Iran. J. Environ. Health. Sci. Eng., 2010, Vol. 7, No. 4, pp. 319-326

BIOREMEDIATION OF A CRUDE OIL - POLLUTED SOIL BY APPLICATION OF FERTILIZERS

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Received 26 August 2009; revised 3 June 2010; accepted 20 August 2010

ABSTRACT
Oil pollution is a worldwide threat to the environment and the remediation of oil-contaminated soils, sediments and water is a major challenge for environmental research. Bioremediation is a useful method for soil remediation, if pollutant concentrations are moderate and non-biological techniques are not economical. The bioremediation consists strategy of actively aerating the soils and adding fertilizer in order to promote oil biodegradation by indigenous microorganisms. The objective of this study was to investigate whether agricultural fertilizers (N, P, K) enhance the microbial degradation of petroleum hydrocarbons in soil. Artificially polluted soil with %1density of crude oil was used and then fertilizers were applied in 3 levels of 0, 1 and 2 ton/ha in 3 replicates. The soils were kept in 30 ºC and 60 percent of field capacity condition for 5 to 10 weeks. To provide the necessary aeration, the soils were tilled twice a week by shovel. Soil sample were analyzed for hydrocarbon-degrading heterophic bacteria count and some soil chemical properties. Residual oil was measured by oil soxhlet extraction method, and gas chromatography. The results showed that the hydrocarbon-degrading and heterotrophic bacteria count in all the treatments increased with time and heterotrophic bacteria population increased from 6×10^3 cfu/g soil to 1.4×10^8 cfu/g soil. Also, soil C/N ratio decreased from 6 to 3. The results indicated that the applied fertilizer increased the degradation of the hydrocarbons compared with the control. Gas chromatography results showed that normal paraffin and isopernoid (Phitane and Pristane) decreased in the range of 45 to 60 percent in all treatments. Furthermore, the results showed that the application of fertilizers at 2 ton/ha rate in oil-contaminated soil lead to greater rates of biodegradation after 5 weeks indicating the feasibility of bioremediation.

Key words: Bioremediation; Fertilizer; Heterotrophic bacteria; Crude oil; Normal paraffin

INTRODUCTION
Commercially explored since the middle of the 19th century, petroleum has been used for many decades for illumination and, on a smaller scale, as lubricant. The invention of the internal combustion engine and its fast adoption in all transport forms enlarged the employment of this natural resource, thus increasing its demand production, transport, stockpiling, and distribution, as well as the raw oil and its by-products. All these activities involve pollution risks that can be minimized, but not totally eliminated, causing several problems for the environment (Pala *et al*., 2006).

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The application of biotechnological processes involving microorganisms, with the objective of solving environmental pollution problems, is rapidly growing, in recent decades, where petroleum and its by-products are concerned. Bioremediation processes, which take advantage of microbial degradation of organic and inorganic substances, can be defined as the use of microorganisms to remove environmental pollutants of soils, water and sediments (Pala *et al*., 2006). The bioremediation process presents countless advantages in relation to other processes employed to remove pollution such as extraction with solvents addition of chemical oxidizers, etc.

(Van Gestel *et al*., 2001; Gogoi *et al*., 2003; Nano *et al*., 2003; Morelli *et al*., 2005; Demnerova´ *et al*., 2005).

One approach to restoring contaminated soil is to make use of microorganisms able to degrade the toxic compounds in a bioremediation process. However, it is known that hydrocarbon biodegradation in soil can be limited by many factors, such as microorganism type, nutrients, pH, temperature, moisture, oxygen, soil properties, and contaminant concentration (Bardi *et al*., 2000; Semple *et al*., 2001; Sabate *et al*., 2004; Ghazali *et al*., 2004; Walter *et al*., 2005; Atlas and Bartha, 2006). These researchers have concluded that the disappearance of crude oil from seawater could be accelerated by the addition of nutrients, such as nitrogen or phosphorus or both.

Recommendations have been advocated for the microbial seeding of oil spills, because bacteria and fungi are the only biological species which have the metabolic capability of utilizing petroleum carbon for cell synthesis (Jobson *et al*., 1974). Also, oil spills result in an imbalance in the carbon–nitrogen ratio at the spill site, because crude-oil is essentially a mixture of carbon and hydrogen. This causes a nitrogen deficiency in an oil-soaked soil, which retards the growth of bacteria and the utilization of carbon source(s).

The effects of nutrients (i.e. NPK), aeration and biostimulation of indigenous soil microorganisms and inoculation of extraneous microbial consortia on the bioremediation of oil contaminated soil have been investigated (Vasudevan and Rajaram, 2001; Gogoi *et al*., 2003; Coulon *et al*., 2005; Ayotamuno *et al*., 2006; Sang-Hwan *et al*., 2007). In addition to a nitrogen deficiency in oil-soaked soil, certain nutrients like phosphorus may be growth-rate limiting (Jobson *et al*., 1974). Furthermore, large concentrations of biodegradable organics in the top layer of agricultural soils deplete oxygen reserves in the soil and slow down the rates of oxygen diffusion to deeper layers (Sang-Hwan *et al.*, 2007).

Crude oil pollution tends to persist in soils until remediation measures, involving the application of nutrients, are resorted to, because oxygen and nitrogen are limiting factors in all types of petroleum degradation. The application of a fertilizer, plus implementing certain agro-technical processes like tilling, were as effective as the use of bioaugmentation with indigenous hydrocarbon-utilizing bacteria (HUB) plus fertilizer application and tilling, in the degradation of the hydrocarbon contaminant. This is because HUB is present in almost all types of soils and would multiply where the right types and concentrations of metabolic feedstock exist (Odokuma *et al*., 2003).

A combination of treatments, consisting of the application of fertilizers and oxygen exposure on bioremediation of a crude oil-polluted agricultural soil was evaluated by Ayotamuno *et al*., (2006). They found that the quantities of fertilizer (NPK) (i.e. 4.7 to 12.5ton/ha), moisture content between 14% and 19% during the wet season and a tillage rate between 2 and 5 times a week, are necessary for an effective bioremediation.

The aims of this study were:

- To evaluate the process of biostimulation achieved by the addition of agricultural fertilizers to petroleum-polluted agricultural soils.
- To determine the total quantity and application rate of the fertilizer and other environmental conditions that would be effective, on the biodegradation process.
- To study the relation of the total heterotrophic bacterial counts to the fertilizer applications.

MATERIALS AND METHODS

Preparation of contaminated soil

A bulk soil from an agriculture field (*Shahid Chamran University, Ahvaz, , Iran*) was taken, air dried and passed through 2mm sieve. Then, the bulk soil was contaminated with crude-oil artificially. The crude-oil from well No: 69 of Maroon oil field (which is paraffin oil with a rate of 1% weight) was sprayed in a way that the whole soil would be polluted homogenically. The soil samples were kept for two weeks and then divided in to 5Kg parts and stored in special containers. The containers were left undisturbed (i.e. in the open air) for 2 weeks. Then the treatments, including additional of different amounts of agricultural fertilizers (urea, ammonium phosphate and potassium sulphate) were applied, but equal rates of tilling were used. The various treatment containers were tilled twice a week with cutlass and shovels to provide the necessary aeration and mixing of nutrients and microbes with the contaminated soil (Ayotamuno *et al*., 2006).

Experimental design and soil treatment

The crude-oil polluted soil was divided into 3 treatment sample-containers, which were extending horizontally 40 cm × 40 cm and of depth 30 cm. The 9 containers were such that the depth and exposed surface-area of the soil, and in turn its temperature, nutrient concentration, moisture content and oxygen availability, could be controlled (Mattewson and Grubbs., 1988). Three containers did not receive any treatment (control); in 6 containers with 5 Kg crude-oil soil, 4 g and 8 g of 20–10–10 NPK fertilizer were applied, respectively, twice during the remediation period, i.e. at two-week intervals. Thus, the equivalent of 1 and 2 ton/ha of fertilizers were applied. The treatment soils were kept under controlled humidity 60 percent of F.C. (field capacity), and temperature condition (30°C), to create appropriate environment for the activity of crude-oil decomposing microorganisms.

In order to remove the effect of the lack of oxygen and preparing aerobic soils conditions, the soils were mixed twice per week for 5 up to 10 weeks. The symbols of the treatments in the experiment were: T0 (control), T1W1 and T1W2 (1 ton of agriculture fertilizer application and 5 and 10 weeks incubation, respectively); T2W1and T2W2 (2 ton of agriculture fertilizer application and 5 and 10 weeks incubation, respectively).

Soil samples

Analysis of soil characteristics

Physicochemical characteristics, such as ECe (Electical Conductivity of soil paste), pH, TPH (total petroleum hydrocarbon), organic carbon, total nitrogen and soil texture were determined in the soils. TPH was measured using the procedure described by Odu *et al*., (1985), while the organic-carbon content was determined by the wet combustion method of Walkly and Black (1934). The total nitrogen was determined using methods adapted from Odu *et al*., (1985). Hydrocarbon-degrading and heterotrophic bacteria were enumerated using MPN method adapted from Wrenn and Venosa (1996). The acquired data from this research in the frame of statistical design was divided split plot in time and were analyzed by SAS software.

Oil sterilizing

In order to sterilize the crude oil, micron Chromafil CA/S %45 syringe filters were used. Thus, at first the oil was pulled with a syringe and then the filter was connected to the syringe tip which was pushed slowly with a controlled pressure downward, to let the oil pass the filter and slowly enter the pipe in the device.

Bacterial enumeration

Hydrocarbon-degrading and heterotrophic bacteria were enumerated using a MPN method adapted from Wrenn and Venosa (1996). Bushnell Haas medium (composition: magnesium soleplate 0.2 g g/L; calcium chloride 0.02 g/L ; monopotassium phosphate 1 g/L; ammonium phosphate dibasic 1 g/L; potassium nitrate 1 g/L and ferric chloride 0.05 g/L) supplemented with 2% (w/w) NaCl was used as the growth medium in 96 well microstate plates. Hydrocarbon sources were added to stimulate the growth of hydrocarbon–degraders. A sample of 3 g of Soil samples was placed in a vial containing 10 mL of Bushnell Haas medium supplemented with 2% (w/w) Nail and mixed to form slurry. One mL of this slurry was placed into a vial containing 9 ml of Bushnell Haas medium supplemented with 2% (w/w) NaCl.

A dilution series was prepared from this sample, from 10^{-1} to 10^{-12}, and used to inoculate plates. and each rarity has three repetition, in a form that for each soil sample 36 experiment pipe should be prepared, after diluting Risasorin identifier in a rate of 90 µl added to pipes and then sterilized crude oil in a rate of 0.2 mL should be added to each pipe and Plates for enumeration of hydrocarbon -degrading bacteria were incubated at room temperature (26-27°C) for 2 weeks. After incubation, all plates were read using a MPN chart to determine positive or negative growth of bacteria, it is necessary to mention that all the needed instruments were sterilized.

Hydrocarbon analysis

The soil samples were placed for TPH measurements in 1 L glass bottles and sealed with aluminum foil. This procedure was undertaken three times to form three replicates. The bags and glass bottles were immediately transferred to the laboratory for analysis.

The concentrations of total extractable matter (TEM) and of each fraction of extractable matter were determined using column chromatography according to the method of Odu *et al*., (1998). The oil was extracted by soxlet using chloroform as a solvent. The total amount of extract containing the total oil compound and some biogenic lipids was estimated by weighing the dry residue after evaporating the solvent. The dry extracts after getting Asphalton in micro liter rates were injected to Gas Choromatography set which made by vinci Technologies company model 2010 that identifying the messengers would be done by FID identifier. A hairy column with a size of 25 m was put inside that, the initial temperature was 50 ˚C and the ultimate temperature was 220 ˚C; temperature programming was done with the rate of 5 ˚C/min. Helium was used of the carrier gas and compressed air and hydrogen gas were used for the flame ionization detector (FID).

RESULTS

The results showed that with increasing fertilizers and soil ventilation during bioremediation and at the time of increasing heterotrophy grows, petroleum degradation has also increased and soil pollution showed a decrease between 45-60 percent. The effect of the 2 ton/ha fertilizers application on petroleum degradation was more than 1 ton/ha fertilizers treatment. Also in the control, because of random ventilation and moisture (60 percent of F.C.), these conditions have been observed suitable for bacteria activity (Figs. 1 and 2).

The results showed a significant difference between the various treatment effects of fertilizers on soil C/N ratio. C/N ratio in the control sample was more than the treatment ones (Fig. 3). With time, C/N ratio of treatments decreased and in the 10th week this proportion was less than the fifth week (Fig. 4). The results from the crude oil-polluted soil treated with fertilizers showed that application of fertilizers treatment had a significant effect on soil bacteria growth (Fig. 5) and that the average bacteria growth in the treatment samples had a statistically significant difference from bacteria growth in the control. Two tons of fertilizers have caused more bacteria growth rather than 1 ton treatment. With increasing time, the population of bacteria has reduced in 10th week compared to that in 5th week (Fig. 6).

The n-alkenes on the basis of their chemical nearness combination are divided into 6 classes as follows: <C13, C13-C16, C17-C21, C22-C25, C26-C29, C29-C36. The first class includes normal alkanes smaller than C13. As these hydrocarbons evaporate in normal conditions, they are not measurable by gas chromatography (Fig. 7). Control sample during 10 weeks had not received nutrition treatment but was under suitable ventilation and moisture as was shown with (TO) in the Figs. 8 and 9, which showed the comparison of normal alkenes classes after agriculture fertilizer application in 5 (T1W1) and 10 (T2W1) weeks.

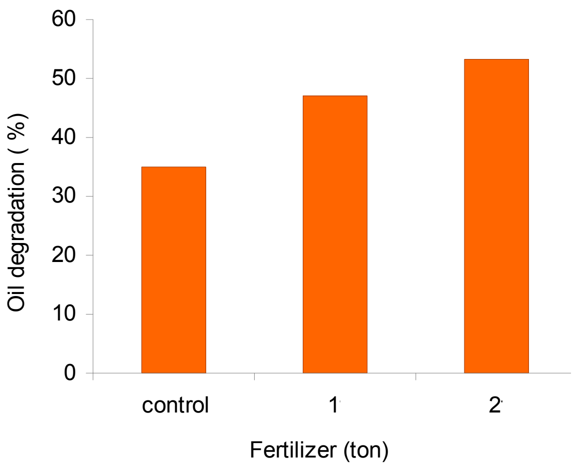

Fig. 1: Effect of fertilizers (NPK) on oil degradation

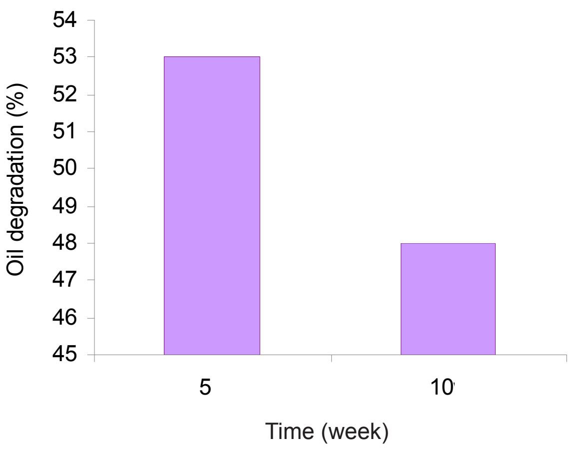

Fig. 2: Effect of time on oil residual

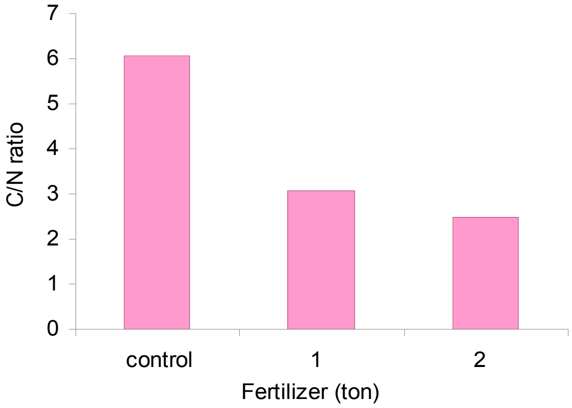

Fig. 3: Effect of fertilizers (NPK) on C/N ratio

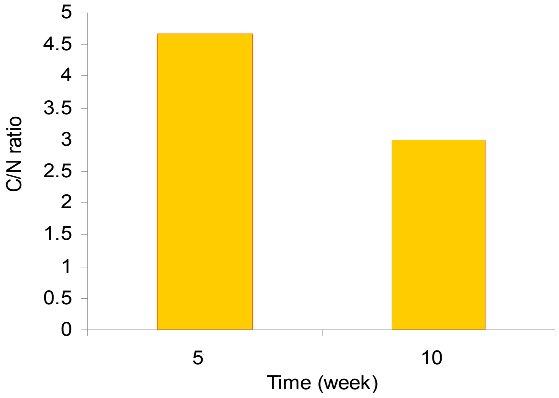

Fig.4: Effect of time on C/N ratio

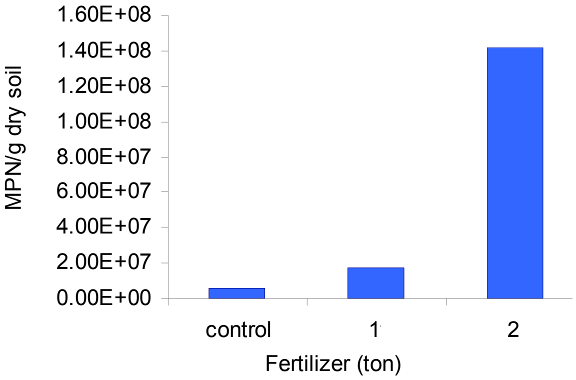

Fig. 5: Effect of fertilizers (NPK) on Bacteria growth

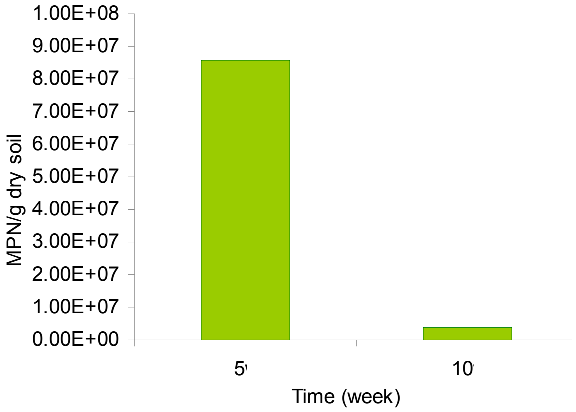

Fig. 6: Effect of time on Bacteria growth

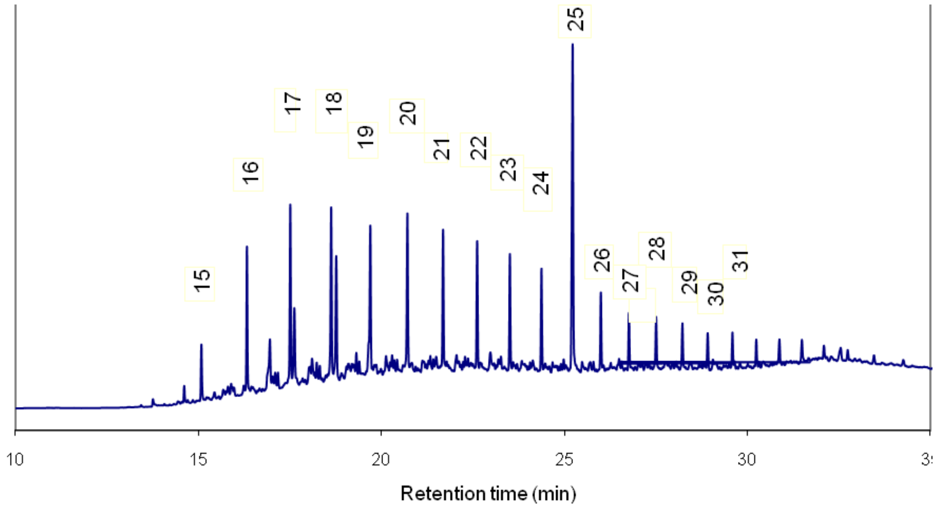

Fig.7: Chromatogram of the oil-contaminated soil before fertilizer application

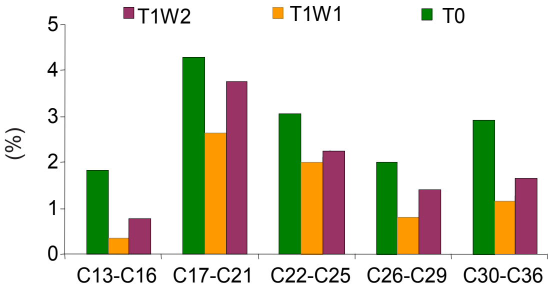

Fig. 8: Percentage of normal alkenes classes after application of 1 ton/ha fertilizer in 5 and 10 weeks

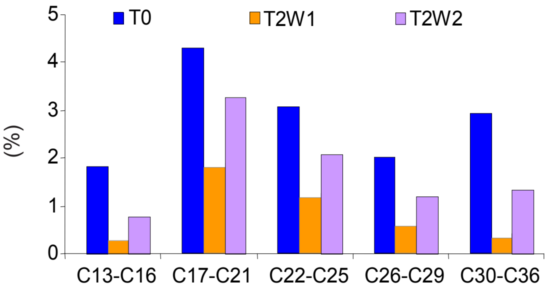

Fig. 9: Percentage of normal alkenes classes after application of 2 ton/ha fertilizer in 5 and 10 weeks

DISCUSSION

The effect of time on petroleum degradation was significant. The petroleum degradation rate decreased with increasing time (Fig. 2) and this observation corresponded with the bacteria growing results. The highest bacteria growing was determined after 5 weeks; because of the presence of fast normal paraffin decomposed and organic nutrition materials, bacteria activity and as a result petroleum degradation was in a maximum rate. Sang-Haw *et al.* (2007) made a similar observation and concluded that hydrocarbon degrading bacterial populations increased rapidly during the first 30 days of 105 days testing period. They proposed this finding that it may be considered as an indicator for the feasibility of oil-polluted soils bioremediation. But, with increasing of time, due to the oil-resistant components with high chain and within less remaining nutrients, the bacteria growth and oil degradation decreased (Schaefer and Juliane, 2007).

Ramsay and partners (2000) in the study of bioremediation on microbial population in oil sediments, observed that continual ventilation and fertilizer increment had considerable effect on the growth of hydrocarbonic degrading bacteria in soil. Van Gestel *et al.* (2001) have reported a significant increment of the oil-polluted soil bioremediation in bacteria population.

In Fig. 1 the average of oil degradation in soil in the treatment samples is observed, which shows significant difference with the control from the statistical point of view. Oil degradation in the lack of treatment samples was less and the oil degradation rate in the treatment samples with 2 ton/ha fertilizer was higher than the treatment ones with 1 ton/ha fertilizer. It is shown that between the oil degradation average in soil in both extraction times (5 to 10 weeks), there is a significant difference, and oil degradation average in soil within 5 weeks was higher than that of 10 weeks (Fig. 2). Sang- Hwan *et al*., (2007) reported similar results that the initial level of oil-polluted soil (9320±344 mg/kg) was reduced by 42-51% in the fertilized soil, whereas, only 18% of the hydrocarbon was eliminated in the non- fertilized soil.

The lack of organic feeding matters, will limit the oil degradation and C/N ratio will increase (Odokuma and Dickson. 2003). As it is observed in Fig. 3, C/N ratio of 6 in control samples reached to 3 in the treatment samples. In the 5th week, there was suitable feeding materials available for bacteria, but with passing of time, the lack of organic maters appeared little by little and limited the bacteria growth and oil degradation as well. With regard to Fig. 3, it may be found out that soil C/N ratio average in the control samples had a statistically significant difference, (the C/N ratio in control samples was more than treatment samples).

In Fig. 3 it is observed that between the ratio of C/N in soil at both analysis times, there has been significant difference and C/N amount in soil in 5 weeks was higher than 10 weeks. Fertilizers in the procedure of getting into organic matter would release feeding materials in the dissolved material and with creating suitable nutrition condition would cause the increment of oil degrader heterotrophic bacteria, oil degradation and decrease of soil C/N ratio. In fact, the bacterial population was readily utilizing the available nitrogen for hydrocarbon degradation; hence the available nitrogen was diminishing with time.

Ayotamuno *et al*., (2006) showed that between the total nitrogen content and remediation period there exists a negative relationship. In the present study, during 5 weeks, because of the presence of normal alkanes and environmental conditions and appropriate feeding, bacteria growth and oil degradation was high, but in 10 weeks, aromatic and remaining asphaltic component, and the lack of nutrition elements caused the decrease of bioremediation process (Schaefer and Juliane, 2007). There were some adverse effects for treated soil with high levels of nutrients, especially for the soil of C/N/P ratio of 100/10/1. Sang-Hwan *et al*., (2007) showed that in fertilized soil, hydrocarbon degrading bacterial population increased rapidly during the first 30 days comparing with non-fertilized soil.

Normal alkanes were calculated for the crude oil of the well NO: 69 of Marroon 3 oil field and Pristan and Phitan were used as interior standards (Gogoi, *et al*., 2003). These combinations are branch-paraffin introducer that is much resistant against biodegradation because of their molecular structure in comparison to normal paraffin's C17 and C18 (De Jonge *et al*., 1997; Atlas and Bartha, 2006). It is observed that the class C13-C16 has included about 12% of the whole crude-oil and the highest volume of normal alkanes and class C26-C29 has included the least volume and only 4% of the whole crude oil (Fig. 8).

Saturates and aromatics fractions of crude oil components are: gasoline (C4-C10), kerosene (C11-C12), diesel-fuel (C13-C20), motor-oil (C21-C40) and residue (> C40) (Atlas and Bartha, 2006). The crude oil used in this study had 23% of diesel-fuel and non–annular isopronoids of Pristan and Phitan organizing 1.34% and 1.55%, respectively.

Hydrocarbons class C13-C16 have had severe changes in the volume of normal alkanes (from %12 in crude oil has reached to 2% in control soil). Class C17-C21 in control has reached to the primary volume in crude oil. But in 3 classes of >C22 hydrocarbons, volume changes of oil degradation were much less (Figs. 8 and 9).

The results confirmed that alkanes section (C13-C21) after oil spray on soil showed a great decrease. Pristan and Phitan also have changed in control rather and the primary oil as 51% and 49 % in volume, respectively, that indicated a high biodegradation in soil. These results are consistent with some other researches like Hunt (1996); De jonge *et al.* (1997); Gogoi *et al.* (2003); Atlas and Bartha (2006) and Das and Murkherjee (2007).

Coulon *et al*., (2005) found that in fertilized soil, all alkane groups showed a percentage of degradation > 85% when the temperature was raised to 20 °C. In Fig. 8 changes in C17-C21 and C26-C29 were very remarkable (from 25% one by has reached to 53% and 46%, respectively). The classes of hydrocarbons C16-C13, C30-C36, C17-C21, C26-C29 and C25-C22, decreased to 65%, 53%, 43%, 46% and 57%, respectively. The less biological degradation was obtained for the class C25-C22 because of the probable heterotrophic bacteria which have shown less tendency to the usage and decomposition of this class. Alkanes of class C13-C16, C29-C29 and C30-C36 had higher degradation by heterotrophic bacteria in comparison to control sample (Figs.8 and 9).

Chemical fertilizer treatments showed about 50% decrease in comparison to control in the medium chain like paraffin fraction, which expresses the lack of tendency of hydrocarbonic degrader heterotrophic bacteria for the degradation of singular normal alkanes. Class C30-C36 had increased significantly from 2% to 62% and showed the microbial tendency for decomposing high chain normal alkanes. Hydrocarbon degradation class C13-C16 was about 74%. Hydrocarbons of median classes of C17-C21, C22-C25, and C26-C29 degraded as 37%, 22% and 69%, respectively. Overally, the applied fertilizer treatments appeared to be effective to accelerate bioremediation of hydrocarbon contaminated soil.

The added fertilizers to oil-polluted soil decreased the C/N ratio from 6 in control sample to about 3 in the fertilizer treatments. Also, the applied treatments caused the oil degradation of 45% to 60%. Hydrocarbon degrader heterotrophic bacteria population in the fertilizer treatments had remarkable increased from 6×10^{3} unit/g soil in the control sample to 2×10^{10} unit/g in fertilizer treatments. Oil degradation and microbial population increment in both treatments in 5 weeks were higher than 10 weeks. These results confirmed that biostimulation of the indigenous soil microorganisms by fertilizers addition resulted in an accelerated biodegradation of oil-contaminated soil.

ACKNOWLEDGEMENTS

The authors appreciate the Soil Biology and Geochemistry laboratories staffs and also deeply thank Dr Alizadeh for the support of analytical instruments.

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