm	30-45 cm	45-60 cm	60-75 cm
pH	7.8	7.7	7.6	7.6	7.5
Silt, %	31.3	33.7	34.2	36.1	35.0
Clay, %	71.5	73.4	68.7	68.2	67.2
Sand, %	4.5	3.2	4.0	3.3	4.1
Texture	Clay	Clay	Clay	Clay	Clay
Total Ca, %	0.4	0.4	0.2	0.3	0.2
P_2O_2, %	0.1	0.07	-	-	-
Total N, %	0.1	0.07	0.06	0.04	0.03
Organic matter, %	2.6	1.8	1.0	0.4	0.1
C/N	7.7	7.4	7.6	7.3	7.1

Figure 1. Chromatograms of crude oil hydrocarbons analysis.



Pri: Pristane; Phy: Phytane; n/iso: n-alkanes/iso-alkanes; R/UCM: resolved n-alkanes/unresolved complex mixture; UCM: unresolved complex mixture.

of 50 cm below the soil surface, allowing leachate collection from F1 and F2. Both fields were immediately tilled after oil treatment and barley (*Hordeum vulgare* L.) cultivation. Sowing was performed 2 d after crude oil spreading during November 2016. Five days later, fields were fertilized with 2.8 kg N, 2.8 kg P_2O_2 and 2.8 kg K_2O, applied to 200 m^2. Soil samples were collected from both fields at different periods (7, 14, and 21 d and 1, 2, 4, 6, 8, 10, and 12-mo) and depths (0 to 75 cm). The samples were homogenously mixed and carefully sorted to remove unwanted soil debris. Barley was harvested in April 2017. Plant density, height, and grain yield were measured in the two fields. Seed samples were collected from the plant when harvested. Drainage water samples were periodically collected from drainage system during rain periods.

Soil samples (50 g) and seeds were dried (60 °C for 12 h) and Soxhlet-extracted for 8 h with 250 mL chloroform. The HC were extracted from water with 50 mL chloroform L^{-1} water, three times in separator funnel for 30 min. The extract was reduced in volume by a rotary evaporator, dried by Na_2SO_4, and concentrated by pure N. The extract was then purified by percolating on florisil column (60 to 100 mesh), evaporated in a pre-weighed dish and weighed. It was then fractionated to aliphatic, aromatic, and polar fractions by successive elution with n-hexane, benzene, and methanol, respectively, on chromatography column. The column was prepared by slurry packing 10 g silica, followed by 10 g alumina and finally 1 g Na_2SO_4 was added to the surface. The fractions were concentrated and transferred to a vial. A sample (1 µL) of aliphatic or polar fraction was analyzed by capillary gas chromatography (GC; Agilent, Santa Clara, California, USA). Helium gas was used as the carrier gas with flow rate of 1.5 mL min^{-1}. The operating temperatures for detector and injector were 350 °C. The silica capillary column was operated under temperature programming, 100 to 320 °C, 3 °C min^{-1}. The aromatic fraction (1 µL) was identified by gas chromatography-mass spectrometry (GC-MS; QP-1000A, Shimadzu, Kyoto, Japan) interfaced with mass-selective detector (MSD) using the same chromatographic conditions. Individual HC were identified based on the retention time and m/z ratio of the HC authentic standards. The HC concentrations were calculated based on the standard calibration curve of each corresponding standard compound. For quality assurance and quality control, method blanks were analyzed. None of the target compounds was detected. The recovery and relative standard deviation for aliphatic HC were in the range of 83% to 92% and 3.9% to 18.6% respectively. The detection limits of the method range from 4.2 to 13.3 µg g^{-1}. For aromatics, the average recoveries of standards varied from 83.5% to 91% and the detection limit ranged from 0.2 to 0.8 ng g^{-1}.

Enumeration and isolation of heterotrophic bacteria (HB) and fungi (HF) and oil-utilizing bacteria (OUB) and fungi (OUF) from soil samples were performed by dilution plate method. Nutrient agar (NG) medium, Sabouraud dextrose agar (SDA) medium and mineral medium (MM) were used to enumerate and isolate the HB, HF and OUB and OUF respectively. A 7-fold serial dilutions were made using 1 g soil and 1 mL from appropriate dilutions were plated in duplicate on culture media by pour plate method. The plates were swirled, allowed to solidify and incubated at 28 ± 2 °C for 7 d for HF and 21 d for OUF and at 37 °C for 24 h for HB and 21 d for OUB. The MM contained 0.1 mL sterile crude oil into a filter paper lining every Petri-dish cover. It was composed of 1 g $NaNO_3$ L^{-1}, 0.6 g KH_2PO_4 L^{-1}, 0.9 g Na_2HPO_4 L^{-1}, 0.2 g K_2SO_4 L^{-1}, 0.4 g $MgSO_4 \cdot 7H_2O$ L^{-1}, 0.7 g $CaCl_2 \cdot 2H_2O$ L^{-1}, 2.5 mL trace element mixture (2.3 g $ZnSO_4$ L^{-1}, 1.8 g $MnSO_4$ L^{-1}, 0.6 g H_3BO_3 L^{-1}, 1 g $CuSO_4$ L^{-1}, 0.4 g Na_2MoO_4 L^{-1}, 0.4 g $CoCl_2$ L^{-1}, 0.7 g KI L^{-1}, 1 g EDTA L^{-1}, 0.4 g $FeSO_4$ L^{-1}, and 0.004 g $NiCl_2$ L^{-1}) and 20 g L^{-1} agar. Chloramphenicol was added (500 mg L^{-1}) to the fungi MM for bacterial growth inhibition.

The bacterial and fungal colonies were enumerated and recorded as colony forming units (CFU) 1 g^{-1} soil sample. Pure cultures were obtained by repeated sub culturing on fresh NG and SDA media. The pure cultures were maintained on agar slants. Each picked individual colony of bacteria and fungi was cultured on liquid MM (50 mL) in 250 mL Erlenmeyer flasks to which 0.1 mL of crude oil was added. After 21 d of incubation in an orbital shaker set at 150 rpm in 37 °C (bacteria) and 28 ± 2 °C (fungi), the strains that showed a visible growth were scored as potentially able to use crude oil. Fungi were identified according to general principles of fungal classification (Gadd et al., 2007). A primary bacterial identification was performed according to their morphological characteristics and biochemical tests (Holt et al., 1994). The bacterial strains molecular identification were done based on 16S rRNA, the DNA extraction kit (Sigma, USA) was used to extract bacterial DNA. The 1500 bp 16S rRNA fragment was amplified by polymerase chain reaction (PCR) using the universal primers (FD1 and RP1). The amplification products were subjected to electrophoresis on agarose gel followed by ethidium bromide staining. The primers were removed and the purified PCR products were sequenced (Macrogen, Seoul, Korea). The obtained sequences were identified by compared them with those of GenBank database using the Blast server at National Center for Biotechnology Information (NCBI, Bethesda, Maryland, USA).

The microbial strains were separately tested for their biodegradation potential in 250 Erlenmeyer flasks containing 150 mL MM and 180 mg crude oil. Control flasks were left without microbes and containing 500 mg $HgCl_2$ L^{-1} to quantify the abiotic losses by evaporation. The flasks were incubated at 37 °C (bacteria) and 28 ± 2 °C (fungi) in an orbital shaker set at 150 rpm for 30 d. The residual HC were then extracted from culture medium with 50 mL chloroform and filtered through sterile 0.45 µm Millipore filter. The extract was reduced in volume to measure the residual oil by spectrofluorometer (RF-540; Shimadzu, Kyoto, Japan) equipped with a DR-3-data recorder. The HC were quantified by measuring the emission intensity at 360 nm, with excitation set at 310 nm and monochromatic slits of 10 nm. The percentage biodegradation of each strain was then determined by the equation:

$$(I - R) \times R \times 100/I \quad [1]$$

where *I* and *R* are the initial and residual HC contents respectively. Thereafter, the extract was separated to aliphatic, aromatic and polar fractions and analyzed by GC and GC-MS using the same previous chromatographic conditions. The percentage biodegradation of each fraction was determined as Equation [1].

Total organic C (TOC) was measured in water samples by wet oxidation method of Walkey and Black. Data statistical analysis was performed by ANOVA, with differences determined by the least significant differences method at the 5% level ($P < 0.05$). Statistical analysis were run with SPSS 16.0 version for Windows program (IBM, Armonk, New York, USA).

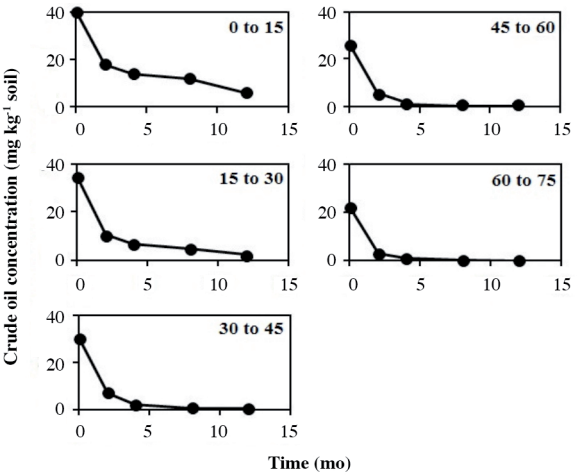
RESULTS AND DISCUSSION

The GC analysis reflected that the F2 soil was composed mainly of biogenic n-alkanes (C25 to C33) with Carbon Preference Index (CPI) value of 8. Most of these HC (1 to 2 mg kg^{-1}) were detected at 0 to 30 cm soil depth, while less amount of them (0.2 to 1 mg kg^{-1}) were existed in 30 to 75 cm depth. The study showed that the crude oil in F1 can be biodegraded by agricultural soil microbial community. After 7 d, HC concentration in soil decreased to 37.5 mg kg^{-1} at 0 to 15 cm depth and 21.1 mg kg^{-1} at 60 to 75 cm depth.

Initially, the GC analysis revealed that only the C14 to C20 n-alkanes were presented in 30 to 75 cm soil depth. This selective migration of lightest HC may due to the chromatographic characteristics of the clays (Gawdzik and Zygadlo, 2010). Thereafter, the n-alkanes in the range of C14 to C30 were detected in all soil depths, indicating that the subsequent vertical migration of HC gradually involved all crude oil. Such selective vertical infiltration of HC has also been shown previously in contaminated soils (Essaid et al., 2011). After 2-mo of experiment, most of the crude oil (10 to 18 mg kg^{-1}) was located at 0 to 30 cm soil depth. The crude oil concentration in this depth decreased continuously over time to 1.5 to 6 mg kg^{-1} after 12-mo (Figure 2).

The n-alkanes disappeared completely between 8 to 12-mo. The C17/pristane, C18/phytane, and (R)/unresolved complex matters Resolved n-alkanes/unresolved complex mixture (R/UCM) ratios decreased. The isoprenoids, pristane

Figure 2. Crude oil concentration at 0-15, 15-30, 30-45, 45-60 and 60-75 cm depths in soil of oil treated field (F1).



(pri) and phytane (phy) and GC resolved aromatic were resistant even after 12-mo with a significant decline in their concentrations were detected. It was reported that the low molecular weight HC tend to be degraded first, leaving behind the much larger molecules which take much longer to break down (Das and Chandran, 2011). The biogenic HC (C25 to C33) and crude oil HC traces did not degraded, although it was found that such HC were completely biodegraded by laboratory studies (Jahangeer and Kumar, 2013). This may be due to the fact that these HC in soil are associated with organic and mineral matter in such a way that they are protected from microorganisms consumption (Jahangeer and Kumar, 2013) (Table 2 and Figure 3).

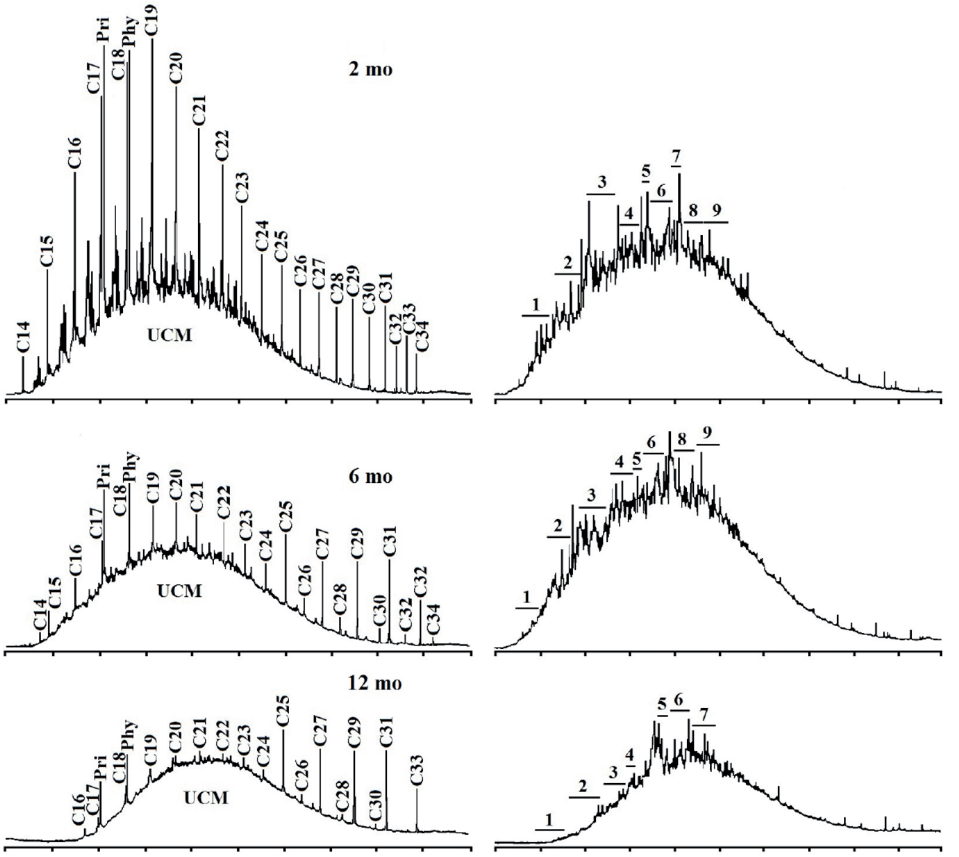
The total residual of UCM consisted of 8% HC. These were relatively the same HC amount existing in UCM of initial crude oil, indicating the high UCM resistance for degradation. The UCM fundamentally consisted of polycyclic alkylated saturated and aromatic HC and T-shaped molecules. These components are known for their resistance to biodegradation and by their harmful environmental effects, especially the carcinogenic polycyclic aromatic HC (PAHs) (Guo et al., 2014).

Table 2. Hydrocarbons ratios indicating biodegradation of crude oil at 0 to 15 cm soil depth.

Time	C17/Pri	C18/Phy	R/UCM
7 d	0.75	0.84	0.18
2 mo	0.63	0.65	0.11
6 mo	0.50	0.51	0.07

Pri: Pristane; Phy: phytane; R/UCM: resolved n-alkanes/unresolved complex mixture.

Figure 3. Chromatograms showing biodegradation of crude oil hydrocarbons in soil of oil treated field (F1) at 0 to 15 cm depth over time.



Pri: Pristane; Phy: phytane; UCM: unresolved complex mixture.

The total HC concentration of all soil depths were calculated and plotted against time (12-mo) (Figure 4). The HC loss rate (biodegradation) for the whole experiment was 5.4 g m^{-2} mo^{-1}. This rate was high at the beginning of the experiment (first 6-mo) (7.9 g m^{-2} mo^{-1}) compared to its end (remaining months) (3 g m^{-2} mo^{-1}). The total HC loss was up to 88% after 12-mo. In general, the biodegradation rate of this study was relatively similar to that reported by Dadrasnia and Agamuthu (2013). However, the previous laboratory studies (Makadia et al., 2011; Omotayo et al., 2012) have indicated that the biodegradation removed about > 80% of petroleum HC. This is suggested other abiotic factors involved in removing HC in the current study as well as the biodegradation process. It has been found that HC vertical flow into the soil may be one of the most abiotic mechanisms that contribute to the HC loss from soil surface (Gawdzik and Zygadlo, 2010).

The results showed that the HC concentration within the soil column (0 to 75 cm depth) ranged from 0.12 μg g^{-1} d^{-1} after a few days of experimental onset to less than 0.02 µg g^{-1} d^{-1} after 12-mo. This means that approximately 9% of the initial loading of HC has migrated to soil depth of 75 cm or more throughout the 12-mo. This occurs in accidental oil spills and in the agricultural soils where a large amounts of surface HC migrate deep into the soil. The HC that penetrate the soil column can biodegrade slowly because of the anaerobic conditions (Essaid et al., 2011).

The GC analysis revealed the presence of HC in the drainage water of F1. These compounds were due to the metabolic products leaching from petroleum biodegradation. The compounds may consist of organic acids and aromatic ketones (Das and Chandran, 2011). The study indicated that there is no significant difference in drainage water TOC of F1 and F2 during the first month of the experiment. In December 2016 and January 2017 the TOC was 3 times higher in F1 (7 to 8 mg L^{-1}) than in F2 (4 to 5 mg L^{-1}) (Figure 5). The HC concentration in drainage water of F1 was about 0.02 mg L^{-1}.

The amount of excess soluble organic C was calculated on the whole experiment and was found to be in the range of 0.14 g m^{-2}. The HC leaching considered abiotic way to crude oil loss from soil surface (Abatenh et al., 2017). The present study has detected the presence of numbers of microorganisms in drainage water. These microbes may play an important role in the HC degradation in drainage water and subsoil.

Figure 4. Crude oil concentration in soil of oil treated field (F1) against the time at 0-75 cm depth.

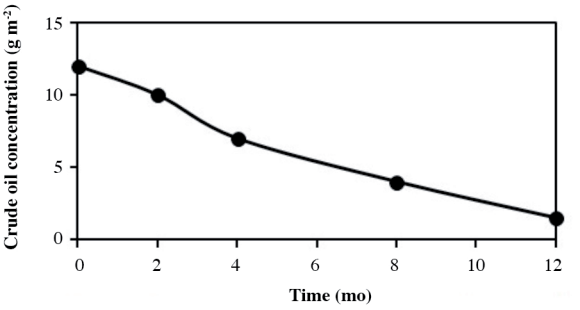
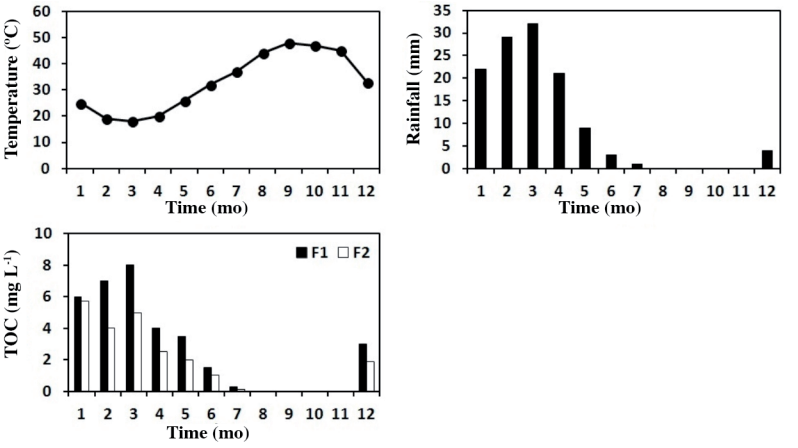


Figure 5. Temperature, rainfall and total organic C (TOC) in drainage water during the time of experiment.



No effects of HC on seedling and density of barley were observed. Li et al. (2013) and Han et al. (2016) found that the germination responses of a number of plants to HC varied greatly depending on the type of plant. A considerable effects of crude oil on the barley physiology were detected. These included a reduction in the leaves number, acquisition of leaves a reddish color, delayed flowering, height reduction, and a significant decrease in grain yield (Table 3). This may be due to phytotoxic effects of petroleum HC (Li et al., 2013). The similar effects were reported by Fowzia Fakhruddin (2018) on the growth of maize in crude oil contaminated soil. However, the petroleum HC in barley seeds were not detected. The GC analysis only revealed a biogenic HC (C20 to C30) in seeds.

The crude oil caused a change in characteristics of F1 soil (0 to 15 cm depth) included an increase in the TOC and pH and decrease in available P and exchangeable K. However, these modified in chemical fertility of soil did not affect the biodegradation process and plant growth. The Ca content and total and mineral N were not affected by crude oil (Table 4). The amount of mineral N added as fertilizer for plant cultivation was appropriate for biodegradation and plant growth. The P and K were also in sufficient amounts to stimulate the microbial degradation and plant growth. The temperature was typical for biodegradation. The temperature is one of the most important factors affecting the microbial HC degradation (Jahangeer and Kumar, 2013). Such effects of crude oil on soil parameters had also been reported by Hajabbasi (2016).

After 8-mo, the current study showed that there was almost an increase of 1000-fold and 10-fold for the OUB and HB, respectively, and by about 100-fold and 50-fold for the OUF and HF, respectively in 0 to 15 cm soil depth of F1, compared to F2 (Figure 6). Where more than 88% of bacteria and fungi were adapted to crude oil utilization. The bacteria and fungi were found in the 15 to 75 cm soil depth (Table 5). It had usually been found that the numbers of aerobic microorganisms decreased with the soil depth (Jahangeer and Kumar, 2013). Tillage and drainage system enhanced soil ventilation and allowed microbial growth in the entire soil. The numbers of OUB and OUF were higher in the 0 to 45 cm soil depth where the concentration of HC was at maximum and in the 45 to 75 cm depth due to selective migration of light HC into soil (Essaid et al., 2011).

In F2, tilling and soil fertilization did not caused a significant change in the numbers of bacteria and fungi at 0 to 15 cm soil depth. The ratios of OUB/HB and OUF/HF remained perfectly stable, about 1%. When the experiment is over, the microorganisms levels in F1 become similar to F2 (Figure 6).

Numerous genera of OUB and OUF were isolated and identified from F1 and F2 soils (Tables 6 and 7). Most of these genera had already been found in the oil contaminated sites elsewhere and identified as oil degraders (Dadrasnia and Agamuthu, 2013; Adams et al., 2015). However, some species such as *Acremonium fusidioides*, and *Cunninghamella echinulata* are not cited as oil degraders by other authors. The percentage biodegradation results of bacterial and fungal strains showed that these strains were able to degrade the petroleum HC at different rates (Tables 6 and 7). The HC assimilation between 32% and 66% and 30% and 46% were detected by active strains of OUB and OUF, respectively.

Table 3. Toxic effect of crude oil hydrocarbons on mature barley.

Character	F2	F1
Plant density, plant m^{-2}	302	290
Plant height, cm	85	76
Plant yield, g 200 m^{-2}	140	121

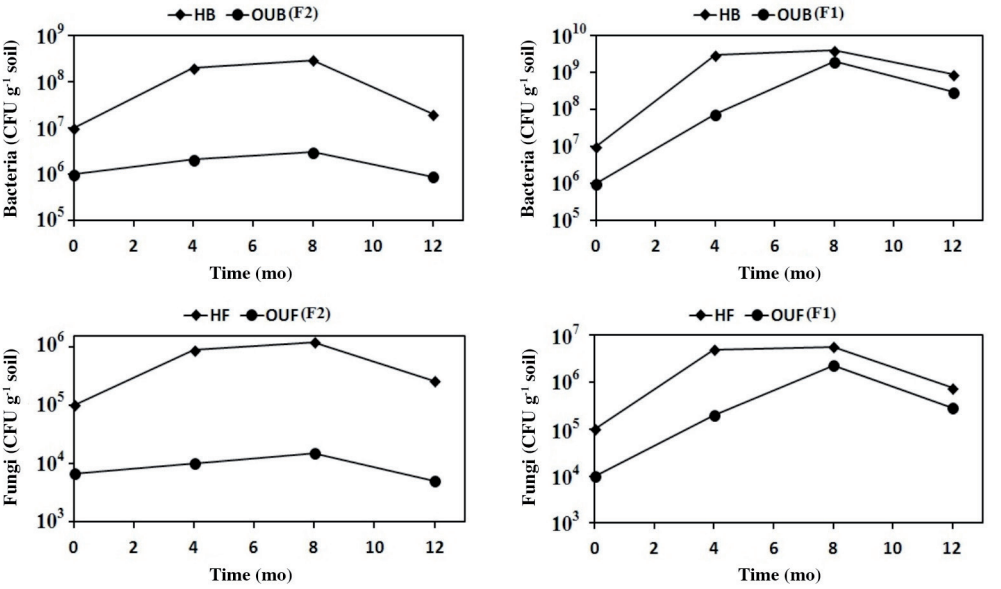
F1: Oil treated field; F2: untreated field (control).

Table 4. Characteristics of soil at 0 to 15 cm depth.

	F2			F1		
Soil property*	7 d	4 mo	12 mo	7 d	4 mo	12 mo
TOC, %	7.1	8.3	8.9	9.5	9.7	9.9
Total Ca, %	0.40	0.39	0.42	0.38	0.37	0.29
pH	7.1	6.9	6.7	7.1	7.3	7.5
P_2O_2, %	0.14	0.12	0.11	0.07	0.08	0.09
K_2O, %	0.20	0.14	0.10	0.18	0.10	0.09
Total N, %	1.0	1.0	0.9	1.2	1.1	0.9
Mineral N, %	0.05	0.06	0.07	0.04	0.07	0.06

F1: Oil treated field; F2: untreated field (control); TOC: total organic C.

Figure 6. Changes in bacterial and fungal numbers at 0-15 cm depth in soils of oil treated field (F1) and untreated field (control) (F2) over time.



HB: Heterotrophic bacteria; OUB: oil-utilizing bacteria; HF: heterotrophic fungi; OUF: oil-utilizing fungi; CFU: colony forming units.

Table 5. Bacterial and fungal counts in oil treated field (F1) and untreated field (control) (F2) at 0-75 cm depth.

		0-15 cm	15-30 cm	30-45 cm	45-60 cm	60-75 cm
		CFU g^{-1} soil				
F2	HB	2×10^8	1.3×10^8	0.5×10^8	0.1×10^8	0.09×10^8
	OUB	2.1×10^6	1.4×10^6	0.8×10^6	0.1×10^6	0.01×10^6
	(OUB/HB) × 100	0.01	0.01	0.01	0.01	0.01
	HF	8.8×10^5	5.7×10^5	2.6×10^5	1.8×10^5	0.5×10^5
	OUF	9.9×10^3	7.2×10^3	4.9×10^3	3×10^3	0.7×10^3
	(OUF/HF) × 100	0.01	0.01	0.01	0.01	0.01
F1	HB	3×10^9	2.2×10^9	1.3×10^9	0.2×10^9	0.07×10^9
	OUB	7.6×10^7	4.7×10^7	2×10^7	0.1×10^7	0.02×10^7
	(OUB/HB) × 100	2.5	0.02	0.01	0.05	0.2
	HF	5×10^6	4.5×10^6	2.1×10^6	1.4×10^6	0.3×10^6
	OUF	20×10^4	13.2×10^4	8.9×10^4	5×10^4	1.3×10^4
	(OUF/HF) × 100	0.04	0.02	0.04	0.03	0.04
	(HB in F1/HB in F2) × 100	1000	1600	2600	2000	700
	(HF in F1/HF in F2) × 100	500	700	800	700	600
	(OUB in F1/OUB in F2) × 100	3600	3300	2500	100	200
	(OUF in F1/OUF in F2) × 100	2000	1800	1800	1600	1800

CFU: Colony forming units; HB: heterotrophic bacteria; OUB: oil-utilizing bacteria; HF: heterotrophic fungi; OUF: oil-utilizing fungi.

It had been shown that bacteria were more active than fungi in the HC biodegradation (Ikuesan, 2017). Such conclusion had also been found by the present study. The mean of HC biodegradation percentage by OUB was 42% and 20% by OUF. However, Omotayo et al. (2012) found that the fungi were more active than bacteria in the HC biodegradation. Most biodegradation studies have shown that the potential for microbial biodegradation isolated from oil-contaminated sites was higher than those obtained from non-contaminated sites (Olajire and Essien, 2014). The same finding was arrived in this study. This suggested that crude oil contamination of soil caused adaptation to microbial population for HC biodegradation (Atlas and Hazen, 2011).

The most active isolated strains in the HC degradation were *Arthrobacter polychromogenes*, *Bacillus cereus*, *Bacillus polymyxa*, *Bacillus subtilis*, *Corynebacterium* sp., *Flavobacterium indologenes*, *Micrococcus* sp., *Pseudomonas fluorescens* (bacteria), *Penicillium chrysogenum*, *Fusarium solani*, and *Aspergillus restrictus* (fungi).

The GC analysis revealed that the alkenes were generally the most biodegraded compounds. The pri and phy were the most resistant to microbial breakdown as shown by the ratios of C17/pri and C18/phy. The UCM breakdown significantly when the overall rate of biodegradation was increased (Tables 6, 7 and 8). The total or partial resistance of microorganisms to biodegrading isoprenoids and cycloalkanes is well known (Gros et al., 2014).

The n- and branched alkanes were partially degraded by *Aspergillus niger*, *Candida utilis*, *Penicillium pinophilum*, *Psilocybe strictipes*, *Rhizopus stolonifer*, and *Saccharomyces cerevisiae*. Whereas, n-alkanes were completely assimilated by *Arthrobacter polychromogenes*, *Bacillus cereus*, *Bacillus polymyxa*, *Bacillus subtilis*, *Corynebacterium* (AZe26), *Corynebacterium* (AZe54), *Corynebacterium* (AZe67), *Flavobacterium indologenes*, and *Pseudomonas fluorescens*, which also utilized 95% of branched alkanes. Aromatic HC were particularly less degraded than alkenes. An exception exists for *C. utilis*, *P. pinophilum*, *Rhizopus oryzae*, and *S. cerevisiae* that have been biodegraded aromatics at the same rate as alkenes.

Polar fraction was difficult to degrade by microorganisms. There was no oil utilizing microbes that can breakdown the polar fraction significantly. This is due to the fact that this fraction consists mainly of complex structure molecules that are resistant to microbial attack (Atlas and Hazen, 2011). These molecules include resins, N, S and oxygen heterocyclic compounds (Minai-Tehrani et al., 2015). However, Jahangeer and Kumar (2013) reported that some of oil utilizers, especially fungi, were well able to degrade the resin molecules. The degradation rates of organic compounds in petroleum mixture vary widely. The n-alkanes biodegradation is more rapid, followed by simple aromatics, whereas cycloalkanes and aromatics degrade more slowly (Das and Chandran, 2011).

Table 6. Oil-utilizing bacteria (OUB), soil depth of isolation of the bacterial strains from oil treated field (F1) and untreated field (control) (F2), and biodegradation of hydrocarbons (HC).

OUB, strain code, and GC pattern	Depth (F1)	Depth (F2)	Alkanes	Aromatics	Polar	Total HC
	cm		%			
Acinetobacter calcoaceticus[C] (AZe45)	-	0-15	32	6	- 43	25 ± 7
Acintobacter baumanii[C] (AZe73)	-	30-45	36	9	-26	26 ± 5
Aeromonas sp.[D] (AZe31)	0-15	0-15	51	18	-7	41 ± 13
Alcaligenes denitrificans[D] (AZe44)	15-30	-	52	18	-18	44 ± 6
Arthrobacter polychromogenes[E] (AZe20)	0-30 and 60-75	-	80	52	0	66 ± 8
Bacillus cereus[E] (AZe79)	15-30	-	61	28	-10	50 ± 9
Bacillus polymyxa[E] (AZe34)	0-30	-	67	32	-37	54 ± 3
Bacillus pumilus[D] (AZe85)	0-15	-	59	25	-15	48 ± 9
Bacillus subtilis[E] (AZe58)	0-15 and 30-45	0-30	75	42	-38	59 ± 5
Corynebacterium[E] (AZe26)	0-15	0-30	63	25	-33	52 ± 5
Corynebacterium[E] (AZe54)	45-60	60-75	77	46	-16	61 ± 6
Corynebacterium[E] (AZe67)	0-45	-	74	38	-53	58 ± 8
Flavobacterium indologenes[E] (AZe66)	0-30	-	62	27	-9	52 ± 9
Micrococcus sp.[E] (AZe55)	0-15	-	67	34	-4	53 ± 1
Moraxella sp.[D] (AZe94)	-	0-15	42	13	-26	35 ± 8
Mycobacterium sp.[D] (AZe72)	0-15	-	47	15	-35	38 ± 7
Nocardia sp.[C] (AZe87)	-	0-30	38	11	-47	37 ± 11
Pseudomonas cepacia[D] (AZe32)	0-30	15-30 and 45-60	57	22	0	46 ± 1
Pseudomonas fluorescens[C] (AZe95)	0-30	30-45	63	30	-22	53 ± 8
Pseudomonas putida[D] (AZe19)	0-15, 30-45 and 60-75	30-45	50	16	0	43 ± 6
Pseudomonas pumilus[C] (AZe84)	-	15-30	37	10	-30	29 ± 4
Pseudomonas vesicularis[C] (AZe2)	-	60-75	33	9	-44	24 ± 7
Rhodococcus sp.[C] (AZe46)	-	15-30	32	7	-19	25 ± 4
Staphylococcus auriculans[D] (AZe63)	-	0-30	40	11	-53	32 ± 8
Vibrio sp.[C] (AZe28)	-	0-15	31	5	-42	22 ± 9
Xantomonas sp.[D] (AZe62)	15-30	-	45	14	-36	34 ± 10

GC: Gas chromatographic.
[A-E]Patterns of gas chromatographic analysis of hydrocarbons after 30 d of biodegradation (see Table 8).

Table 7. Oil-utilizing fungi (OUF), soil depth of isolation of the fungal strains from oil treated field (F1) and untreated field (control) (F2), and biodegradation of hydrocarbons (HC).

OUF and GC pattern	Depth (F1)	Depth (F2)	Alkanes	Aromatics	Polar	Total HC
	cm		%			
Acremonium fusidioides[C] (Nicot) W. Gams	0-15	-	39	29	-5	28 ± 4
Acremonium kiliense[D] Crutz	15-45	-	40	31	-12	26 ± 8
Acremonium strictum[D] W. Gams	0-15	-	45	33	-22	30 ± 4
Aspergillus ustus[C] (Bain) Thom and Church	-	0-15	23	16	-10	13 ± 5
Aspergillus restrictus[D] G. Smith	0-15	-	47	32	-22	40 ± 6
Aspergillus terreus[C] Thom	-	0-15	20	11	-5	10 ± 4
Aspergillus niger[B] Van Tieghem	-	15-30 and 60-75	11	5	-13	8 ± 10
Candida utilis[A] Minter	-	0-15	8	10	-10	7 ± 11
Cladosporium herbarum[C] (Persoon:Fries) Link	45-75	0-15	30	22	-22	22 ± 3
Cunninghamella echinulata[C] (Thaxt.) Thaxt. ex Blakeslee	0-30	0-15	32	26	-2	21 ± 4
Cunninghamella elegans[C] Lendner	15-30	-	37	21	-15	31 ± 5
Fusarium solani[D] (Martius) Saccardo	0-15	-	43	30	-14	41 ± 7
Microascus cinereus[C] (Emile-Weil and Gaudin) Curzi	-	0-15	20	12	-6	9 ± 13
Penicillium chrysogenum[D] Thom	0-15	-	54	32	-28	46 ± 9
Penicillium pinophilum[B] Gilman and Abbott	-	0-15 and 45-60	10	11	-13	5 ± 11
Psilocybe strictipes[B] Singer and A.H. Smith	0-30	-	16	10	-11	6 ± 12
Rhizopus oryzae[C] Went and Prins. Geerl.	15-30	-	21	22	-20	11 ± 3
Rhizopus stolonifer[A] Vuillemin	-	60-75	7	8	-15	4 ± 10
Sordaria fimicola[C] (Roberge ex Desm.) Ces. and De Not.	15-30	0-15	39	27	-30	27 ± 2
Saccharomyces cerevisiae[A] Meyen ex E.C. Hansen	-	0-45	9	9	-10	4 ± 12
Talaromyces flavus[C] (Klocker) Stolk and Smson	0-15	-	38	25	-24	22 ± 5
Trichoderma harzianum[C] Rifai	-	0-15	25	14	-22	15 ± 6
Trichoderma koningii[C] Oudemans	15-45	-	37	24	-11	23 ± 7
Trichoderma pseudokoningii[C] Oudemans	0-15 and 30-45	15-30	36	18	-11	20 ± 4
Trichoderma viride[C] Pers.	30-45	15-60	34	20	-23	28 ± 5

GC: Gas chromatographic.
[A-E]Patterns of gas chromatographic analysis of hydrocarbons after 30 d of biodegradation (see Table 8).

Table 8. Gas chromatographic (GC) patterns, hydrocarbons (HC) biodegradation and HC ratios indicating crude oil biodegradation.

	GC pattern A	GC pattern B	GC pattern C	GC pattern D	GC pattern E
% Biodegradation (% B)	1 to 9	10 to 19	20 to 39	40 to 59	60 to 80
% B of n-alkanes	36 ± 2.1	85 ± 5.3	100	100	100
% B branched alkanes	9 ± 1.1	37 ± 3.4	56 ± 6.7	89 ± 5.5	95 ± 2.4
% B cycloalkanes	5 ± 0.7	14 ± 2.6	35 ± 7.3	57 ± 9.4	66 ± 3.6
C17/Pri	1.34 ± 0.13	0.26 ± 0.02	-	-	-
C18/Phy	1.55 ± 0.01	0.28 ± 0.03	-	-	-
n/iso	0.90 ± 0.2	0.32 ± 0.03	-	-	-
R/UCM	0.43 ± 0.2	0.23 ± 0.1	0.18 ± 0.05	0.15 ± 0.03	0.09 ± 0.02

Pri: Pristane; Phy: phytane; n/iso: n-alkanes/iso-alkanes; R/UCM: resolved n-alkanes/unresolved complex mixture.

CONCLUSIONS

The study has shown that agricultural lands contaminated with low oil concentration can allow the growth of plants with minor adverse effects and does not require fertilization or soil treatment other than those required for traditional farming operations. They ensure hydrocarbons (HC) biodegradation in high efficiency with lesser environmental impact. However, some of HC remain stationary in the soil as organic matter. The vertical infiltration of HC in the soil plays an important role in removing of these contaminants. When petroleum HC contaminate soil, they stimulate the oil degrading microorganisms activity. The microbial degraders from oil-contaminated soil utilize the HC more than those from non-contaminated soil. The microbial degraders are found in the entire 0 to 75 cm soil depth as a result of soil ventilation. The

bacteria were more efficient in HC degradation than fungi. Alkanes were the most degraded HC. Isoprenoids, cycloalkanes and aromatics were less degraded. Polar compounds were resistant to microbial attack. Biogenic HC were not broken by microbes. The release of HC metabolic compounds by degraders was demonstrated. Attention should be given to environmentally stable carcinogenic polycyclic aromatic HC.

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