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Crude Oil Biodegradation in Shatt Al-Arab River, Iraq

KEYWORDS: Shatt Al-Arab River, oil biodegradation, Bioremediation, UV mutated bacteria

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ABSTRACT *Oil degrading bacteria in Shatt Al-Arab River were widely distributed to constitute a big percentage of heterotrophic bacteria. Their numbers and biodegradation potential were higher during summer and in sediment than during winter and in water. They belonged to the genera Micrococcus, Vibrio, Flavobacterium, Corynebactrium, Pseudomonas, Arthrobacter, Bacillus, Staphylococcus, Nocardia, Aeromonas, and Acintobacter. The most active degraders degraded the crude oil by more than 50 % over a 21 days period. The UV mutated degrader (P. putida) and Arthrobacter sp. mixed culture degraded the crude oil by 92 % over the same period. The rates of biodegradation in open water were limited by temperature and available nutrients. The adding of nutrients and oil degraders cultures to crude oil contaminated site was enhanced biodegradation. All components of crude oil were biodegraded at different rates. The biodegradation of aromatic hydrocarbons were much lower than n– and branched alkanes.*

INTRODUCTION

The production, transportation and distribution of petroleum during the past century resulted in hydrocarbons contamination becoming a major environmental problem. Petroleum has contaminated waters, soils, threatening human heath and damaging the environment (NRC, 2003). However, most of the environmental inputs of petroleum are accommodated largely due to capacities of microorganisms to biodegrade hydrocarbons (Atlas, 1981; Harayama *et al.*, 2004). Such hydrocarbons degrading microorganisms are ubiquitously distributed in the environment, and that the rates of hydrocarbon biodegradation are limited by abiotic environmental factors (Van Hamme *et al.*, 2003). The persistence of petroleum pollutants depend on the quantity and quality of the hydrocarbon mixture and on the properties of the affected ecosystem. In one environment, petroleum hydrocarbons can persist almost indefinitely, whereas under another set of conditions, the same hydrocarbons can be completely biodegraded within a few hours or days (Atlas, 1995).

This paper will report the potential of Shatt Al-Arab River ecosystem for petroleum biodegradation. Shatt Al-Arab River is the most important river and the only source of freshwater in the arid surroundings of southern Iraq. It is the prime freshwater source and pours about $5x10^9$ m^3 nutrient rich water into the Arabian Gulf each year (Al-Saad, 1995). Its water is liable to contain petroleum contaminants because of numerous operations. Farid *et al.* (2008) reported that the oil refinery effluents and losses during loading operations have been identified as the major sources of oil contamination in the water of Shatt Al-Arab River which empties into the North West Arabian Gulf. Little is known about hydrocarbons biodegradation process in Shatt Al-Arab River environment. The most previous studies in the river focused on the isolation and distribution of hydrocarbons utilizing microorganisms (Shamshoom *et al*, 1990). There is a need to understand the fate and behavior of hydrocarbon contaminants in this ecological system. Therefore, the present study was achieved. The results obtained in this study can service as a baseline for future biodegradation studies in the region.

The objective of present work was to 1- determine the density of oil degrading bacteria in the water and sediment samples of Shatt Al-Arab River. 2- isolate the indigenous oil degrading bacteria from the river area. 3- determine in vitro the hydrocarbons biodegradation potential of a variety of samples and isolates. 4- know the seasonal variations exist in the petroleum biodegradation potential. 5- mutate the indigenous oil degrading bacteria to obtain on bacterial inoculum with higher ability to degrade crude oil hydrocarbons. And finally, 6- evaluate in situ the effects of adding mixed cultures of exogenous oil degrading bacteria and nutrients on the bioremediation of crude oil.

MATERIALS AND METHODS

Description of study area and sampling

The Shatt Al-Arab River originates from the confluence of the two major rivers of Iraq (Tigris and Euphrates) at Qurna. Karun River, the only tributary of the Shatt Al-Arab River, joins its eastern bank south of Basrah City (Figure 1). The length of the Shatt Al-Arab River from Qurna i.e. its place of origin, to its mouth in Arabian Gulf, extends about 175 km. Its with varies at different points, ranging from 0.4 km at Basrah City to 1.5 km at its mouth. The water depth increases in general towards the Gulf with a maximum of 12.2 m. The water level is, however, affected by the high and low tides of the Arabian Gulf where the average tidal range is about 1.7 m. Shatt Al-Arab water characterized as being well mixed with limited vertical stratification of temperature and chlorinities. The water of Shatt Al-Arab mouth may be traced as far as 5 km into the Arabian Gulf. The discharge of this river reaches the waters of Kuwait Bay during the flood season.

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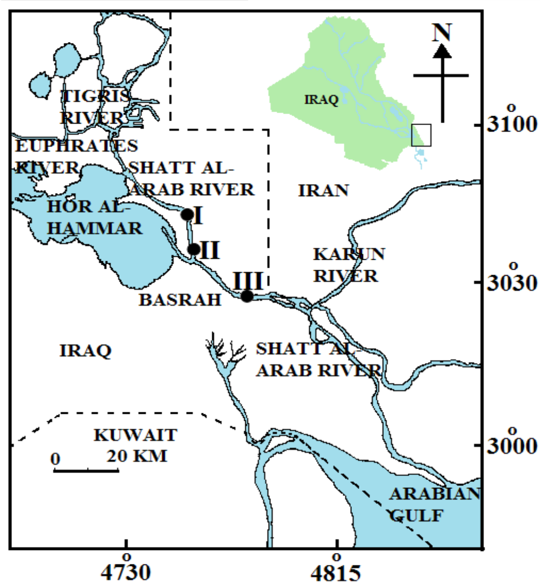

Figure 1: Map of Shatt Al-Arab River showing the position of stations

Three stations on the Shatt Al-Arab River were chosen for this study. The locations of these stations were shown on the map. Samples of water and sediment were collected from the study stations during winter and summer of 2012. The water collector device and van veen grap sampler were employed to collect the samples of water and sediment respectively. The samples were then transferred to laboratory by sterile bottles (water) and containers (sediment). The temperature, salinity, and pH of samples were measured. The samples were processed for bacterial enumeration within a few hours of collection.

Enumeration and isolation of bacteria
Enumeration of heterotrophic bacteria was done by mixed 1 ml of water or 1 g of sediment with 9 ml of sterile normal saline. The suspension was left to settle for 5–10 min. Six ten-fold dilutions were prepared and 0.1 ml from appropriate dilution was spreaded on to nutrient agar media (Difco) by sterile spreader. The plates were incubated for 48 hr. at 37 °C.

Enumeration and isolation of oil degrading bacteria were performed by inoculated 1 ml of water or 1 g of sediment in 250 ml Erlenmeyer flasks. Each flask contained mineral salts medium composing (per liter): NaCl, 0.3 g; $(NH_4)_2 SO_4$, 0.6 g; KNO_3, 0.6 g; KH_2PO_4, 0.25 g; $K2HPO_2$, 0.75 g; $MgSO_4.7H_2O$, 0.15 g; LiCl, 20 µg; $CuSO_4.5H_2O$, 80 µg; $ZnSO_4.H_2O$, 100 µg; $Al_2 (SO_4)_3.16H_2O$, 100 µg; $NiCl.6H_2O$, 100 µg; $CoSO_4.7H_2O$, 100 µg; KBr, 30 µg; KI, 30 µg; $MnCl_2.4H_2O$, 600 µg; $SnCl_2.2H_2O$, 40 µg; $FeSO_4.7H_2O$, 300 µg; and distilled H_2O, 1000 ml, (pH 7.5). After sterilization, 0.1 ml of sterile weathered Basrah regular crude oil to each 100 ml of medium was added into the flasks as the sole source of carbon and energy. Control flasks were left untreated with samples. All flasks were incubated at 37 °C in an orbital shaker set at 150 rpm. for 7–28 days. The bacterial growth of liquid cultures were monitored at 7–28 days intervals by plating 0.1 ml of appropriate dilution of liquid cultures on nutrient agar media (Difco). The plates were incubated at 37 °C for 48 hr., after which each type of colony appearing on the agar was recorded and picked up. The grown colonies were purified, enumerated, and examined microscopically. Pure cultures were further inoculated onto tube slants containing respective media and were kept stock cultures. These cultures were maintained at 4 °C and subcultured every 6–8 weeks.

Identification of bacteria was carried out according of their morphological characteristics and biochemical tests (Cowan and Steel, 1975; Holt *et al.*, 1994). The list of parameters used in characterization of bacteria is represented in Table 4.

Determination of biodegradation potential of samples
Several methods were used to determine the hydrocarbons biodegradation potential of the water and sediment samples. These methods involved exposing the samples to crude oil hydrocarbons in vitro, and measuring the oxygen consumption, the carbon dioxide production, and the changes in the concentration and weight of crude oil hydrocarbons every 7 days intervals up to day 21. Controls treatment was achieved without inoculated with samples.

To determine the oxygen consumption the procedure of Atlas *et al.* (1978) was used, 1 ml of water or 1 g of sediment samples were placed in a Gilson respirometer with 0.1 ml of sterile weathered Basrah regular crude oil. The rate of oxygen consumption was then measured.

To measure CO_2 production the procedure of Isinguzo and Bello (2005) was used, 90 ml of Matale's enrichment media, 0.1 ml of sterile weathered Basrah regular crude oil and 1 ml of water or 1 g of sediment samples were added in one liter covered jars. The jars were supplied with 20 ml vial containing 1 g of BaO_2 and 1 ml of sterile water to absorb the CO_2 formed during the biodegradation. The vials were withdrawn to analyze the quantity of CO_2 liberated.

To measure the concentration of crude oil hydrocarbons, the hydrocarbons were extracted from the starter liquid cultures media with 50 ml n–hexane three times followed the procedure described by Chaineau *et al.* (1999). The extracts were then filtered through sterile 0.45 µm Millipore filter to remove the culture materials. To these extracts 10 g of anhydrous sodium sulfate were added to remove excess water. The hexane extracts were reduced in volume to less than 5 ml by using a rotary vacuum evaporator. Further concentrate was made by stream of pure nitrogen for analysis by Shimadzu RF-540 spectroflurometer equipped with a DR-data recorder. The hydrocarbons were quantified by measuring the emission intensity at 360 nm, with excitation set at 310 nm and monochromatic slits of 10 nm.

To determine the change in weight of crude oil hydrocarbons, the gravimetric method 5520E in Standard Methods was used (American Public Health Association, 1992).

Determination of biodegradation potential of bacteria
The isolated bacteria from water and sediment samples were tested for their ability to degrade of crude oil hydrocarbons (Table 6). Each isolate was inoculated in 250 ml Erlenmeyer flasks contained the oiled mineral salts media. Control flasks were left without microbes. The flasks were incubated at 37 °C in an orbital shaker set at 150 rpm. for 7–21 days. The growth of bacteria was monitored and the hydrocarbons were extracted from the liquid cultures to determine the crude oil concentration by spectroflurometer._

Mutation of bacteria
Six bacterial species (*Pseudomonas aeruginosa*, *Pseudomonas putida*, *Pseudomonas cepacia*, *Pseudomonas fluorescens*, *Bacillus cereus* and *Bacillus subtilis*) were subcultured on to nutrient agar plates, incubated for 6 hr., suspended in the 20 ml of phosphate buffer (pH_7) (NaH_2PO_4, 6.0 g; Na_2HPO_4, 9.52 g; 1L distilled water) and diluted to 10^{-4} and then subjected to UV radiation for different periods (5 min. and 30 min.). Another replicates of cultures were incubated for 12 hr. and then subjected to UV radiation for 60 min. Different rates and stages of irradiation were designed to cover the different phases of exponential growth of different bacterial cells in order to effectively induce mutation. The UV light source was a germicidal lamp (254 nm). The output of the lamp was 10 erg/mm/sec. The irradiation was only applied

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when the fluency of lamp was in a stable maximum. After, each bacterium that cultured on nutrient agar media were observed morphologically for phenotypic expression and examined for their ability to degrade of crude oil hydrocarbons. When irradiation was over, mixed cultures of UV subjected and non subjected bacteria were made and then they inoculated in oiled mineral salts media for test their potential for hydrocarbons biodegradation (Table 6).

Bioremediation experiments

Bioremediation of crude oil was studied in situ into four water experimental pools each one measuring (2000×2000×1000 cm). All pools were designed in station II during summer. Four treatments were tested on the pools. The first pool was supplied by spreading 1000 ml of weathered Basrah regular crude oil. The second one was supplemented with 1000 ml of crude oil, 300 g from each of $(NH_4)_2So_4$ and K_2HPO_4 as nutrients, and mixed culture of oil degrading bacteria (*Pseudomonas* sp., *Pseudomonas putida* (mutant), *Micrococcus* sp., *Corynebacterium* sp., *Arthrobacter* sp. and *Bacillus* sp.). The third one was supplemented with crude oil and mixed culture of oil degrading bacteria. The fourth pool was left unoiled without any other treatment and saved as a background control. The water samples were taken from each pool every 7 days intervals up to day 21 of treatment. Then, the oil degrading bacteria were enumerated on the agar oiled mineral salts media (the same composition of above liquid media with addition 20 g of agar) incubated at 37 °C. and, the hydrocarbons were extracted and their concentrations were determined.

Hydrocarbons were extracted from water samples following the procedure of Cripps (1989), According to which, 100 ml of nangrade carbon tetrachloride (CCl_4) were used in two successive 50 ml extractions and the extracts were combined. The mixture was vigorously shaken to disperse the CCl_4 thoroughly throughout the water sample. The shaking was repeated several times before decanting the CCl_4. A small amount of anhydrous sodium sulphate was added to these extracts to remove excess water. The CCl_4 extracts were reduced to volume less than 5 ml by using a rotary vacuum evaporator. The reduced extracts were carefully pipette into a precleaned 10 ml volumetric flask, making sure that any residual particles of sodium sulphate were excluded and evaporated to dryness by a stream of pure nitrogen. The flasks were then rinsed with a fresh hexane. The rinsing was used to make the sample volume up to exactly 5 ml. The concentrated extracts were then fractionated by column chromatography. A column filled with 8 g each 5 % water deactivated alumina (100-200 mesh) was placed at the top and silica gel (100-200 mesh) at the bottom which were extracted with methylene chloride for 36 hr., dried at 130 °C for 24 hr., and deactivated with deionized water. The extract was then applied to the head of the column and eluted with 50 ml of hexane to isolate the aliphatic fraction and 50 ml of benzene to isolate the aromatic one. The both fractions were reduced to a suitable volume prior to analysis by a Perkin-Elmer Sigma 300 capillary gas chromatography in which the helium gas was used as a carrier gas with a linear velocity of 1.5 ml min^{-1}. The operating temperatures for detector and injector were 350 °C and 320 °C, respectively. The silica capillary column was operated under initial, final and rate temperatures that programmed as follows: Initial temperatures were 60 °C for aliphatic fraction for 4 min and 70 °C for aromatic fraction for 0 min, while final temperatures were 280 °C for aliphatic fraction and 300 °C for aromatic fraction for 30 min and rate was 4 °C/min for both aliphatic and aromatic fractions. Quantification of peaks and identification of hydrocarbons were done by a Perkin-Elmer computing integrator model LC-100.

RESULTS

Environmental parameters

Water temperature, pH, and salinity of Shatt Al-Arab River were reported in Table (1). The winter water samples had an average temperature of 15 °C, pH of 7.4, and salinity of 3.8 ‰. Summer samples had an average temperature of 34 °C, pH of 8.1, and salinity of 5.0 ‰.

TABLE -1
ENVIROMENTAL PARAMETWRS OF SHATT AL-ARAB RIER

Season	Parameter	Station			
		I	II	III	average
Winter	Temperature °C	16	16	14	15
	pH	7.5	7.3	7.5	7.4
	Salinity ‰	4.9	3.8	2.8	3.8
Summer	Temperature °C	35	34	34	34
	pH	8.1	8.1	8.2	8.1
	Salinity ‰	6.1	4.9	4.2	5.0

Heterotrophic and oil degrading bacterial counts

Table (2) showed the numbers of bacterial population in Shatt Al-Arab River. Oil degrading bacteria were found to comprise a big percentage of the total heterotrophic bacteria. Generally, the percent oil degraders were ranged from 64 % to 93 %. Sediment samples generally had higher counts of heterotrophic and oil degrading bacteria than water samples, but not necessarily higher percentage of oil degrading bacteria. The summer counts of heterotrophic and oil degrading bacteria in water and sediment samples were typically two orders of magnitude higher than comparable winter counts. The highest numbers of heterotrophic and oil degrading bacteria were found in samples collected from station II. Station III samples had lower numbers of heterotrophic and oil degrading bacteria. In water and sediment samples the percent oil degraders was higher in station 2 than other stations.

TABLE -2
BACTERIAL COUNTS (COLONY FORMING UNITS ml^{-1} or g^{-1}) IN SHATT AL-ARAB RIER

Sample	Season	Station	HB	ODB	P
Water	Win-ter	I	$7.8x10^4$	$6.5x10^2$	0.83
		II	$9.9x10^4$	$8.2x10^2$	0.82
		III	$7.1x10^4$	$5.7x10^2$	0.80
	Sum-mer	I	$5.0x10^6$	$3.2x10^4$	0.64
		II	$7.7x10^6$	$7.2x10^4$	0.93
		III	$2.7x10^6$	$2.3x10^4$	0.79
Sediment	Win -ter	I	$2.9x10^5$	$2.3x10^3$	0.79
		II	$3.9x10^5$	$3.6x10^3$	0.92
		III	$2.2x10^5$	$2.0x10^3$	0.90
	Sum-mer	I	$3.9x10^7$	$2.7x10^5$	0.69
		II	$4.5x10^7$	$4.1x10^5$	0.91
		III	$1.9x10^7$	$1.3x10^5$	0.68

HB= Heterotrophic bacteria, ODB= Oil degrading bacteria, P= Oil degrading bacteria/ Heterotrophic bacteria %

Biodegradation potential of samples

Growth of oil degrading bacteria in crude oil exposed samples was significantly different between types of samples (Table 3). Sediment samples generally had higher growth of oil degrading bacteria than water samples. Summer growth of oil degrading bacteria in water and sediment samples was higher than winter growth. The highest growth of oil degrading bacteria was found in the samples collected from station 2. Generally, the growth of oil degrading bacteria in samples increased with incubation time. After 21 days of incubation, $11.4x10^2$ and $11.1x10^3$ fold increase in water samples oil degrading bacteria and $55.1x10^2$ and $52.2x10^4$ fold increase in sediment samples oil degrading bacteria compared with the 7 days of incubation were observed during winter and summer respectively. After 28 days of incubation, the growth of oil degrading bacteria in samples decreased.

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Rates of O_2 consumption, CO_2 production and crude oil percents weight and concentration losses of samples were shown in Figure (2, 3, 4, and 5). Significant differences between type of samples and times of incubation were found. Generally, sediment samples showed the greatest values of O_2 consumption, CO_2 production and crude oil percents weight and concentration losses. The lowest values were found in water samples. Summer sediment and water samples showed the highest values than winter. Samples collected from station II had the highest values than another stations. Increase in rates of O_2 consumption, CO_2 production, and crude oil percents weight and concentration losses were observed with increasing of incubation time up to day 21.

TABLE -3
OIL DEGRADING BACTERIAL NUMBER CHANGE DURING DIFFERINT INCUBATION PERIODS

Sample	Season	Week	I	II	II	Control
Water	Winter	1	$2.8x10^2$	$3.3x10^2$	$2.0x10^2$	0
		2	$5.3x10^2$	$6.7x10^2$	$4.1x10^2$	0
		3	$6.3x10^2$	$7.9x10^2$	$5.3x10^2$	0
		4	$4.7x10^2$	$5.8x10^2$	$3.9x10^2$	0
	Summer	1	$1.1 x10^4$	$1.9 x10^4$	$0.8x10^4$	0
		2	$2.8x10^4$	$4.1x10^4$	$2.0x10^4$	0
		3	$4.9x10^4$	$6.2x10^4$	$3.8x10^4$	0
		4	$2.2x10^4$	$3.1x10^4$	$1.4x10^4$	0
Sediment	Winter	1	$6.6x10^2$	$9.2x10^2$	$5.1x10^2$	0
		2	$1.4x10^3$	$1.9x10^3$	$1.1x10^3$	0
		3	$2.5x10^3$	$3.0x10^3$	$2.1x10^3$	0
		4	$0.9x10^3$	$1.3x10^3$	$0.6x10^3$	0
	Summer	1	$7.1x10^4$	$9.8x10^4$	$4.9x10^4$	0
		2	$1.5x10^5$	$2.0x10^5$	$1.0x10^5$	0
		3	$2.4x10^5$	$3.1x10^5$	$1.9x10^5$	0
		4	$1.5x10^5$	$2.1x10^5$	$1.2x10^5$	0

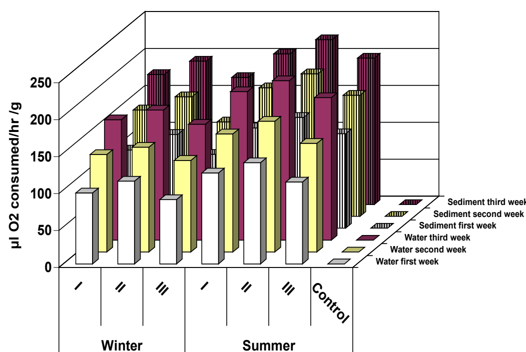

Figure 2: O_2 consumption rates of oil exposed samples collected from Shatt Al-Arab river

Oil degrading bacteria
The oil degrading bacteria identified from stations water and sediment samples were belonged to the genera *Micrococcus*, *Vibrio*, *Flavobacterium*, *Corynebactrium*, *Pseudomonas*, *Arthrobacter*, *Bacillus*, *Staphylococcus*, *Nocardia*, *Aeromonas*, and *Acintobacter*. Members of the same bacteria genera in water and sediment samples were observed (Table 4). Table (5 and 6) showed the distinguishing characteristics of bacteria.

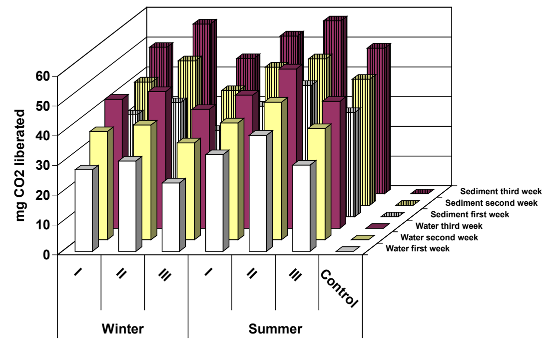

Figure 3: CO_2 production from biodegradation of oil in samples collected from Shatt Al-Arab river

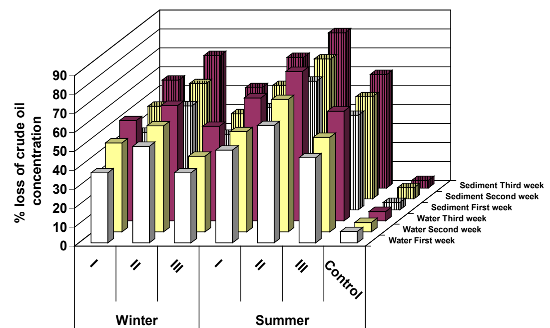

Figure 4: Perccent concentration loss of oil in samples collected from Shatt Al-Arab river

Biodegradation potential of bacteria
The bacterial isolates biodegraded the crude oil at different rates (Table 7). The crude oil percent concentration loss ranged from 64 % for *Pseudomonas* sp. to 32 % for *Vibrio* sp. The most active isolates in the utilization of crude oil were *Pseudomonas* sp., *Corynebactrium* sp., *Arthrobacter* sp., *Micrococcus* sp., *Pseudomonas aeruginosa*, *Pseudomonas putida*. and *Bacillus* sp. In these isolates, the crude oil percent concentration loss was more than 50 %.

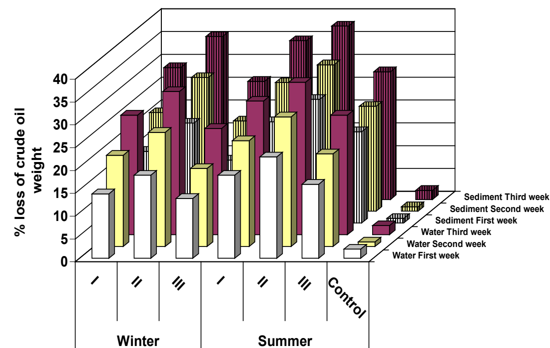

Figure 5: Perccent weight loss of oil in samples collected from Shatt Al-Arab river

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TABLE -4
OIL DEGRADING BACTERIA ISOLATED FROM SHATT AL-ARAB RIER

Sample	Station		
	I	II	II
Water	*Micrococcus* sp.	*Micrococcus* sp.	*Micrococcus* sp.
	Vibrio sp.	*Vibrio* sp.	*Vibrio* sp.
	Flavobacterium sp.	*Flavobacterium* sp.	*Flavobacterium* sp.
	Corynebactrium sp.	*Corynebactrium* sp.	*Corynebactrium* sp.
	Pseudomonas sp.	*Pseudomonas* sp.	*Pseudomonas* sp.
	Pseudomonas putida	Pseudomonas putida	Pseudomonas putida
	Pseudomonas fluorescens	Pseudomonas fluorescens	Pseudomonas aeruginosa
	Arthrobacter sp.	*Arthrobacter* sp.	*Bacillus* sp.
	Bacillus sp.	*Bacillus* sp.	Bacillus cereus
	Bacillus cereus	Bacillus cereus	*Nocardia* sp.
	Staphylococcus sp.	*Nocardia* sp.	*Acintobacter* sp.
	Nocardia sp.	*Acintobacter* sp.	*Aeromonas* sp.
	Acintobacter sp.	*Aeromonas* sp.	
Sediment	*Micrococcus* sp.	*Micrococcus* sp.	*Micrococcus* sp.
	Vibrio sp.	*Vibrio* sp.	*Vibrio* sp.
	Flavobacterium sp.	*Flavobacterium* sp.	*Flavobacterium* sp.
	Corynebactrium sp.	*Corynebactrium* sp.	*Corynebactrium* sp.
	Pseudomonas sp.	*Pseudomonas* sp.	*Pseudomonas* sp.
	Pseudomonas aeruginosa	Pseudomonas aeruginosa	Pseudomonas putida
	Pseudomonas cepacia	Pseudomonas fluorescens	Pseudomonas fluorescens
	Arthrobacter sp.	*Arthrobacter* sp.	*Bacillus* sp.
	Bacillus sp.	*Bacillus* sp.	Bacillus cereus
	Bacillus cereus	Bacillus subtilis	Bacillus subtilis
	Nocardia sp.	*Nocardia* sp.	*Staphylococcus* sp.
	Aeromonas sp.	*Aeromonas* sp.	*Aeromonas* sp.
	Acintobacter sp.		*Acintobacter* sp.

Mutation of bacteria
From six species of oil degrading bacteria (*Pseudomonas aeruginosa*, *Pseudomonas putida*, *Pseudomonas cepacia*, *Pseudomonas fluorescens*, *Bacillus cereus* and *Bacillus subtilis*) were subjected to UV radiation. The two species (*Pseudomonas aeruginosa* and *Pseudomonas putida*) were only mutated and one species (*P. putida*) was exhibited a high ability to degrade crude oil. The crude oil percent concentration loss by *P. putida* mutant was 66 %. The mixed culture of mutant bacteria and *Arthrobacter* sp. was found to degrade the crude oil better than other mixed cultures (Table 6). The ability of this culture to degrade crude oil within reasonably desired period (21 days) was observed 92 %.

TABLE -5
DISTINGUISHING CHARACTERISTICS OF BACTERIA

Character	*Micrococcus* sp.	*Vibrio* sp.	*Flavobacterium* sp.	*Corynebacterium* sp.	*Pseudomonas* sp.	*Arthrobacter* sp.	*Bacillus* sp.	*Staphylococcus* sp.	*Nocardia* sp.	*Aeromonas* sp.	*Acintobacter* sp.
Cell shape	Spherical	Short rods	Rods	Diplo-bacillus	Rods	Irregular rods	Rods	Spherical	Branched filaments	Rods	Rods
Motility	-	+	d	-	+	d	+	-	-	d	-
Gram stain	+	-	-	+	-	+	+	+	+	-	-
Albert's stain				+		-					
Spores formation	-	-	-	-	-	-	+	-	d	-	-
Pigmentation	-		+		+			-	d	d	
Oxidase test		+			+					+	-
Catalase test	+	+	+	+	+	+	+	+		+	+
Glucose fermentation	-	+	+	+	-	-		+		+	
Lactose fermentation			+	+	-	-				+	
Hydrolysis of starch		d	d				d				
Hydrogen peroxide formation	-							-			
Gas production	-	-			-			-		d	-
H_2S produce		-									-
Nitrate reduction							d				
Indol		d									-

d= +or-

Bioremediation experiments
The numbers of oil degrading bacteria were significantly different between four pools samples (Table 7). Oiled pools generally had higher counts of oil degrading bacteria than unoiled ones. The nutrients supplementing pool counts of oil degrading bacteria was higher than comparable unfertilized pool. Figure (6 and 7) showed the changes in crude oil components of exogenous bacteria and/or nutrients exposed pools. Gas chromatographic analysis only evaluated n-alkanes in the range of C_{14}-C_{30} as well as pristane and phytane compounds. The chromatographic data showed that the low molecular weight n-alkanes C_{14}-C_{16} were approximately totally metabolized whereas the other n-alkanes C_{17}-C_{30} were less degraded. Pristane and phytane were more resistant to biodegrade than n-alkanes. The data also showed that biodegradation was not restricted to paraffin compounds, but that the aromatic compounds were also degraded. The evaluated aromatic compounds was fluorine, anthracene, floranthene, pyrene, benzo {a} anthracene, chrysene, benzo {b} fluoranthene, benzo {k} fluoranthene, benzo {a} pyrene, benzo {ghi} perylene and indino {1, 2, 3 ed} pyrene. The data showed that the higher weight polycyclic aromatic hydrocarbons (floranthene, pyrene, benzo {a} anthracene, chrysene, benzo {b} fluoranthene, benzo {k} fluoranthene, benzo {a} pyrene, benzo {ghi} perylene and indino {1, 2, 3 ed} pyrene.) were more resistant to bacterial attack whereas lower weight polycyclic aromatic hydrocarbons (fluorine and anthracene) were less resistant. Increase in the rates of hydrocarbons biodegradation were observed in the pool supplementing with nitrogen and phosphorus nutrients and oil degrading bacteria than other pools. The biodegradation rates of n– and branched alkanes were higher than of aromatic hydrocarbons._

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DISCUSSION

Shatt Al-Arab river environment were found to posses the potential for petroleum biodegradation. Oil degrading bacteria were found to be widely distributed. They constitute a big percentage of the indigenous heterotrophic bacteria. Atlas (1981) and Brakstad and Lødeng (2004) reported that the numbers of oil degrading microorganisms present in an ecosystem determines in part the ability of that ecosystem to degrade petroleum pollutants. The numbers of oil degrading bacteria were found to vary in relation of water temperature. When the temperature was high during summer the bacterial counts were significantly higher than that when the temperature was low during winter. The low numbers of oil degrading bacteria found in winter water and sediment samples may indicate a restricted ability to degrade petroleum in those samples (Atlas, 1981). The shifts in hydrocarbons utilizing microorganisms in natural habitats due to temperature changes had previously been reported by Coulon *et al*. (2007) and Venosa and Holder (2007). Temperature represents one of the most important factors affected the rates of hydrocarbons microbial metabolism (Ferguson *et al*., 2003; Delille *et al*., 2009). The variation in the numbers of oil degrading bacteria between the stations may depend on variations in the levels of nutrients and petroleum contamination (Boopathy, 2000). The higher count and percent of oil degrader found in water and sediment samples of station II may indicate that this area had previously been exposed to hydrocarbons. Panda *et al*. (2013) reported that the numbers of hydrocarbons utilizing microorganisms in an ecosystem have found to correspond to the presence of petroleum. The low percent hydrocarbon utilizer was indicative of a pristine environment (McGenity *et al*., 2012). The levels of hydrocarbon utilizing microorganisms can be used as an indicator of the presence of petroleum pollutants (Yakimov *et al*., 2007; Elloumi *et al*., 2008).

Biodegradation experiments in liquid cultures showed that there are actual changes in the numbers of oil degrading bacteria of crude oil exposed samples during 21 days of incubation. During this period, the numbers of water and sediment samples oil degrading bacteria increased. This indicated that these bacteria had shown a rapid adaptation to degradation of petroleum hydrocarbons. Such conclusion had also been reported by Cerniglia and Heitkamp (1989) Chikere *et al*. (2009). Macnaughton *et al*. (1999) observed a strong increase in numbers of bacteria after an oil treatment. However, after 28 days of incubation, the bacterial numbers declined down. The response of bacteria to crude oil hydrocarbons was quite immediate, without the time lag associated with a long period of enzymatic adaptation. After exhaustion of easily degradable compounds, the remaining crude oil components were progressively more recalcitrant to biodegradation, so the bacterial numbers decreased. In liquid cultures, metabolic by products like fatty acids, ketones, or alcohols may accumulated and inhibit the biodegradation potential of the microbiota (Oudot, 1984; Chaineau *et al*., 1995). This could also explain part of the decrease in the numbers of oil degrading bacteria after 28 days of incubation.

TABLE -6
DISTINGUISHING CHARACTERISTICS OF BACTERIAL SPECIES

Character	Pseudomonas putida	Pseudomonas fluorescens	Pseudomonas aeruginosa	Pseudomonas cepacia	Bacillus cereus	Bacillus subtilis
Cell shape	Rods	Rods	Rods	Rods	Rods	Rods
Motility	+	+	+	+	d	+
Gram stain	-	-	-	-	+	+
Albert's stain	-	-	-	-		
Spores formation					+	+
Oxidase test	+		+			
Catalase test	+	+	+	+	+	+
Hydrolysis of starch	-		-	d		
Gelatin hydrolysis	d		+	-		
NO^-_3 to NO^-_2				-	+	+
Growth in anaerobic agar					+	-
Maximum temperature for growth, C°					35-45	45-55
Minimum temperature for growth, C°					10-20	2-20
Egg yolk reaction					-	+
Casein hydrolyzed					+	+
Tyrosine decomposed					-	+
Fluorescent pigments	+	+	d			
Growth at 41 C°	-	-	+	d		
Arginine dihydrolase	+		+	-		
Denitrification	-	d	+	-		
Glucose	+		+	+		
Sucrose		d				
Geraniol	-		+			
Ethanol		d				
Saccharate		d		+		

Petroleum biodegradation potential measurements of samples showed that water and sediment samples had the ability to metabolize crude oil. The fact that there was rapid increase in the rates of oxygen consumption when crude oil was added indicates that these samples were actively degrading hydrocarbons when collected and that the samples were capable of immediately beginning to degrade petroleum pollutants.

The CO_2 production experiments showed that samples were capable of mineralization of petroleum, i.e. conversion of hydrocarbons to CO_2 and H_2O or complete removal of petroleum hydrocarbon pollutants.

TABLE -7
BIODEGRADATION POTENTIAL OF OIL DEGRADING BACTERIA AFTER 21 DAYS OF INCUBATION

Bacteria	% loss of crude oil concentration
Micrococcus sp.	57
Vibrio sp.	32
Flavobacterium sp.	35
Corynebactrium sp.	60
Pseudomonas sp.	64
Pseudomonas putida	54
Pseudomonas aeruginosa	55
Pseudomonas cepacia	46
Pseudomonas fluorescens	37
Arthrobacter sp.	59
Bacillus sp.	51
Bacillus subtilis	39
Bacillus cereus	43
Staphylococcus sp.	36
Nocardia sp.	46
Acintobacter sp.	36
Aeromonas sp.	40

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Pseudomonas putida (mutant)	66
Pseudomonas putida (mutant) + *Arthrobacter* sp.	92
Pseudomonas putida + *Arthrobacter* sp.	66
Pseudomonas sp. + *Arthrobacter* sp.	76
Pseudomonas sp. + *Corynebactrium* sp.	76
Arthrobacter sp. + *Corynebactrium* sp.	70
Pseudomonas sp. + *Bacillus cereus*	66

TABLE -8
BACTERIAL COUNTS (COLONY FORMING UNITS ml^{-1} or g^{-1}) IN EXPERIMENTAL POOLS

Week	First pool	Second pool	Third pool	Fourth pool
1	$3.0x10^4$	$7.2x10^9$	$2.2x10^6$	$3.7x10^2$
2	$4.2x10^4$	$8.1x10^9$	$4.2X10^6$	$4.2x10^2$
3	$6.2X10^4$	$9.8x10^9$	$5.3x10^6$	$4.2X10^2$

Evaluation of weight and concentration losses of crude oil measured by gravimetric and fluorescence spectrophotometric techniques showed that the reduction in crude oil weight and concentration can extensively be detected in water and sediment samples. This confirmed that crude oil undergo biodegradation. The weight loss showed, though, that abiotic parameters can greatly influence the rates of crude oil biodegradation.

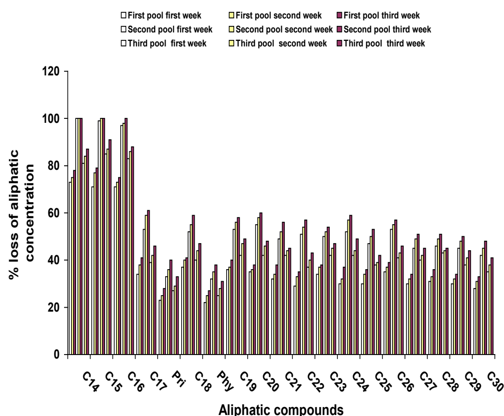

Figure 6: Aliphatic compounds degradation in oiled pools during 21 days of remediation process

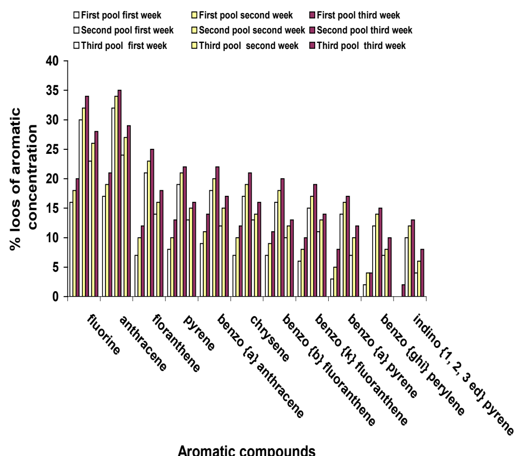

Figure 7: Aromatic compounds degradation in oiled pools during 21 days of remediation process

Biodegradation experiments indicated that the activity in water samples was generally lower than sediment samples. This was in agreement with conclusion of Cerniglia and Heitkamp, (1989). Summer samples were found to have fairly high biodegradation potential. The samples collected in winter were also capable to biodegrade crude oil but in lower rates than summer samples. The variation in the crude oil biodegradation potential between summer and winter samples may dependent in part on variations in the levels of hydrocarbon degrading microorganisms and in part on abiotic factors. Several investigators have reported seasonal fluxes in oil degrading microorganisms and hydrocarbons degradation rates with the highest activities in summer and the lowest activities in winter (Pierce *et al.*, 1975; Lee and Ryan, 1983; Cerniglia and Heitkamp, 1989). Lee and Ryan (1983) suggested that high summer activity in water and sediment exposed to hydrocarbons was due to both a higher density of bacteria and to higher metabolic activity of individual bacteria at the higher temperature. The experiments showed that station II had high biodegradation potential. This area characterized by having a high percent and numbers of oil degrading bacteria capable to metabolizing hydrocarbons.

A variety of oil degrading bacterial isolates were recovered from recent water and sediment samples. Some bacteria were Gram-negative and belonged to genera already known for their ability to degrade petroleum hydrocarbons: *Pseudomonas*, *Flavobacterium*, *Aeromonas*, *Acintobacter*, and *Vibrio*., other bacteria were Gram-positive including several genera of bacteria that were also known as being able to utilize petroleum hydrocarbons: *Micrococcus*, *Arthrobacter*, *Corynebactrium*, *Bacillus*, *Staphylococcus*, and *Nocardia* (Cerniglia, 1992; Geiselbrecht *et al.*, 1998; Van Hamme *et al*, 2003; Brakstad and Bonaunet, 2006). Biodegradation potential experiments showed that the bacterial isolates were able to degrade crude oil at different rates and the most active isolates were *Pseudomonas* sp., *Corynebactrium* sp., *Arthrobacter* sp., *Micrococcus* sp., *P. aeruginosa*, *P. putida*, and *Bacillus* sp.

UV radiation was commonly used to generate mutant strains of microorganisms. Its wavelengths were preferentially absorbed by nucleotides of DNA and by aromatic amino acids of proteins, so it has important biological and genetic effects (Witkin, 1976). Of about six bacterial species subjected to genetic mutation in our experiments only two mutated and one (*P. putida*) exhibited high ability to degrade crude oil. The present experiments demonstrated that the mixed culture of mutant bacteria (*P. putida*) and *Arthrobacter* sp. given the greatest capability to degrade crude oil compared with another mixed cultures.

The bioremediation experiments revealed that the biodegradation in crude oil supplemented pool was limited by available concentrations of nitrogen and phosphorus. The addition of these nutrients was found to stimulate microbial activity and enhance biodegradation. In addition to, the adding (seeding) of bacteria capable to degrade hydrocarbons to the oiled pool initiate the degradative process. Atlas (1995) and Ruberto *et al*. (2003) reported that there are two approaches taken for the bioremediation of petroleum pollutants are the addition of microorganisms (seeding) that are able to degrade hydrocarbons and the modification of the environment, for example adding fertilizers or aerating the contaminated sits. Bioremediation experiments confirmed that crude oil can actually undergo biodegradation in situ. Biodegradation resulted in the complete degradation of low molecular weight n-alkanes and extensive degradation of high molecular weight n-alkanes. The larger molecules are considered to be less degradable than smaller ones (de Jonge *et al.*, 1997). Del Arco and de Franca (2001) reported that the susceptibility of microbes to degradation of n-alkanes compounds was inversely proportional to increasing carbon numbers. Isoprenoid pristane and phytane were more resistant to microbial attack. Branched alkanes are more re-

sistant to microbial degradation than n-alkanes due to their molecular structure (Van Hamme *et al*., 2003). Aromatic compounds, including polynuclear aromatic compounds, were also found to undergo biodegradation but their degradation rate was lower than n-alkanes and isoalkanes and was correlated with the number of rings. This was already observed in oil biodegradation studies (Harayama *et al*., 1999; Kanaly and Harayama, 2000) and in bioremediation experiments (Atlas, and Cerniglia, 1995; Hozumi *et al*., 2000). The higher molecular weight polycyclic aromatic hydrocarbons are known to be more persistent (Boonchan *et al*., 2000).

CONCLUSION

The potential of Shatt Al-Arab River for oil biodegradation is due in a large part to a wide variety of oil degraders which are widely and abundantly distributed in its water and sediment and include variety genera of bacteria *Micrococcus*, *Vibrio*, *Flavobacterium*, *Corynebactrium*, *Pseudomonas*, *Arthrobacter*, *Bacillus*, *Staphylococcus*, *Nocardia*, *Aeromonas*, and *Acintobacter*. This biodegradation potential is being with spatial and seasonal variations dependent on variations in levels of oil degraders and abiotic parameters, such as temperature and available of essential nutrients which may restrict oil biodegrading activity. By overcoming environmental limitation, such as by adding fertilizers to overcome nitrogen and phosphate limitations, the activities of indigenous degraders can be stimulated, and the rates of oil degradation accelerated. Seeding cultures of undefined mixtures of microorganisms or very specific cultures of microorganisms with defined metabolic capacities can also initiate the degradative process. Individuals hydrocarbons of crude oil were totally or partially biodegraded: low molecular weight n-alkanes were completely degraded by oil degraders while the high molecular weight n-alkanes are less degradable. Branched alkanes are resistant to biodegradation compared with n-alkanes. Aromatic hydrocarbons, including polynuclear aromatic compounds are more resistant to microbial attack than n- and branched alkanes. Further experiments are required with petroleum hydrocarbons in Shatt Al-Arab River environment to determine the full degradative potential of microorganisms and the mechanisms by which they metabolize the hydrocarbons.

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