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EFFECT OF PETROLEUM POLLUTION AMOUNT ON BIOLOGICAL TREATMENT OF HYDROCARBON COMPOUNDS IN SANDY-LOAM SOIL

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Abstract

Biological treatment of sandy-loam soil polluted with different levels (19, 28 or 37 g/kg) of petroleum has been investigated. The soil were supplemented with a mineral sol and a mixed culture of petroleum degraders in association with the native microorganisms. The soil was periodically moistened and tilled. The petroleum degraders and residues were then monitored and estimated over time periods. Significant bacterial and fungal growth was observed from 10^5 and 10^4 CFU/g on the 1st week to 10^{10} and 10^6 CFU/g on the 4th week, respectively. On 4th week, the biodegradation in the soil contaminated with a primary petroleum concentrations of 15, 22 and 29 g/kg achieved the following levels: 27.3 %, 22.8 % and 17.8 % (total petroleum); 96.3 %, 65.2 % and 54.2 % (n-alkanes-(NA)); 98.0 %, 59.0 % and 57.0 % (pristane-(PR)); 35.0 %, 20.0 % and 16.0 % (phytane-(PH)); and 29.3 %, 18.5 % and 11.4 % (polycyclic aromatic compounds (PAC)), respectively. The biological degradation was inversely proportionate to the increase in the petroleum pollution level and vice versa. Low molecular weight compounds were more biodegradable than high molecular weight. The biodegradability of hydrocarbons was as follows: NA> PR> PH> PAC.

Keyword **:** Biological treatment, Sandy-loam soil, Petroleum pollution, Hydrocarbon

Introduction

Petroleum on our planet is widely used in chemical industries and energy production. Uncontrolled disposal of petroleum products has a negative impact on land and water. Petroleum pollution may occur as a result of oil spills from oil storage tanks, cracked oil pipelines, oil refineries and petrochemical plants, oil transportation accidents and the explosion of oil structures, etc. (Zhang *et al*., 2012; Ahmed & Fakhruddin, 2018).

Soil contamination with petroleum is an important issue that has received international attention nowadays because it causes serious impacts on humans and the environment (Laffon *et al*., 2016). With an growing interest in conserving ecosystems, many chemical and physical processes have been used to remove petroleum pollution from soil. Nevertheless, these methods are costly and only transfer pollution from one location to another (Gargouri *et al*., 2015).

Biological treatment of oiled soil was established as an effective, economical, diversified and environmentally friendly process (Guerra *et al*., 2018). This process takes advantage of the ability of microorganisms to degrade and remove organic chemicals, and endeavor to enhance rates of natural biological degradation. The method is based on improving the bioprocesses of treating or reducing levels of hazardous substances in the polluted places. The basis of biological treatment of organic chemicals is the mineralization of pollutants into final innocuous compounds of carbon dioxide and water (Xu *et al*., 2018). Consequently, bioremediation is a good, effective, environmentally harmless and inexpensive alternative compared to physical and chemical treatment processes that mainly depend on burning, evaporation and stabilization of pollutants. Bio-treatment of oiled soil usually includes two strategies: biological augmentation (adding petroleum degraders to the soil) and biological stimulation (adding nutrients and other microbial growth limiting substances) that can be achieved separately or together (Ali, 2019).

Studies have shown that the effectiveness of biodegradation of petroleum hydrocarbons in the soil system depends on several factors like the history of oil pollution, type of soil, oil composition and level, and environmental parameters that include temperature, salinity, pH, nutrient, oxygen, moisture content, etc. (Varjani & Upasani, 2017).

The concentration of oil is the important factor that determines the rate and potential for biodegradation of hydrocarbons. Salleh *et al*. (2003) showed that the efficiency of the microbial degradation of petroleum sludge appeared in the petroleum concentration from 1.25 % to 5 % and was better at the concentration of 5 %. Concentrations of petroleum above 5 % reduce the number of microorganisms due to toxic effect. Tarabily & Khalid (2002) explained that the efficacy of oil degraders is completely stopped by increasing the petroleum level from 50 %. As well as oil toxicity, high levels of oil may prevent the growth of microorganisms by disturbing the ratio of carbon-(C): nitrogen-(N): phosphorous-(P) and prohibiting the exchange of oxygen. High concentrations of volatile hydrocarbons are harmful to petroleum degraders as a result of their high toxicity (Xu *et al*., 2018). Salleh *et al*. (2003) reported that the rate of microbial degradation of hydrocarbons depends on the composition and concentration of the petroleum. In biological treatment, the petroleum concentration impacts biodegradation, and a very high or very low concentration reduces the biodegradation performance. Chen *et al*. (2017) has shown that the increase in petroleum concentration leads to a decrease in the biodegradation of the bacterial consortium as free or immobilized.

Although many studies and reviews have addressed the effects of environmental factors on the biological treatment

of petroleum (Al-Hawash *et al*., 2018), the effect of petroleum properties such as petroleum concentration have not gained much concern from the authors. Eco-factors and petroleum properties do not influence the biological treatment process independently, but they have a combined impact (Chen *et al*., 2017), so it is important to study them with the same interest and details. In general, there are few studies dealing with the effect of petroleum concentration on biological treatment and to understand this effect in different ecosystems, much research is needed. Thus, the present study was carried out to assess the effect of different levels of petroleum on the biological treatment of sandy-loam soil deliberately contaminated with petroleum. The soil was equipped with exotic microorganisms, in addition to the existing local microorganisms, and microbial growth conditions were provided.

Materials and Methods

A triplicate bio-treatment trials was carried out in a 46×29×32 cm polyethylene bioreactor in the laboratory. Soil (60 kg) was placed in the bioreactor and mixed with 8 l of mineral sol to get a soil layer 20 cm high. After that, 15 kg of soil was mixed with 19, 28 or 37 g/kg of crude oil, then added to the surface of the previous soil layer to form a height of 10 cm. So the height of the two soil layers together (whole soil) in the bioreactor is 30 cm. a mixed culture of petroleum degraders was then added to the reactor. The top layer of the soil was constantly moistened with water and mineral sol to compensate the vaporized water and depleted nutrients. The soil was also plowed daily with a small manual shovel up to a depth of 10 cm to ensure oxygen supply to the microbes. This experiment stimulates the biological treatment of soil exposed to a petroleum spill accident. For the purpose of detecting the loss of petroleum compounds by non-biological factors and petroleum transported to the subsoil layer in the bioreactor, parallel trials were performed in the same circumstances previously described with inhibiting the growth of microorganisms by adding a sol of NaN_3 (0.5 %) to the reactor. Taking into account the absence of a large petroleum filtration in the soil layers 15 and 30 cm, soil samples were collected from 5 spots of equal distance from the soil layer 10 cm in the reactor. These 5 soil sub-samples were then mixed and homogenized in one sample for hydrocarbons analysis and microbial count.

Oil used in the current study is light Nahran-Umar (NU) crude oil of API gravity >34 equipped from the company of Iraqi South Oil. Some of the properties of this crude oil were reported by Ali *et al*. (2013). Analysis of NU crude oil by the gas chromatography-(GC) showed that oil consists of 47.61 % saturated hydrocarbons, 33.34 % aromatic hydrocarbons and 20.19 % polar compounds. Polar compounds are usually deemed to be one of the most resistant parts of crude oil to the biodegradation (Ali, 2019).

Studied soil (sandy-loam) was taken from the Al-borjesia region located in Basrah Governorate, in southern Iraq. Some of the physical and chemical characteristics of the soil can be listed as follows: Sand (g/kg)= 863.72, silt (g/kg)= 55.34, clay (g/kg)= 80.99 (hydrometer method) (Van Reeuwijk, 2002), pH= 7.90 (PH-meter, model 361), organic carbon (g/kg 0.43 (Walkley and Black method) (Walkley & Black, 1934), organic matter (g/kg)= 0.67 (gained by multiplying the content of organic carbon by a 1.724 factor), total nitrogen (g/kg)= 0.05 (Kjeldahl procedure) (Kjeldahl, 1883), C:N ratio= 8.60 (obtained by dividing the concentration of organic carbon by the concentration of total nitrogen), $CaCO_3^-$, (g/kg)= 94.00 (titration method) (Horváth *et al*., 2005), P (mg/kg)= 7.22 (Mehlich III method) (Mehlich, 1984), Ca^{+2} (m/ *M*l)= 6.84, Mg^{+2} (m/*M*l)= 3.16, Na^+ (m/*M*l)= 6.70, K^+ (m/*M*l)= 1.04 (ammonium acetate (1N) extraction method, pH= 7.0, Ca and Mg were measured by atomic-absorption-spectrophotometer, AA-70000/Shimadzu and Na and K with flame-photometer, model 130), $CO_3^=$ (m/*M*l)= 0.00, $HCO_3^=$ (m/*M*l)= 1.89 (titration method) (Richards, 1954), $SO_4^=$ (m/*M*l) = 9.63, Cl^- (m/*M*l) = 6.47 (ionic-chromatography, Dionex IC 2000, USA), electrical conductivity (dS/m)= 2.60 (conductivity-meter, model 304), and cation exchange capacity (Cmole(+)/kg)= 3.47 (distillation and titration methods after leaching the saturated soil with ammonium by sodium chloride, 10%) (Van Reeuwijk, 2002). The soil was polluted with oil and the bio-treatment was then studied with an primary petroleum levels of 15, 22 and 29 g/kg of soil.

The mixed culture of petroleum degrading microorganisms used in this study were antecedently isolated from petroleum polluted agricultural land in Abu-Al-Khaseeb region in Basrah Governorate. The mixed microbial culture was also previously activated in a conical flask (500 ml) filled with a mineral medium (100 ml) consisting of Na_2HPO_4 (g/l)= 1.4, NH_4NO_3 (g/l)= 1.0, KH_2PO_4 (g/l)= 0.5, $MgSO_4.7H_2O$ (g/l)= 0.1, $MnSO_4.H_2O$ (g/l)= 0.03, $CaCl_2.2H_2O$ (g/l)= 0.02, and crude oil (v/v)= 1%. Cultivation was achieved in an shaker incubator (150 rpm) at 30 °C for 48 hr.

Total petroleum hydrocarbons were extracted from the soil (60 g) by Soxhlet with chloroform (200 ml) (Okop & Ekpo, 2012). The extract was then reduced, saponified and concentrated. The petroleum hydrocarbons were estimated by the gravitational method. The extract was further fractionated into aliphatic (NA), aromatic (PAC), and polar compounds by column chromatography on silica gel and alumina (10 g) (60 to 200 mesh), and anhydrous sodium sulfate (1 g). The NA, PAC and polar was eluted with hexane, benzene and methanol (50 ml) respectively. The fractions were concentrated and analyzed (1 µl) by Agilent-GC-(6890) equipped with mass-spectrometry detector-(5973) operating in electron effect mode-70 eV. The splitless mode was used to inject the samples at a temperature of 60 °C and the temperature of oven was then elevated to 280 °C-(PAC) or 300 °C-(AN) by 5 °C/minute and kept isothermally at each final temperature to 20 minutes. The HP, 5MS (5% phenyl-methyl-siloxane) capillary column with 30 m×0.25 mm id×0.25 µm film thickness was used. Ion source and analyzer (quadrupole mass) temperature were hold on 230 °C and 150 °C, respectively. The carrier gas (helium) used was at 99.99 % purity and 2.7 ml/minute flow rate. The temperature of injector and transfer line were kept in 250 °C and 280 °C, respectively. The retention times and quantification ions of AN and PAC at the samples were determined by standard solutions of AN-(C_{14}-C_{34}) and PAC-(naphthalene-(Na), acenaphthylene-(Ac), fluorine-(Fl), phenanthrene-(Ph), anthracene-(An), fluoranthene-(Fu), pyrene-(Py), benz(a)anthracene-(Ba), chrysene-(Ch), benzo(k)fluoranthene-(Bf), benzo(a)pyrene-(Bp), indeno(1,2,3,cd)pyrene-(Ip), and benzo(g,h,i)perylene-(Be)) (Supelco-USA). The mode of full scan and ion fragment-(m/z 85) was utilized for the quantitative analysis of AN. For

each PAC, the molecular ion fragment was chosen to define and quantify the PAC in the mode of eclectic ion monitoring. Furthermore, the mode of full scan was also used to identify the PAC and to emphasize the compounds. In order to estimate the concentration of compounds in the samples on the basis of measuring the above standard solutions at various concentrations, calibration curves were created for all compounds. The blank procedure for quality control and assurance was implemented, as the target compounds were not disclosed. Average PAC recovery rates varied from 80 % to 100 %. Relative standard deviations of PAC were between 9 % to 17 %. The recovery rates for AN were around 67 % to106 % (an average of 85 %).

The standard dilution plate technique was utilized to estimate petroleum microbial degraders. Stock prepared from suspending 1 g of soil sample in sterile distilled water (9 ml) and then shaken at 150 rpm for 1 hour. Of this stock, serially diluted (10^{-2} to 10^{-7}) ware achieved and 0.1 ml of each one were inoculated on solid mineral salt medium composing of NaCl (g/l)= 10, K_2HPO_4 (g/l)= 1.2, KH_2PO_4 (g/l)= 0.83, $MgSo_4.7H_2O$ (g/l)= 0.42, $NaNO_2$ (g/l)= 0.42, KCl (g/l)= 0.29, Agar (g/l)= 15, pH= 7.1 for bacteria and pH= 5.6 for fungi, and crude oil (ml)= 2 (as source of carbon and energy and available to cultures by vapour phase transmit). Streptomycin (0.5 ml) was added to media of fungi to repress growth of bacteria. Culture plates (3 replicate) were incubated at 37 °C to 4 day (bacteria) and at 30 °C to 7 day (fungi). The numbers of bacteria and fungi were then enumerated. After obtaining the mean values and taking into account the dilution factor, the total number of colony forming units-(CFU) per gram of soil was determined. The petroleum degraders colonies were picked randomly and sub-cultured to purify on nutrient agar (bacteria) and sabouraud dextrose (fungi) plates. The plates were incubated at 30 °C to 24 hours for bacteria and at 30 to 3 day for fungi to obtain pure colonies. The isolates were then distinguished employing the Carpenter (1977), and Bargey's (1994) techniques.

The trials were conducted in triplicate and data were reported as the mean ± one standard deviation-(SD). The results were also undergone to a one-way ANOVA statistical analysis by using IBM SPSS-(Statistical Package for Social Science) software, version 22. Mean values were separated and compared utilizing the least significant difference-(LSD) test at 5% level of significance. All graphs were executed by Microsoft-Excel 2007.

Results and discussion

After 4 weeks of bio-treatment of sandy-loam soil contaminated with different levels of petroleum in the bioreactor, the cumulative losses of petroleum decrease with an increase in the quantities of petroleum polluted to the soil. Biodegradation in soil contaminated with primary petroleum concentrations (15 or 22 g kg^{-1}) reached a similar level (36% and 34%, respectively) on the 4th week of bio-treatment, indicating the forbearance of microorganisms to these lower primary petroleum concentrations. Whereas, there is less efficacy for microorganisms in soil contaminated with an initial petroleum concentration of 29 g/kg, which may be due to the sensitivity of some microorganisms to the high concentration of petroleum in the degradation process (Fig. 1).

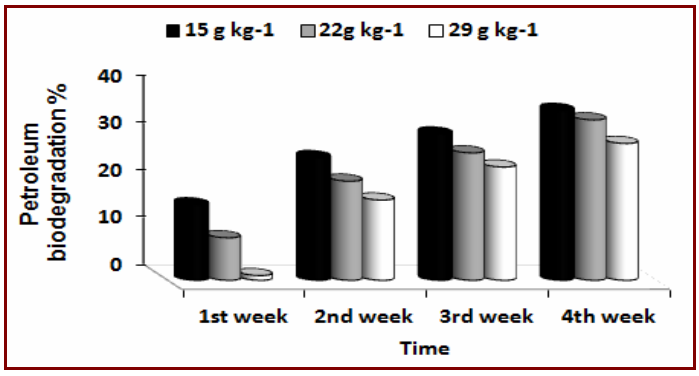

Fig. 1: Biodegradation of petroleum in soil polluted with various petroleum levels.

The GC analysis just estimated the extent of AN from C_{13} to C_{30} and PAC including Na, Ac, Fl, Ph, An, Fu, Py, Ba, Ch, Bf, Bp, Ip and Be (Fig. 2 and 3) which are comparatively prevalent in the studied crude oil. The results of the analysis of the soil contaminated with the lowest level (15 g/kg) of the petroleum revealed that the NA from C_{13} to C_{16} are completely degraded, while the AN between C_{17} to C_{32} are less biodegraded. Light PAC consisting of four or less benzene rings are more susceptible to biological degradation than those heavy PAC containing more than four benzene rings. The range of biodegradation in soil contaminated with petroleum levels (22 and 29 g/kg) relies on the petroleum hydrocarbon components, while the effectiveness of the degradation of the microorganisms is declined. Thus, the vulnerability to biodegradation is directly proportional to the decrease in the number of carbon atoms in AN and the benzene rings in PAC. On 4th week of biological treatment in the bioreactor, the values of microbial degradation of PR ranged from 98.0 % (15 g/kg) to 57.0 % (29 g/kg), which also seemed to be influenced by the level of petroleum. While the values of PH were from 35.0 % to 16.0 % (Fig. 2), suggesting that the PH is resistant to biological degradation. Biodegradation of branched alkanes-(BA) is inhibited by the existence of AN (Wentzel *et al.*, 2007). Nevertheless, a number of investigations have indicated that it could be more biodegradable (Rojo, 2009). This study also demonstrates these findings. The data of the current study are consistent with the results of other studies (Das & Chandran, 2011; Nzila, 2018; Unimke *et al.*, 2018; Ali, 2019), which showed that the various petroleum compounds biodegrade differently depending on the level of petroleum pollution. Low-molecular-weight-(LW) AN are degraded faster than high-molecular-weight-(HW) AN. The AN are more susceptible to biodegradation than BA, which in turn are more resistant. The potential for PAC microbial degradation increases as their benzene rings decrease. PAC were the most rebellious to the microbial attack from both the BA and AN.

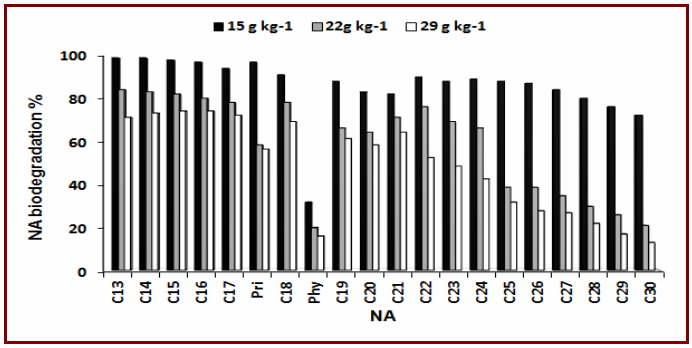

Fig. 2: Biodegradation of NA in soil polluted with various petroleum levels on 4th week of bio-treatment.

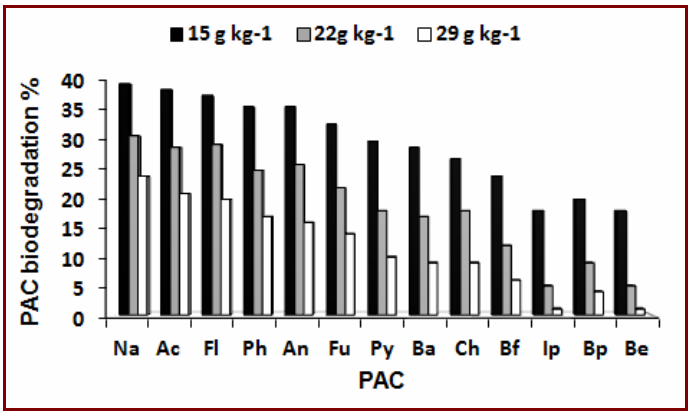

Fig. 3: Biodegradation of PAC in soil polluted with various petroleum levels on 4th week of bio-treatment.

The petroleum compounds bioavailability in the soil is a critical factor in controlling rates of biological degradation (Varjani and Upasani, 2017; Unimke *et al*., 2018). Current data gained from the use of low levels of petroleum may indicate the adsorption of petroleum into the solid-phase, which enables microorganisms to bind directly with petroleum hydrocarbons. The decrease in biodegradation in the case of using high levels of petroleum may be due to the partial propagation of petroleum into the solid-phase and the loss of the N and P elements. The continuous supply of soil with water and mineral solution (nutrients) to compensate them in bioreactors with high levels of petroleum may also not be enough to achieve optimum efficacy.

The results of Table 1 indicate to existence real changes in the counts of microorganisms in soil contaminated with different concentrations of petroleum during the study period. On the 4th week of bioremediation, there is an increase in growth to 10^{10} CFU/g for bacteria and 10^{6} CFU/g for fungi compared with the 1st week of the process (10^{5} CFU/g for bacteria and 10^{4} CFU/g for fungi). Indeed, inoculation was carried out only once at the starting of the process, resulting in the reproduction of microorganisms only due to the utilization of petroleum hydrocarbons. This strategy robustly suggests implementation of this process at the field level, as it greatly diminish the expenses of inoculum production represented by reagents used for microbial acclimation and automatic ventilation.

Table 1: Alterations in the microorganisms numbers (CFU/g) in soil polluted with various petroleum levels.

Petroleum level	Bio-treatment time	Bacteria	Fungi
15 g kg^{-1}	0 week	$4.2\pm0.5\times10^{5}$	$2.2\pm0.2\times10^{4}$
	1st week	$2.6\pm0.3\times10^{7}$	$5.3\pm0.4\times10^{4}$
	2nd week	$5.8\pm0.4\times10^{7}$	$1.6\pm0.5\times10^{5}$
	3rd week	$1.4\pm0.7\times10^{8}$	$4.6\pm0.4\times10^{5}$
	4th week	$3.0\pm0.3\times10^{10}$	$3.8\pm0.5\times10^{6}$
22 g kg^{-1}	0 week	$3.6\pm0.6\times10^{5}$	$3.1\pm0.3\times10^{4}$
	1st week	$3.7\pm0.5\times10^{7}$	$7.4\pm0.6\times10^{4}$
	2nd week	$7.7\pm0.6\times10^{8}$	$8.6\pm0.5\times10^{4}$
	3rd week	$2.4\pm0.3\times10^{9}$	$7.1\pm0.2\times10^{5}$
	4th week	$1.7\pm0.4\times10^{10}$	$4.3\pm0.4\times10^{6}$
29 g kg^{-1}	0 week	$4.5\pm0.2\times10^{5}$	$2.7\pm0.4\times10^{5}$
	1st week	$6.8\pm0.4\times10^{6}$	$3.8\pm0.3\times10^{5}$
	2nd week	$8.0\pm0.2\times10^{8}$	$1.4\pm0.3\times10^{5}$
	3rd week	$9.2\pm0.6\times10^{8}$	$4.1\pm0.6\times10^{6}$
	4th week	$4.5\pm0.5\times10^{10}$	$8.0\pm0.5\times10^{6}$

The estimation of petroleum losses by non-biological factors, which were evaluated by the gravimetric method, exhibited a decrease in the heavy components of petroleum by about 5.3±0.3% in the 1st week of bioremediation and about 8.2±0.4 % after that week. These results are involved in the computation of biological degradation. The efficacy of the bio-exterminator employed in control trials was demonstrated by the absence of microorganism growth registered in its existence. Regarding the possibility of petroleum filtering through the soil system during the biological treatment period (4 weeks), it was found that most of the petroleum remains in the upper layer (10 cm) (Table 2).

Table 2: Petroleum heavy constituents in various layers of soil in the 4th week of bio-treatment.

Soil depth	Soil petroleum level		
	15 g/kg	22 g/kg	29 g/kg^{-1}
10 cm	$13.0^{a}\pm0.2^{b}$	20.1±0.4	25.9±0.3
15 cm	0.02	0.03	0.04
20 cm	0.02	0.02	0.02

a= 4 trails mean value; b= SD of mean

Conclusions

It can be concluded from the data of the current study that the biological degradation of petroleum hydrocarbons in sandy-loam soil was directly proportional to the low level of petroleum pollution in the soil. Petroleum hydrocarbons are perfectly or partly degraded. The LM-NA is almost completely degraded while the HM-NA is less degraded. BA are more renitent than NA. PR was more biodegradable than PH. PAC was less biologically degraded than BA and NA. Non-biological factors can also impact the rates of petroleum hydrocarbon degradation.

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