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EFFECT OF PETROLEUM OIL SPILLED IN THE DESERT OF KUWAIT ON THE MICROBIAL FLORA OF THE SOIL

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ABSTRACT

During the invasion of Kuwait, Iraqi invaders destroyed oil wells. As a result 60-80 million barrels of crude oil cotaminated about 25 km2 in the desert of Kuwait. The residual oil in three soil samples in the polluted area, was measured 10 months after the spill. The results show no significant loss in the saturated hydrocarbons, while the aromatic hydrocarbons were more degraded .

Oil-degrading bacteria were significatly greater the polluted soil samples. They rapidly increased in number and their R/C ratios ranged from 14.7 - 108.2. In contrast the R/C ratios of total bacteria, actinomycetes, fungi, cellulose decomopsers and thermophiles were in the range of 0.1 - 8.5 .

Three genera of bacteria were recorded in the control (non-polluted) soil sample; Arthrobacter (64.3%), Corynebacterium (16.9%) and Pseudomonas (3.6%). In the polluted samples, shifts in the diversity of genera were observed; Pseudomonas was more frequent (78.6%)

than Flavobacterium (21.4%) in soil sample (3)' in soil sample (2) Arthrobacter developed more (53.8%) than Pseudomonas; while in soil sample (1) all the bacterial isolatates were Pseudomonas .

The genus Streptomyces was more frequent than the genus Nocardia in all soil samples (except soil sample 3). On the other hand Nocardia sp. were developed more in the less polluted sample (3) (52.9%) than in the other samples .

The white and the blue series of Streptomyces were more sensitive to the exposure to oil pollutants, they disappeared from all the contaminated samples. In contrast the green series was the most resistant one, it was more frequent in the highly polluted samples, suggesting of being an indicator of highly polluted soil samples .

INTRODUCTION

Petroleum addition to an ecosystem will result in changes in the size and composition of the indigenous microbial community that are able to adapt and utilize the new substrate. *Petroleum* percolation through soil reduces aeration and upsets the carbon/ inorganic nutrients balance for the indigenous populations. Toxic components of petroleum may selectively inhibit members of the microbial community producing shifts in population size and species diversity within the soil. The most widely documented response of microbial communities to exposure to petroleum oil is a rapid increase in the size of hydrocarbon utilizing component of the commu-

nity. A great deal of knowledge are available on the response of oil degraders when oil is added to environment, but few are available about the reaction of the community as a whole .

The magnitude of the response is variable between communities, depending upon the ecosystem, and nature and amount of hydrocarbons. Pollutants that inhibit the growth or metabolism of one segment of the community may serve as growth substances for another portion and have no influence on a third group, i.e petrolum pollutants can serve as both a nutrient and potential toxicant .

Petroleum hydrocarbons does not necessarily manifest itself in soils where biodegradtion conditions are favourable. The most toxic components may volatilize or become immobilized by orption to soil organic matter .

Iraqi invaders during their invasion to Kuwait destroyed petroleum oil wells, as a result, 60-80 million barrels of oil were crushed from the wells to cover an area about 25 km2 in the desert of Kuwait and more that 70 oil lakes are formed. About one third of the soil in this area is contaminated with 5-20% of the oil. A program of soil remediation is needed to reduce the oil contents of these soils to restore the ability of Kuwait's land for plant and animal producion .

The first step in soil remediation is to study the potential impacts of oil pollutants in soils, includes measurements of either the

effect on the integrated response of the community, metabolic activity or the response of specific segment of the community such as cellulose degraders or various specific taxonomic groups (Buckley, 1981). Accordingly the aim of the present work is to study the effect of the spilled oil on the size and commposition of some groups of the natural microbial community in the polluted soil samples .

MATERRIALS AND METHODS

Collection of soil samples

Soil samples were collected under aseptic condition from three different sites in the polluted desert area. The first soil sample was collected from a site of 7.4% oil content, the second sample was collected from a site 5 m from the first one and it is polluted with 5.9% of the oil. The third soil sample was obtained from a site 8 m from the second one, it contains 2.7% of the oil pollutants. A control soil sample was collected from ono-polluted site in the same area. No vegetation was observed in sites of sampling. Each sample was a mixture of at least three samples collected from different spots in the same site. Samples were collected from 5-15 cm depth, ten months after the contamination of the soil .

Determination of the residual oil

For determination of the residual oil in each soil sample, 25 gm air-dired soil was shaken twice with 200 ml of chlorofrm. The

extracts were pooled, dried over anhydrous sod. sulphate and evaporated. The residue in each sample was weighted .

Determination of the loss of petroleum hydrocarbons

To determine the loss of hydrocarbons in the polluted soil samples a known weight of the hexane soluble fraction of the extracted oil residue was fractionated into saturates and aromatic hydrocarbons by successive elution with n-hexane and benzene on a silica gel column as described by Oudot (1984). For control experiment the same weight of hexane soluble fraction used in the above experiments was obtained from Kuwaiti crude oil, and fractionated as above. The loss of the saturated and aromatic hydrocarbons can be calculated .

Microbiological Methods :

Total viable bacteria, actinomycetes, fungi, cellulose decomposers and thermophiles were counted using the usual dilution plate method. Nutrient agar (Difco) supplemented with 0.1% yeast extract and 0.4% soluble - starch was used for counting total bacteria and thermophiles. For counting actiomycetes and fungi inorganic salts starch agar (ISP4) and malt extract agar were used respectively. Cellulose decomposers were counted as described by Diab and Al-Gounaim (1984). Incubation periods were 5-7 days at 30°C for total bacteria, actionmycites and fungi; 15 days at 30°C for cellulose decomposers and 2-3 days at 50°C for thermophiles. From a

plate showing maximum counts of bacteria and actinomyctes, all colonies were isolated, purified and subcullured on suitable media .

For counting oil-degrading bacteria, 50 gm of soil sample was suspended in 100 ml disilled water and shaken for 15 minutes. Ten milliliters of the supernatant was filtered on a Sterile filter membrane (0.4 μm). The membrane was removed and located on a plate of oil-silica gel medium containing crude oil as the only carbon source, and prepared as described by Walker and Colwell (1979). Four plates were used for each sample. The plates were incubated at 30°C for a period of 21 days, after which the developed colonies were counted and expressed as counts/g air-dried soil (free from oil). All colonies developed were isolated purified and subcultured on a suitable medium and left for further studies.

Identifications of bacteria and actinomycetes isolated from the four soil samples were carried out using the methods recommended to use Bergey's Manual of Determinative Bacteriology (1974), and Bergey's Manual of Systematis Bcteriology Vol 1,2 and 4 (1984, 1986, 1989) .

RESULTS AND DISCUSSION

The area from which the soil samples were collected represents a sandy soil with pH 7.8 (for the control sample and 6.5 to 6.8 for the polluted soil samples .

Chloroform extractable materials differ according to the degree of oil pollution. Soil samples (1) and (2) contained chlorform extractable materials 7.4% and 5.9% respectively, they may be considered as heavily polluted samples. On the other hand soil samle (3) was leass contaminated one, it contained 2.7% oil pollutants. The control soil sample, was unpolluted sample, its content of the chlororm extractable materials did not exceed 0.01% .

Results of the analysis of the residual petroleum oil fractions extraced from the contaminated soil samples 10 months after the spill (Table 1) show that no signifcant loss of the saturated hydrocarbons . Maximum loss was 9.1% from sample (3) and minimum loss was 4.5% from sample (2). The low loss of the saturated hydrocarbon in there soil samples may be attributed to the low concentrations of phosphorus and nitrogen nutrients in the desert soil . This often limits the growth and activity of hydrocarbon utilizing microorganisms. On the other hand it is of interest to observe that under these conditions, aromatic hydrocarbons were more degraded than the saturated hydrocarbons, especially in sample (2) in which 17.5% loss was recorded. Oudot et al. (1989) contaminated agricultural soil with gas oil 4 kg m^{-2} andstudied the infiltration and biodegradation of this oil. They found that after one year, the saturated fraction in the surface layer (0-15 cm) was only slightly degraded. Atals (1981) reported that without added nutrients, aromatic hydrocarbons were more readily attack the saturated hydrocarbons by

Table (1) : Loss of the saturated and the aromatic hydrocarbons from the contaminted soil samples, 10 months after the spill. The residual oil in each sample is given .

Soil sample	Residual oil (%)	Saturated hydrocarbons loss (%)	Aromatic hydrocarbons loss (%)
1	7.4	7.3	14.2
2	5.9	4.5	17.5
3	2.7	9.1	11.6
4	0.01	---	---

soil and marine microbes. Addition of P and N nutrients stimulated degradation of saturated hydrocarbons more than the aromatic hydrocarbons. Diab (1991) found that in the absence of P and N nutrients an isolate of *Pseudomonas* sp and of *Nocardia sp* were able to degrade more armatic hydrocarbons than saturated hydrocarbons of petroleum oil .

From the above results, it may be suggested that before the remediation treatment, the contaminated desert soil must be left without the addition of nutrients for a pariod enough to stimulate the biodegradation of the aromatic fractions of the soil pollutants .

Results of the concentrations of the different groups of microorganisms (Table 2) show that the maximum counts of total bacteria ($86.3 \pm 6.4 \times 10^3$ / g soil) and actionmycetes ($26.3 \pm 1.0 \times 10^3$ / g soil) were recored from the less cotaminated soil sample (3). Regarding the concentration of fungi in this soil sample, it can be observed that there is a decrease in their counts ($2.7 \pm 0.1 \times 10^2$ / g soil) as compared to the counts recorded from the control non-polluted soil sample ($14.3 \pm 0.1 \times 10^2$ / g soil).

Increasing oil pollutants to 5.9% (sample 2) resulted in sharp decrease in total bacteria ($3.9 \pm 0.2 \times 10^3$) as compared to ($20.7 \pm 1.5 \times 10^3$ in the control sample), actinomycetes ($0.8 \pm 0.04 \times 10^3$ as compared to $3.1 \pm 0.2 \times 10^3$) and fungi ($0.9 \pm 0.1 \times 10^2$ as compared to ($14.0 \pm 0.1 \times 10^2$). On the other hand increasing the oil pollutants

Table (2) : Counts / g soil of the different groups of microorganism in the polluted soil samples as compared to their counts in a contorl non-polluted sample. R/C = ratio of the counts in the polluted sample to that in the control sample (R/C = more than one, indicates possitive response).

Organisms	Soil samples			
	1	2	3	Control sample
Total bacteria.	31.6 ± 0.6	3.9 ± 0.2	86.3 ±6.4	20.7 ± 1.5
Counts x 10^3	1.5	0.2	4.2	
R/C				
Actinomycetes	0.73 ± 0.03	0.8 ± 0.04	26.3 ± 1.0	3.1 ± 0.2
counts x 10^3	0.2	0.3	8.5	
R/C				
Oil degraders	7.0 ± 0.2	2.5 ± 0.2	18.4 ± 1.2	0.17 ± 0.01
Coutns x 10^2	41.2	14.7	108.2	
R/C				
Fungi	16.7 ± 0.2	0.9 ± 0.1	2.7 ± 0.1	14.3 ± 0.1
Counts x 10^2	1.1	0.1	0.2	
R/C				
Cellulose decomposers	4.6 ± 0.2	2.8 ± 0.1	4.8 ± 0.4	6.2 ± 0.2
Counts x 10^2	0.8	0.5	0.8	
R/C				
Thermophiles	7.6 ± 0.6	3.0 ± 0.1	3.5 ± 0.4	6.0 ± 0.3
Counts x 10^2	1.3	0.5	0.6	
R/C				

to 7.4 % resulted in slight increase in the concentrations of total bacteria and fungi. While, actinomyctes sharply decreased to reach $0.73 \pm 0.03 \times 10^3$ / g soil). Bacteria tend to respond more rapidly to oil contamination of soil, whereas fungi may be inhibited initially (Pinholt et al., 1978). Conversely, Jensen (1975) reported that the activity of fungi tends to persist long after bacterial activity has tapered off. Perry and Cerniglia (1973) proposed that though fungi are lower in numbers, they adapt more rapidly to adverse environmetal condition, such as limiting N and P or low moisture and pH. Davis and Westlake (1978) suggested that filamentous fungi may enhance oil biodegadtion in soil indirectly their mycelial invasion of the oil, thus providing increased surface contact area for bacteria capable of initiating hydrocarbon biodegradation. The same suggestion may be given also to actinomycetes Jensen (1975) reported that a number of actinomycetes have been shown to have hydrocarbon-degrading ability, (though these organisms do not seem to compete as successfully in contaminated soil) . Their slower growth, however, may infer a more dominant role in the later stages of hydrocarbon biodegradation .

Cellulose decomposers and thermophiles (Table 2) were less stimulated in the polluted soil samples as compared to the nonpolluted control sample. Their R/C ratios in most cases were less than one indicating negative response to oil contamination. Walker et al. (1974, 1975) concluded that oil would decrease utilization of chitin,

cellulose, lipids and protein, because the proportional representation of these populations within the total community was decreased by a crude and refined oil. On the other hand Kator and Herwig (1977) reported that oil had no effect on the population size of chitinolytic and cellulytic microorganisms in salt marsh water and sediments .

The results of the counts of oil-degarading microorganisms (Table 2) show that this group of microorganims was significantly greater in the polluted soil samples than in non-polluted control one. Maximum counts of oil-degrading bacteria were recorded from the less polluted soil sample (3) ($18.4 \pm 1.2 \times 10^2$ / g soil), this was followed by a count of ($7.0 \pm 0.2 \times 10^3$ / g) soil from the heavily polluted sample (1). As comparison, the lowest counts ($2.5 \pm 0.2 \times 10^2$ / g soil) of such organisms were recorded from the heavily polluted sample (2), although the higher percentage of oil degraders was recorded from this soil sample (3.4%). The results in Table (2) indicate that oil-degrading microorganism as indicated from their R/C ratios were of great positive response to exposure to oil contamination than the other groups of microorganisms. They rapidly increased in numbers, and their R/C ratios ranged from 14.7 (for soil sample 2) to 108.2 (for soil sample 3). In contrast the R/C ratios of fungi and all other groups of bacteria were in the range of 0.1 - 8.5 .

The most widely documented response of microbial community to exposure to oil is rapid increase in the size of hydrocarbon uti-

lizing component of the community. The phenomenon is wide spread among communities from variety of ecosystems. An accompanying response is an increase in the total bacterial number (Walker and Colwell, 1973; Hood et al., 1975; Hortwits and Atlas, 1977; Kator an Herwig, 1977 Colwell et al., 1978; Buckley, 1980). These results have similaries to our results obtained from soil sample (1) and (3). However, in soil sample (2) no accompanying positive response was observed (R/C = 0.2). This may find a support in the finding of Song and Bartha (1990) who found that an increase of the most probable numbers of hydrocarbon degraders was accompanied by a decline in other aerobic heterotrophs. These observations may be attributed to the release of harmful substances into the environment during the process of oil degradation. This may be supported by the results found in Table (1), in which it is observed that the highest amount of aromatic hydrocarbons (17.5%) was dagreded in soil (2).

It is difficult to generalize about the microbial response of soils subjected to oil contamination, because of great diversity of geographical and climate regions that do not readily lend themselves to comparison. Atlas et al. (1978) reported that both Pruhose Bay crude oil diesel fuel oil spilled on an Arctic coast plain soil produced an overall increase in microbial numbers for up to seven years. Other investigators (Jones et al., 1970; Gudin and Syratt, 1975; Sextone and Atlas, 1977, 1978; Pinholt et al., 1979) also found that peroleum application to soils increased activity and total

microbial counts as well as shifts to the composition of the microbial community. In contrast, Antoniewski and Schaefer (1972) reported no significang change in microbial numberds of hydromull soil contaminatrd either with crude oil or with a paraffinic hydrocarbon mixture. Odu (1972) reported an initial decline in both microbial numbers and respiration in a Sandy Nigerian soil contaminated with crude oil. Wayndham and Costerton (1981) reported no significant differences in total bacterial counts between control and oil sand sites .

Eighty two bacterial isolates were obtained from the polluted soil samples and 28 isolates form the control (non-polluted one). Results in Table (3) show that all strains isolated from soil samples (1) and (3) (28 isolates from each sample) were Gram negative rods. In soil (2) and in the control non polluted soil sample (4), the gram negative rods were only 46.2% and 3.6% respectively. On the other hand more than 50% of the isolates from soil sample (2) and more than 80% from the control sample (4) were gram positive rods. Gram variable rods were recorded only from the control sample .

Results of the identification of the isolated bacteria revealed the presence of three genera in the ono-polluted soils ample, they are *Pseudomonas* (3.6%) Arthrobacter (64.3%) and *Corynebacterium* (17.9%). In the less polluted soil sample, *Pseudomconas* (78.6%) and *Flavobacterium* (21.4%) were present. When the pol-

Table (3) : Distribution of the different forms and genera of bacteria isolated from the different soil samples. G-R: Gram negative rods, G + R : Gram positive rode and G±R = Gram variable rods.

Sample No.	Total No. of isolates	Gram reaction (%)			Genera (%)			
		G-R	G±R	G±R	*Pseudomonas*	*Flavo-bacterium*	*Arthro-bacter*	*Coryne baceterium*
1	28	100	--	--	100	--	--	--
2	26	46.2	53.8	--	46.2	--	53.8	--
3	28	100	--	--	78.6	21.4	--	--
Total	82	82.9	17.1	--	75.7	7.3	17.0	--
4	28	3.6	82.1	14.3	3.6	--	64.3	17.8

lutants increased to 5.9% in sample (2), a shift in the diversity of genera was observed, *Pseudomnas* (48.2%) and *Arthrobacter* (53.8%) were recorded. Another shift was observed in soil sample (1), in which all the Gram negative rods isolated were *Pseudomonas* strains. These results indicate the dominance of *Pseudomonas* strains these polluted soil samples. Jensen (1975) reported that oil treated soils possessed lower bacterial species richness than untreated soil. Population of *Arthrobacter*, *Corynebacterium*, *Mycobacterium* and *Nocardia* showed strong positive responses to oil contamination. Soil *Pseudomonads* represent anther bacterial group with a major role in soil biodegradation. *Pseudomonas* strains are often isolated from oil contaminated environments (Cooper and Hedrock, 1976; Diab, 1991 a) and have shown to degrade a wide variety of hydrocarbons including aromatic hydrocarbons (Traxler, 1962; Diab, 1991 b).

As for actinomycetes (Table 4), 53 isolates were isolated from the polluted soil samples and 21 isolates from the control non-polluted one. 79.2% of the isolates in the polluted samples and nearly 85.7% in the control sample were belonging to the genus *Stretomyces*. The genus *Nocadia* was more frequent (52.9) in soil sample (3) than in the other soil samples .

Classification of the genus *Streptomyces* to colour series (Table 4) show that five series were recorded from the control sample, the most frequent one was the grey series (47.4%). This was

Table (4): Distribution of the genera Nocardia and Streptomyces in the different soil samples. Distribution of the colur series of Streptomyces are also given. Gn: grean series, Gr: gray series, W = white seria, Y = yellow series, B = blue series and un = unidentified ser .

Sample	Nocardia		Streptomyces							
	No.of isolate	%	No.of isolate	%	Genera (%)					
					Gn	Gr	W	Y	B	Un.
1	--	--	16	100	93.8	6.2	--	--	--	--
2*	1	5	18	90	78.9	21.1	--	--	--	--
3	9	52.9	8	47.1	45.4	27.3	--	9.1	--	18.2
Total	10	18.9	42	79.2	76.1	17.4	--	2.2	--	4.3
4**	2	9.5	18	85.7	10.5	47.4	10.5	--	21.1	10.5

* One *Streptosporangium* strain was isolated .

** One *Streptoverticllium* strain was isolated .

followed by the blue series (21.1%). Other series were represented by 10.5% each. In the polluted soils samples there was a shift the diversity of the series; the dominant series was the green one (76.1%); white and the blue series disappered; the yellow and the unidentified series recorded only in the less contaminated soils ample (3) and the grey series decreased as the pollutants increased to reach 6.2 % in the highly polluted soil sample (1) .

It appears from the above results that the most resistant series of Streptomyces to oil exposure was the green series, it reached 93.8 % in the highly polluted sample (1) On the other hand and the most sensitive were the white and the blue series , this was followed by the unidentified series and the grey series .

All of the actinomycete and bacterial strains isolated from the different samples in the present work were selected and kept for further studies to evaluate their role and activities in the bioremediation process of these polluted desert soil .

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تأثير التلوث النفطى على فلوا الكائنات الدقيقة بالصحراء الكويتية

الملخص العربى

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* كلية التربية الأساسية بالكويت

* كلية العلوم قسم النبات جامعة الأزهر (بنات)

فى أثناء الغزو العراقى للكويت و تفجير آبار البترول حدث تلوث بيئى فتلوثت مساحة قدرها ٢٥ كم^{2} من الصحراء الكويتية بحوالى ٦٠ - ٨٠ مليون برميل من الزيت الخام .

وبعد مرور عشر شهور وحدوث بعض التحلل فى الزيت بالتربة الملوثة تم تجميع ٣ عينات ملوثة وعينة غير ملوثة (قياسية)وتعيين كمية الزيت المتبقية فى كل تربة فأظهرت النتائج أنه لايوجد نقص محسوس من مركبات الهيدوكربون المشبعة بينما كانت المركبات الهيدوكربونية الحلقية أكثر عرضة للتحلل البيولوجى .

وكان تركيز البكتريا المحللة للبترول أعلى فى التربة الأكثر تلوثا بالزيت الخام .

وكانت النسبة بين الأعداد فى العينات الملوثة إلى الأعداد وفى العينة الغير ملوثة بين (١٤ر٧ إلى ١ر٨ر٦) .

بينما كانت هذه النسبة (١ر - ٥ ر٨) لكل من العدد الكلى للبكتريا والأكتينوميسينات والفطريات ومحللات السليولوز و الكائنات الحية للحرارة .

وقد سجل تواجد ثلاث أجناس من البكتريا فى التربة غير ملوثة هم :

الأرثروبكتر (٣ر٦٤ ٪) الكواراينيكتريم (٩ر١٦٪) بينما كانت السودموناس (٦ر٣٪) . وقد وجد تحول عكسى فى العينات الملوثة فكان جنس السودموناس الأكثر شيوعا حيث سجل ٦ر٧٨ ٪ من العزلات وكان الأرثروبكتر ٨ر٥٣ ٪ محققاً بذلك سيادة على جنس السودومانوس فى التربة رقم (٢) بينما كان جنس الفلافوبكتريم (٤ر٢١ ٪) فى التربة رقم (٣) .

بينما كان الجميع معزولات تنتسب لجنس السودومانوس التربة رقم (١) . اما بالنسبة للاكتينوميسبنات كان جنس الأستربتوميسس هو الأكثر شيوعا ثم جنس النوكارديا فى جميع أنواع التربة ماعدا التربة رقم (٣) حيث ساد بها جنس النوكارديا .

وبالنسبة لمجموعات الألوان من جنس الأستربتوميسس فقد سجلت أيضاً اختلافاً فى التوزيع نتيجة لهذا التلوث البيئى حيث كانت مجموعتى الأستربتوميسس البيضاء والزرقاء أكثر حساسية لهذا الحدث وسجل اختفائها فى جميع أنواع التربة الملوثة وكانت المجموعة الخضراء هى المجموعة السائدة وهى تسجل بذلك تحملاً لهذه الظروف البيئية مما يمكن إعتبارهم دليلاً مرشداً لمثل هذا التلوث .