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Petroleum bioremediation – a multiphase problem

Eugene Rosenberg, Rachel Legmann, Ariel Kushmaro, Ran Taube, Ellik Adler[1] & Eliora Z. Ron
Department of Molecular Microbiology and Biotechnology, The George S. Wise Faculty of Life Sciences, Tel-Aviv University, Tel-Aviv, Israel; [1] Marine and Coastal Environment Division, Office of the Environment, Jerusalem, Israel

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Abstract

Microbial degradation of hydrocarbons is a multiphase reaction, involving oxygen gas, water-insoluble hydrocarbons, water, dissolved salts and microorganisms. The fact that the first step in hydrocarbon catabolism involves a membrane-bound oxygenase makes it essential for microorganisms to come into direct contact with the hydrocarbon substrate. Growth then proceeds on the hydrocarbon/water interface. Bacteria have developed two general strategies for enhancing contact with water-insoluble hydrocarbons: specific adhesion mechanisms and production of extracellular emulsifying agents. Since petroleum is a complex mixture of many different classes of hydrocarbons, of which any particular microorganism has the potential to degrade only part, it follows that the microorganisms must also have a mechanism for desorbing from 'used' oil droplets.

The major limitations in bioremediation of hydrocarbon-contaminated water and soil is available sources of nitrogen and phosphorus. The usual sources of these materials, e.g. ammonium sulfate and phosphate salts, have a high water solubility which reduces their effectiveness in open systems because of rapid dilution. We have attempted to overcome this problem by the use of a new controlled-release, hydrophobic fertilizer, F-1, which is a modified urea-formaldehyde polymer containing 18% N and 10% P as P_2O_5. Microorganisms were obtained by enrichment culture that could grow on crude oil as the carbon and energy source and F-1 as the nitrogen and phosphorus source. The microorganisms and the F-1 adhered to the oil/water interface, as observed microscopically and by the fact that degradation proceeded even when the water phase was removed and replaced seven times with unsupplemented water – a simulated open system. Strains which can use F-1 contain a cell-bound, inducible enzyme which depolymerizes F-1.

After optimizing conditions in the laboratory for the use of F-1 and the selected bacteria for degrading crude oil, a field trial was performed on an oil contaminated sandy beach between Haifa and Acre, Israel, in the summer of 1992. The sand was treated with 5 g F-1 per kg sand and inoculated with the selected bacteria; the plot was watered with sea water and plowed daily. After 28 days the average hydrocarbon content of the sand decreased from 5.1 mg per g sand to 0.6 mg per g sand. Overall, there was an approx. 86% degradation of pentane extractables as demonstrated by dry weight, I.R. and GLC analyses. An untreated control plot showed only a 15% decrease in hydrocarbons. During the winter of 1992, the entire beach (approx. 200 tons of crude oil) was cleaned using the F-1 bacteria technology. The rate of degradation was 0.06 mg g^{-1} sand day^{-1} (10 °C) compared to 0.13 mg g^{-1} sand day^{-1} during the summer (25 °C).

Introduction

Hydrocarbons are a ubiquitous class of natural compounds. Not only are they found in petroleum-polluted areas, but small concentrations are present in most soils and sediments (e.g. Giger & Blumer 1974; Stephenson 1966). It is, therefore, not surprising that hydrocarbon-oxidizing bacteria are located in virtually all natural areas, although with large variations in cell concentration (Rosenberg 1991). Why then is petroleum pollution a major ecological problem? The reason is that for microorganisms to degrade hydrocarbons, they must have available sources of oxygen, nitrogen and phosphate, elements which are not present in sufficient quantities in crude oil and petroleum products.

The use of hydrocarbons as substrates for bacterial growth presents special problems to both the microorganisms using them as a source of carbon and energy (Table 1) and to investigators in the field of hydrocarbon microbiology.

There are two essential characteristics that define hydrocarbon-oxidizing microorganisms:

1. membrane-bound, group-specific oxygenases and
2. mechanisms for optimizing contact between the microorganisms and the water-insoluble hydrocarbon.

Several reviews have been published on the microbial metabolism of straight-chain and branched alkanes (e.g. Singer & Finnerty 1984), cyclic alkanes (e.g. Perry 1981) and aromatic hydrocarbons (e.g. Cerniglia 1984). It has been established that the first step in the degradation of hydrocarbons by bacteria is the introduction of both atoms of molecular oxygen into the hydrocarbon. In the case of aromatic hydrocarbons, ring fission requires a dihydroxylation reaction and the subsequent formation of a *cis*-dihydrodiol (Gibson 1968) and is carried out by a membrane-bound enzyme system (Crutcher & Geary 1979). Further oxidation leads to the formation of catechols that are substrates for another deoxygenase that catalyzes ring fission (Dagley 1971).

In general, alkanes are terminally oxidized to the corresponding alcohol, aldehyde and fatty acid (McKenna & Kallio 1965). Fatty acids derived from alkanes are then further oxidized to acetate and propionate (odd-chain alkanes) by inducible oxidation systems. Different microorganisms exhibit different group specificities. For example, some grow on alkanes of six to ten carbons in chain length, whereas others grow on long chain alkanes. Some of the oxygenases are encoded on plasmids and others on chromosomal genes. Subterminal oxidation apparently occurs in some bacterial species (Markovitz 1971).

The low solubility of hydrocarbons in water, coupled to the fact that the first step in hydrocarbon degradation involves a membrane-bound oxygenase, makes it essential for bacteria to come into direct contact with the hydrocarbon substrates. Two general biological strategies have been proposed for enhancing contact between bacteria and water-insoluble hydrocarbons:

1. specific adhesion mechanisms and
2. emulsification of the hydrocarbon.

In order to understand the special cell surface properties of bacteria that allow them to grow on hydrocarbons, it is necessary to consider the dynamics of petroleum degradation in natural environments. Following an oil spill in the sea, the hydrocarbons rise to the surface and come into contact with air. Some of the low molecular weight hydrocarbons volatilize; the remainder are metabolized relatively rapidly by microorganisms which take up soluble hydrocarbons. These bacteria do not adhere to oil and do not have a high cell-surface hydrophobicity (Rosenberg & Rosenberg 1985). The next stage of degradation involves microorganisms with high cell-surface hydrophobicity, which can adhere to the residual high molecular weight hydrocarbons.

Table 1. Requirements for biodegradation of petroleum.

A.	Microorganisms with:
	1. Hydrocarbon-oxidizing enzymes
	2. Ability to adhere to hydrocarbons
	3. Emulsifier-producing potential
	4. Mechanisms for desorption from hydrocarbons
B.	Water
C.	Oxygen
D.	Phosphorus
E.	Utilizable nitrogen source

In the case of *A. calcoaceticus* RAG-1, this adherence is due to thin hydrophobic fimbriae (Rosenberg et al. 1982). Mutants lacking these fimbriae failed to adhere to hydrocarbons and were unable to grow on hexadecane. Other bacteria exhibit high cell surface hydrophobicity as a result of a variety of fimbriae and fibrils, outer-membrane and other surface proteins and lipids. Bacterial capsules and other anionic exopolysaccharides appear to inhibit adhesion to hydrocarbons (Rosenberg et al. 1983).

Desorption from the hydrocarbon is a critical part of the growth cycle of petroleum-degrading bacteria. Petroleum is a mixture of thousands of different hydrocarbon molecules. Any particular bacterium is only able to use a part of the petroleum. As the bacteria multiply at the hydrocarbon/water interface of a droplet, the relative amount of nonutilizable hydrocarbon continually increases, until the cells can no longer grow. For bacteria to continue to multiply, they must be able to move from the depleted droplet to another one. *A. calcoaceticus* RAG-1 has an interesting mechanism for desorption and for ensuring that it only reattaches to a droplet of fresh oil (Rosenberg et al. 1989). When cells become starved on the 'used' hydrocarbon droplet or tar ball, they release their capsule. The capsule is composed of an anionic heteropolysaccharide, with fatty acid side-chains, referred to as emulsan (Rosenberg 1986). The extracellular, amphipathic emulsan attaches avidly to the hydrocarbon/water interface, thereby displacing the cells to the aqueous phase. Each 'used' oil droplet or tar ball is then covered with a monomolecular film of emulsan. The hydrophilic outer surface of the emulsan-coated hydrocarbon prevents reattachment of the RAG-1 cells. The released capsule-deficient bacteria are hydrophobic and readily adhere to fresh hydrocarbon substrate.

Many hydrocarbon-degrading microorganisms produce extracellular emulsifying agents. In some cases, emulsifier production is induced by growth on hydrocarbons (Hisatsuka et al. 1971). Mutants which do not produce the emulsifier grow poorly on hydrocarbons (Itoh & Suzuki 1972). Pretreatment of oil with emulsifying agents can both inhibit and stimulate oil biodegradation (e.g. Foght et al. 1989; Nakahara et al. 1981). As discussed above, emulsification may be a by-product of a cell/hydrocarbon detachment process.

Acinetobacter sp. HO1-N accumulates extracellular membrane vesicles of 20–50 nm in diameter when grown on hexadecane (Kappeli & Finnerty 1980). The isolated vesicles partition exogenously supplied hydrocarbons in the form of a microemulsion. These vesicles appear to play a role in the uptake of alkanes. Recently, Miller & Bartha (1989) have been able to overcome the difficulties involved in the transport of water-insoluble, solid hydrocarbons by using unilamellar vesicles. A *Pseudomonas* isolate grew on octadecane (C_{18}) and hexatri-acontane (C_{36}) with K_s values of 2,450 and 2,700 mg/liter, compared to 60 and 41 mg/liter, respectively, when the hydrocarbon was presented in the form of liposomes. The data clearly demonstrate the importance of transport in the microbial metabolism of recalcitrant hydrocarbons.

The low solubility of hydrocarbons in water, coupled to the fact that the first step in hydrocarbon degradation involves a membrane-bound oxygenase, makes it essential for bacteria to come into direct contact with their hydrocarbon substrates.

As mentioned above, microorganisms which have the genetic potential to bind, emulsify, transport and degrade hydrocarbons are widely distributed in Nature. Thus, the rate limiting step in biodegradation of hydrocarbons is not the lack of appropriate microorganisms. Depending upon the particular environmental situation, the extent of degradation depends on the availability of moisture, oxygen and utilizable sources of nitrogen and phosphorus. Thus, effectiveness of a bioremediation program depends on defining the limitations and overcoming them – in a practical way.

The requirements for oxygen and moisture is not a problem in oil spills in aquatic environments. However, on land, oxygen and water are often rate-limiting. If oil has not penetrated too deeply into the ground, then watering and tilling are often advantageous. These problems have been discussed theoretically by Atlas (1991), and Bartha et al. (1990) have provided some recent useful data on the subject.

The major limitation in the biodegradation of hydrocarbons on land and water is an available

source of nitrogen and phosphorus. In theory, approximately 150 mg of nitrogen and 30 mg of phosphorus are consumed in the conversion of 1 g of hydrocarbon to cell material. The nitrogen and phosphorus requirements for maximum growth of hydrocarbon oxidizers can generally be satisfied by ammonium phosphate. Alternatively, these requirements can be met with a mixture of other salts, such as ammonium sulfate, ammonium nitrate, ammonium chloride, potassium phosphate, sodium phosphate and calcium phosphate. When ammonium salts of strong acids are used, the pH of the medium generally decreases with growth. This problem can often be overcome by using urea as the nitrogen source. All of these compounds have a high water solubility which reduces their effectiveness in open systems because of rapid dilution. In principle, this problem can be solved by using oleophilic nitrogen and phosphorus compounds with low C:N and C:P ratios. For example, it was found that a combination of paraffinized urea and octyl phosphate was able to replace nitrate and inorganic phosphate, respectively (Atlas & Bartha 1973).

Recently, commercial nitrogen and phosphorus-containing fertilizers that have an affinity for hydrocarbons are being developed for treating oil pollution. The most extensively studied example is the oleophilic fertilizer Inipol EAP 22, an oil-in-water microemulsion, containing a N:P ratio of 7.3:2.8 (Glaser 1991). Inipol EAP 22 is composed of oleic acid, lauryl phosphate, 2-butoxyl-1-ethanol, urea and water. The fertilizer was used in large quantities to treat the oil-contaminated shoreline following the 1989 oil spill in Prince William Sound, Alaska. Although a number of articles (reviewed by Pritchard 1993) have reported that the fertilizer treatment was successful, several questions still remain, mainly because of a lack of appropriate controls. There are at least three problems with Inipool EAP 22. First, it contains large amounts of oleic acid, which serves as an alternative carbon source, thereby further increasing the C:N ratio in the environment. Second, it contains an emulsifier which could be harmful to the environment. Third, as soon as the fertilizer comes into contact with water, the emulsion breaks, releasing the urea to the water phase where it is not available for the microorganisms.

We report here, laboratory and field experiments using a new controlled-release, hydrophobic fertilizer, referred to as F-1.

Isolation of oil-degrading bacteria using fertilizer F-1 as the source of nitrogen and phosphorus

A mixed bacterial culture was obtained by enrichment culture procedure using crude oil as a carbon and energy source, F-1 as a nitrogen and phosphorus source and sea water as the source of other minerals. Fresh sea water and tar served as the enrichment inoculum. After three transfers of 1 ml into 20 ml fresh medium, turbid cultures were obtained during overnight incubation. Microscopic examination demonstrated a mixed bacterial population and emulsified oil droplets. When plated on marine agar, several different colony types appeared from which three different strains were isolated for further studies. Each of these pure cultures was able to grow on and emulsify oil utilizing F-1 as its sole nitrogen and phosphorus source. The bacteria were referred to as ER strains RL3 (ER-LR3), RL4 (ER-RL4) and RT (ER-RT).

Growth of mixed cultures

The kinetics of growth of the mixed culture on crude oil and acetate is shown in Figs. 1 and 2. The mixed culture had a doubling time of approximately 2 h, reaching stationary phase after 40 h at 2×10^8 cells/ml. The pH of the growth medium decreased from an initial value of 7.8 to a minimum of 6.8 at 24 h and then increased to a final value of 7–7.2. The turbidity of the culture rose sharply up to 32000 Klett units (K.U.), mostly because of emulsification of the oil. In order to estimate how much of the turbidity was contributed by the cells, the culture was grown on 0.5% acetate medium reaching 500 K.U. and 2×10^9 cells/ml. Assuming that the K.U./cell was the same in both media, then

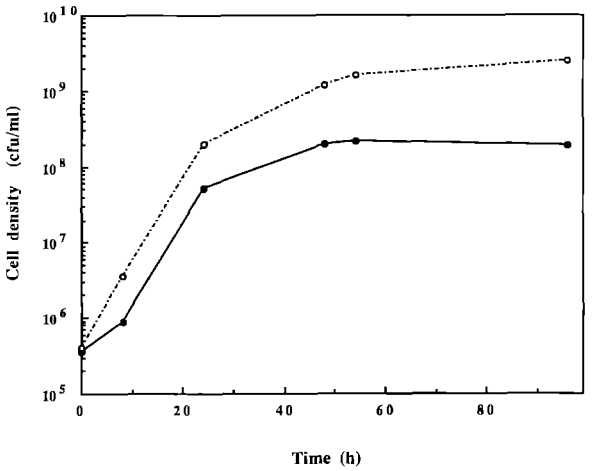

Fig. 1. Growth kinetics of the mixed culture on crude oil (●) and acetate (○) media, using F-1 as the source of nitrogen and phosphorus. To 125 ml flasks containing 10 ml sterile sea water, 0.05 ml crude oil and 0.01 ml of the enrichment culture (three days growth) were added 0.01 ml fertilizer F-1. After incubation with mixing at 25 °C, approximate dilutions were plated for viable cell density (CFU/ml) on Marine agar (18.7 g Marine broth MA-2216 [Difco Labs], 10g NaCl and 16 g agar per liter).

2×10^8 cells/ml in the oil medium would account for only 50 K.U. out of the 3200 K.U. measured. The remainder would be due to the emulsified oil.

The main interest of the mixed culture is in its ability to utilize fertilizer F-1 as the sole nitrogen and phosphorus source when grown on crude oil. Thus, it was essential to demonstrate that the

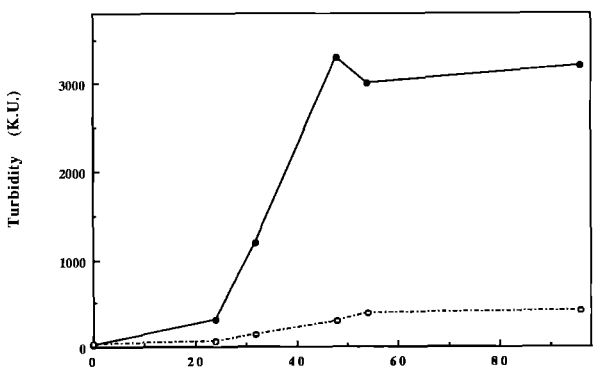

Fig. 2. Growth and emulsification of crude oil by the mixed culture, using F-1 as the source of nitrogen and phosphorus. The experiment was performed as described in Fig. 1, using 0.5% in crude oil (●) and 0.5% acetate (○) as the carbon source.

Table 2. Growth of the mixed culture as a function of fertilizer F-1 concentration.[a]

Fertilizer (%)	Turbidity (K.U.)	Cell density (c.f.u./ml)
0.0	22	3.0×10^6
0.05	800	1.4×10^8
0.1	790	1.6×10^8
0.2	1200	1.8×10^8
0.5	1250	2.1×10^8
1.0	2000	4.0×10^8
1.6	2650	4.0×10^8

[a] The experiment was carried out as described in Fig. 1 using different concentrations of F-1 and incubation for 72 h.

culture's growth was dependent on the fertilizer. Accordingly, the mixed culture was grown on different concentrations of fertilizer as is shown in Table 2. The data show that at the lowest level of fertilizer tested – 0.05%, the culture reached 1.4×10^8 cells/ml and 800 K.U., compared to 3.0×10^6 cells/ml and 22 K.U. without fertilizer. Further increase in the concentration of F-1 led to slight increases in both cell density and turbidity.

As demonstrated above, emulsification of the endogenous crude oil did not proceed in parallel with the viable cell numbers. Thus, it was interest-

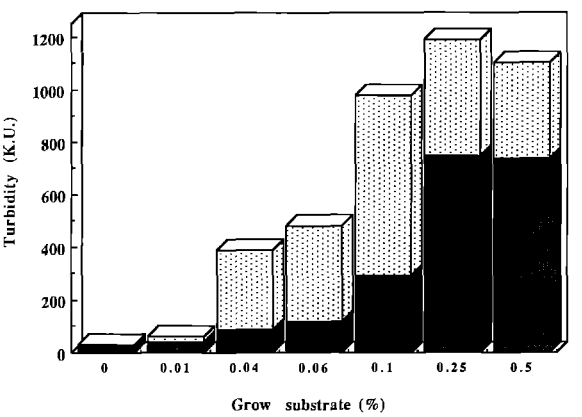

Fig. 3. Emulsifying potential of the mixed culture. To 125 ml flasks containing 10 ml sterile sea water and 0.1% F-1. Different concentrations of crude oil were added. After inoculation with 10 µl of the enrichment culture, the flasks were incubated with mixing at 25 °C for 72 h. Samples were removed for determination of turbidity (dark bars). Crude oil was then added to the flask to reach a final concentration of 1%, mixed at 25 °C for 1 h and then turbidity determined (spotted bars).

ing to determine how much oil could be emulsified when it was added at the end of the growth experiment (Fig. 3). The data demonstrate that the cultures have the potential to emulsify higher amounts of oil than were present in the growth media. This effect was especially pronounced when 0.1% crude oil was used as the substrate. The turbidity in that experiment rose to 980 K.U. with addition of 0.9% crude oil, compared to 290 K.U. at the end of the growth period. When higher concentrations of oil were used as growth substrate, e.g. 0.5%, then the increase in turbidity following addition of oil and shaking was reduced.

Table 3. Characterization of strains.

Property	Strain		
	RL3	RL4	RT
Colony on marine agar			
diameter	2 mm	<1 mm	1–2 mm
color	white	white	yellowish
Bacterial shape	rod	rod	rod
Dimensions (μm) of cells			
from marine agar (16 h)	0.3/1.5	0.6/1.2	0.6/1.0
from broth, on oil (72 h)	0.5/1.6	0.5/2.2	0.44/1.1
Motility	+	+	+
Flagellar arrangement			
from marine agar (16 h)	peritrichal	polar	none
from broth, on oil (72 h)	none	polar	none
Growth temperature, °C			
4 °C (100 h)	–	–	–
20–25 °C (24 h)	+	+	+
37 °C (24 h)	+	+	+
41 °C (100 h)	–	–	–
NaCl (6%) tolerance	+	+	+
NaCl requirement for growth	–	+	+
Lipase	+	+	+
Oxidase	+	+	+
Catalase	+	+	+
Starch hydrolysis	–	+	+
Plasmids	–	2 > 60 kb	2 > 60 kb 1–3 kb
Antibiotic sensitivity			
ampicillin	S[b]	S	S
tetracycline	R[b]	R	R
penicillin G	S	S	R
erythromycin	S	S	S
nalidixic acid	S	R	R

[a] Determined by electron microscopy.
[b] S is sensitive and R is resistant.

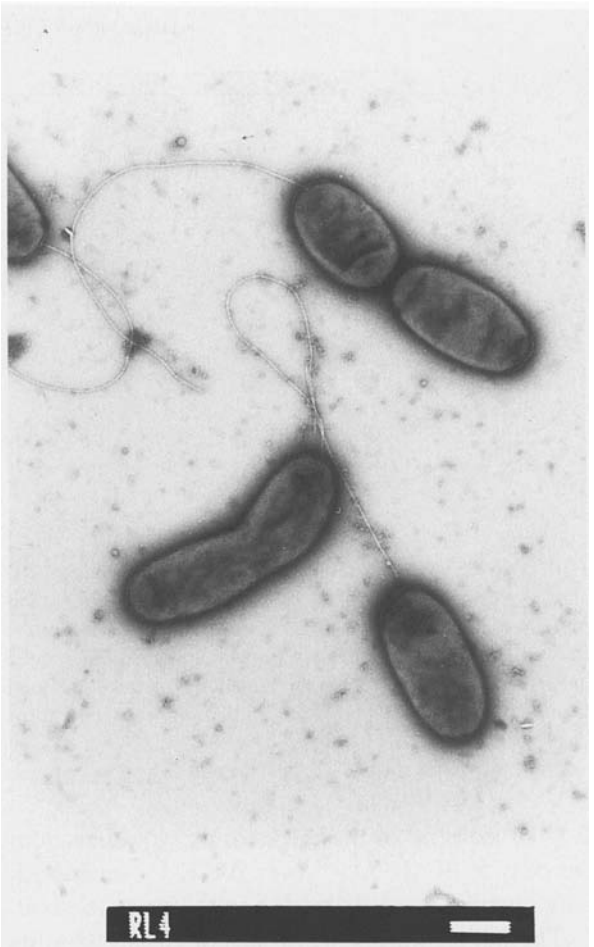

Fig. 4. Electron microscopy of the pure strains: RL3 (A), RL4 (B) and RT (C).

Characterization of the pure culture

The three pure bacterial strains that were isolated from the mixed culture were able to grow on and emulsify crude oil, utilizing fertilizer F-1 as a nitrogen and phosphorus source. The general characteristics of the three strains are summarized in Table 3. All of the strains are gram negative, aerobic, motile, oxidase positive, urease negative and rod shaped. Electronmicrographs of the strains are shown in Fig. 4. The strains have been tentatively identified as *Pseudomonasceae* genus, *Gluconbacter* (strain RT), *Pseudomonadaceae* genus *Pseudomonas* (strain RL4) and *Pseudomonas alcaligenes* (strain RL3)

The kinetics of growth and emulsification of the three strains are shown in Figs. 5–7. All the strains

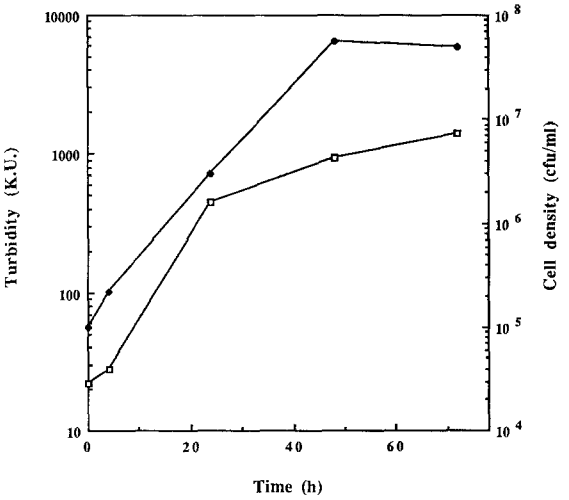

Fig. 5. Kinetics of growth and emulsification of crude oil by strain RL3. Sea water (20 ml) containing 0.5% crude oil and 20 mg fertilizer F-1 was inoculated with 20 µl of strain RL3 (three days growth). The culture was incubated at 25 °C with shaking; samples were removed for the determination of viable count (●) and turbidity (□).

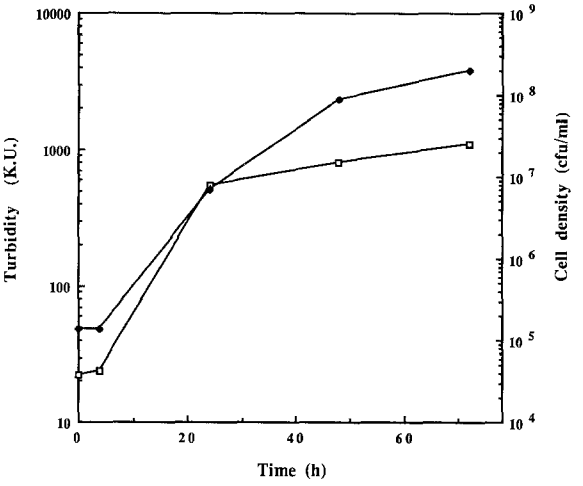

Fig. 6. Kinetics of growth and emulsification of crude oil by strain RT. The experiment was performed as described in Fig. 5.

have the ability to grow and emulsify oil, using F-1 as a nitrogen and phosphorus source. The strains showed similar doubling times, approximately 3h. Strain RT reached the highest growth density (2×10^8 c.f.u./ml), although its emulsification (1000 K.U.) ability was almost the same as the other two strains. The pH in the growth media (in all th strains) decreased from an initial value of 7.8 to about 7.0 at the end of the growth period. Here, as in the case of the mixed culture, the turbidity of the cultures came mostly from the emulsified oil.

Table 4 shows the ability of the pure cultures to grow on different hydrocarbons. The nitrogen and phosphorus source was fertilizer F-1. All of the strains grew on decane, hexane, hexadecane, kerosine, crude oil and gas oil and failed to grow on toluene, naphthalene and heptane. Only strain RL3 grew on xylene. Tetradecane, iso-octane and paraffin oil served as substrate for strains RL3 and RL4, but not for strain RT. The failure of the strains to grow on toluene, naphthalene and heptane could be the result of the toxicity of these hydrocarbons when present at 0.5%. Lower concentrations were not tested.

When grown on crude oil medium, all three pure cultures adhered to the oil. An example of strain RL3 adhering to an oil droplet is shown in Fig. 8.

Adhesion of fertilizer F-1 to crude oil

A key property of F-1 is that it is insoluble in water and binds to the hydrocarbon/water interface. This phenomenon is demonstrated qualitatively in Fig. 9 and quantitatively in Table 5. When the powdered F-1 was added to a suspension of crude oil in

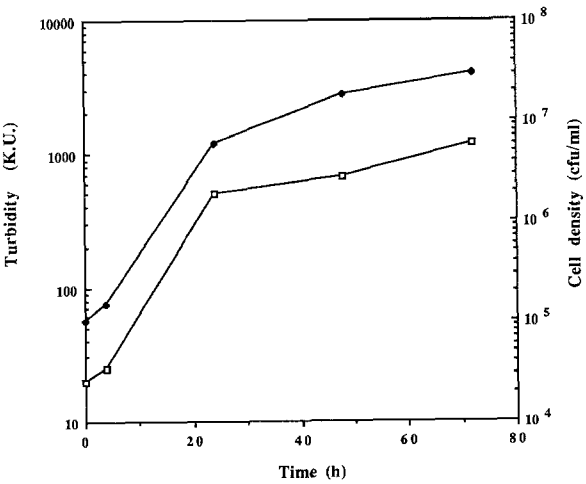

Fig. 7. Kinetics of growth and emulsification of crude oil by strain RL4. The experiment was performed as described in Fig. 5.

sea water, the fertilizer adhered to the oil and floated to the surface.

One of the major problems with supplementing a contaminated oil spill in the sea or in a moist environment with water soluble nitrogen and phosphorus compounds is that they will not be concentrated near the oil where growth must occur. Rather, the compounds will spread and dilute in the open system. It was therefore interesting to test whether or not this problem could be overcome by use of the fertilizer F-1. The procedure that was used compared growth and emulsification (turbidity) after the fertilizer or soluble nutrients and oil were mixed and the aqueous phase was removed and replaced with fresh sea water. The data (Table 5) clearly show that F-1 can support growth on crude oil even after the aqueous phase had been removed and replaced twice with fresh sea water containing no additions. None of the cultures that received ammonium sulfate and phosphate buffer, and treated in a similar manner, grew significantly. This demonstrates that the soluble nutrients were efficiently removed by the wash out process. Strain *A. calcoaceticus* RAG-1 (Rosenberg et al. 1982) which grew well on crude oil supplemented with ammonium sulfate and phosphate salts, failed to grow on F-1 as its course of nitrogen and phosphorus, no matter whether a wash out procedure took place or not. The pure strains RL3, RL4 and RT and the mixed culture grew after the dilution procedure, reaching more than 10^7 cells/ml and high values of turbidity. The mixed culture showed the highest ability to grow and reached 1×10^8 cells/ml and 900 K.U. Strain RL3 was almost as effective as the mixed culture in growth yield and emulsification of the crude oil.

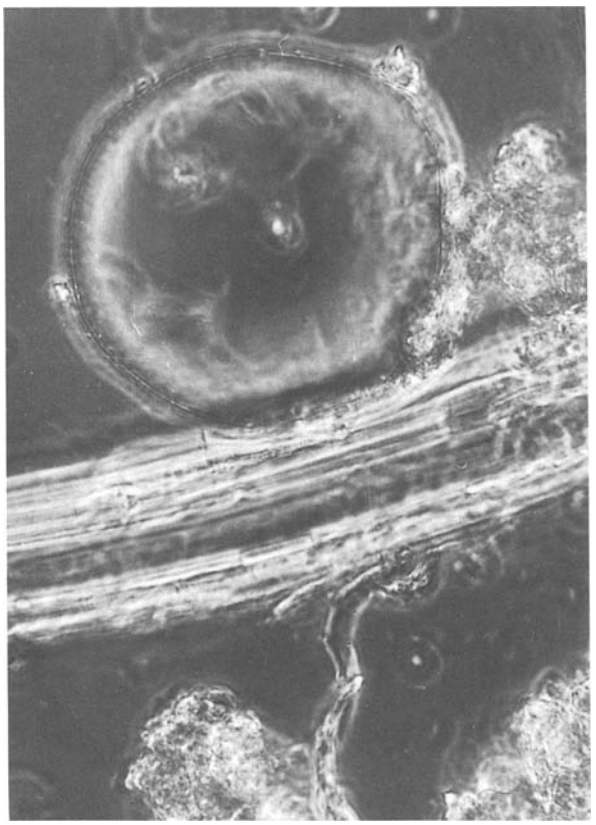

Fig. 8. Adhesion of RL3 to oil. The phase micrograph was taken from an RL3 culture grown for 3 days on crude oil/F-1 medium. The diameter of the oil drop is approximately 25 μm.

Table 4. Hydrocarbon utilization by pure strains.

Hydrocarbon[a]	Strains		
	RL3	RL4	RT
Decane	+	+	+
n-hexane (vapor)	+	+	+
Toluene	–	–	–
Xylene	+	–	–
Naphthalene	–	–	–
Hexadecane	+	+	+
Tetradecane	+	++	–
Heptane	–	–	–
Kerosene	+	+	+
Crude oil	+	+	+
Gas-oil	+	+	+
Iso-octane	+	+	–
Paraffin oil	++	++	–

[a] Except for n-hexane, growth was tested in shake flasks containing 0.5% of the hydrocarbon and 0.1% F-1.

Isolation and characterization of the inducible cell-bound activity for F-1 utilization

Regarding fertilizer F-1, it is important to understand the mechanism by which the isolated culture utilized F-1 as its sole nitrogen and phosphorus source. Preliminary experiments indicated that the F-1-utilizing strains had the enzymatic capabilities

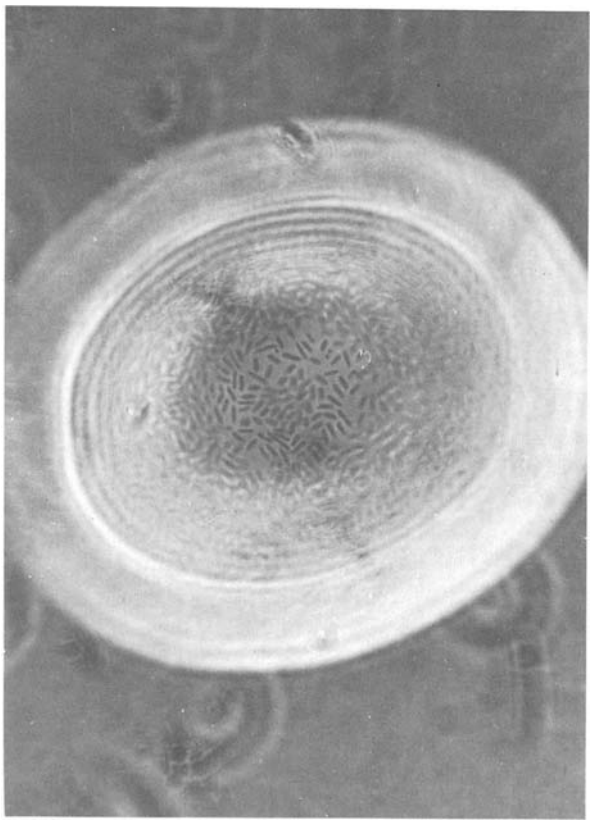

Fig. 9. Adhesion of F-1 to an oil droplet. The photograph was taken through a phase microscope from an RL3 culture grown on crude oil/F-1 medium.

of degrading it. The determination of 'F-1-ase' activity of the pure strains was based on a bioassay for degrading the F-1 to water soluble, low molecular weight compounds. The quantity of low molecular weight F-1 was estimated by a bioassay using strain RL3. The sample to be tested (whole bacteria or bacterial extracts) was initially dialyzed in order to remove low molecular weight nitrogen and phosphorus compounds. It was then incubated with the insoluble fraction of F-1 in a dialysis bag, which was put in water containing cells of an indicator strain (RL3 bacteria) but no nitrogen source. If the sample is active, the F-1 in the dialysis bag will be degraded to low molecular weight compounds that will dialyze out of the bag and enable the bacteria outside the bag to grow. One unit of F-1-ase activity was defined as the activity resulting in bacterial increase of 10^6 cells.

The data presented in Table 6 demonstrate the F-1-ase activity of strains RL3, RL4 and RT. The activity was mainly associated with the cells and not the extracellular fluid.

Table 7 shows that the F-1-ase activity of cells is influence by the source of nitrogen and phosphorus in the growth medium. Cells grown on media containing F-1 had higher activities of F-1-ase than cells grown on ammonium sulfate and phosphate buffer. Strain RL2 yielded the highest specific activity of F-1-ase in broken cell preparations.

Table 5. Growth of bacteria on crude oil and F-1 following removal of water soluble nutrients.[a]

Strain	Cell density (c.f.u./ml)				Turbidity (K.U.)	
	(time 0)		(time 120 h)		(time 120 h)	
	UF-1	AS	UF-1	AS	F-1	AS
RL4	5.4×10^5	6.5×10^5	9.0×10^7	4.0×10^3	550	22
RL3	8.0×10^5	4.0×10^5	9.0×10^7	2.0×10^6	860	25
RT	3.0×10^5	4.0×10^5	6.0×10^7	2.0×10^6	490	20
RAG-1	1.0×10^3	1.0×10^3	1.0×10^3	1.0×10^3	20	20
Mixed culture	3.0×10^5	5.0×10^5	1.0×10^8	1.0×10^6	900	23

[a] F-1 medium contained 0.2% fertilizer (equivalent to 0.05% nitrogen). AS medium contained 0.1% ammonium sulfate (equivalent to 0.2% nitrogen) and 10 mM phosphate buffer. Both media contained 0.5% crude oil as a carbon source. Each 20 ml medium was mixed well and then allowed to stand for 5 min allowing the crude oil and the aqueous phase to separate. The aqueous phase was then removed as completely as possible and replaced with 20 ml fresh sea water. The procedure of removal of the remaining water soluble nutrients was repeated once more. Each flask was then inoculated with 40 µl of a pure or a mixed culture and incubated for 120 h at 20 °C with shaking. Viable counts were determined after inoculation (time 0) and at 120 h. Turbidity was measured at 120 h.

Laboratory experiments using F-1 and the mixed culture to treat oil-contaminated beach sand

The effect of temperature on the biodegradation of crude oil-contaminated sand is shown in Fig. 10. After 30 days at 20 °C and 30 °C, the amount of remaining pentane-extractable hydrocarbon had decreased to 45% and 55%, respectively, of the initial value. At 60 °C, 85% of the hydrocarbon was still present, indicating that the evaporation of the heavy crude oil that was used in the experiment was not a major factor.

Experiments demonstrating the requirement for added bacteria and fertilizer F-1 on biodegradation of crude oil in beach sands is summarized in Table 8. When both bacteria and fertilizer were added, 45–54% of the pentane-extractable hydrocarbons were degraded in 10–15 days. Without addition of fertilizer or bacteria, only 9% was degraded in 15 days. The optimum amount of fertilizer was approximately 5–10 mg per g sand. The Ashdod sand showed a strong requirement for added bacteria, 45% degradation with bacteria, compared to 8% without added bacteria. It should be noted that soil chronically polluted with crude oil can have a relatively high indigenous concentration of useful bacteria, suggesting that the requirement for added bacteria will vary, depending on the condition of the soil.

Table 6. F-1-ase activity of pure cultures.[a]

Fraction assayed	F-1(ase) activity (units)
Control	190
RL3 broth	1,000
RL3 cells	16,000
RL4 broth	800
RL4 cells	17,000
RT broth	1,200
RT cells	18,000

[a] The control was either sea water or a culture of RAG-1. For strains RL3, RL4 and RT, cultures were prepared on Acetate medium containing 0.1% F-1. After 3 days incubation, the cells were separated from the culture broth by centrifugation at 10,000 × g for 15 min. The cells were then resuspended in sea water to 10^9 per ml and assayed as described in the text. The cell-free broth was concentrated 150-fold prior to the assay.

Field experiment using F-1 and the mixed culture to treat oil-contaminated Haifa beach sand

In August 1991, approximately 100 tons of heavy crude oil was accidentally spilled about 3 km north of Zvulon Beach between Haifa and Akko in Israel. The oil-contaminated sand was collected into piles and subsequently spread over the beach.

Prior to the field trial, a simulation trial for bioremediation of the Haifa contaminated beach sand was performed in the laboratory, The experiment was performed on one kilogram samples of the hydrocarbon-contaminated sand from Haifa beach in plastic containers. The results (Fig. 11) indicated that in a period of about 5 weeks the concentration of hydrocarbons was reduced by 10% without any treatment. Plowing and wetting resulted in a 20% reduction of hydrocarbon, while the microbial treatment reduced the hydrocarbons by about 70%. Initially, two plots of 50 m^2 were selected, one for the experiment and the second to serve as a control.

In the field trial the experimental plot was inoculated with 20 l of the mixed culture containing strains RL3, RL4 and RT. Fertilizer F-1 (38 kg, in the form of a fine powder) was then added as the source of nitrogen and phosphorus. The experiment plot was watered from the adjacent sea (water temperature was 27 °C). The plot was then tilled by hand with the help of a simple rake to the depth of about 5 cm. The control plot was left undis-

Table 7. Induction of F-1-ase activity by F-1.

Strain	N,P Source[a]	Protein (mg/ml)	F-1(ase) activity	
			U/ml	U/mg protein
RL2	F-1	0.33	1,400	4,200
RL3	AS	0.04	40	1,000
RL4	F-1	0.23	290	1,230
RL4	AS	0.02	5	230
RT	F-1	0.30	1,000	3,300
RT	AS	ND	ND	ND

[a] The strains were grown in Acetate medium containing either 0.1% F-1 or 0.2% ammonium sulfate and 20 mM phosphate buffer (AS). After 3 days of growth, the cells were harvested, sonicated and assayed as described in the text.

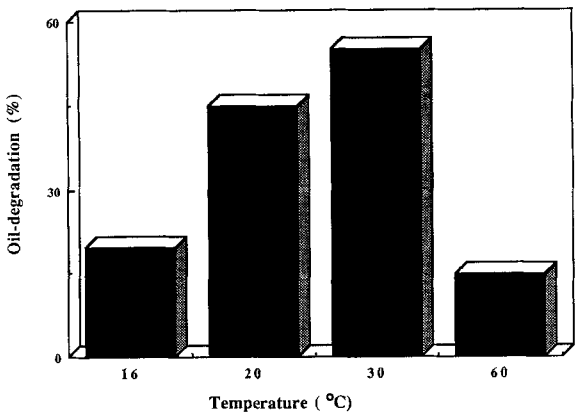

Fig. 10. Temperature dependence of enhanced biodegradation of hydrocarbon-contaminated sand from Haifa beach. To 300 g of hydrocarbon-contaminated sand (7.15 mg/g sand) from Haifa beach in 100 cm^2 plastic containers were added F-1 (10 mg/g sand), followed by addition of 2 ml of the 'mixed culture' (1×10^8). The containers were incubated for 30 days at various temperatures. The sand was plowed and kept moist by addition of sea water daily. The amount of residual hydrocarbon remaining in the sand was determined by dry weight following pentane extraction.

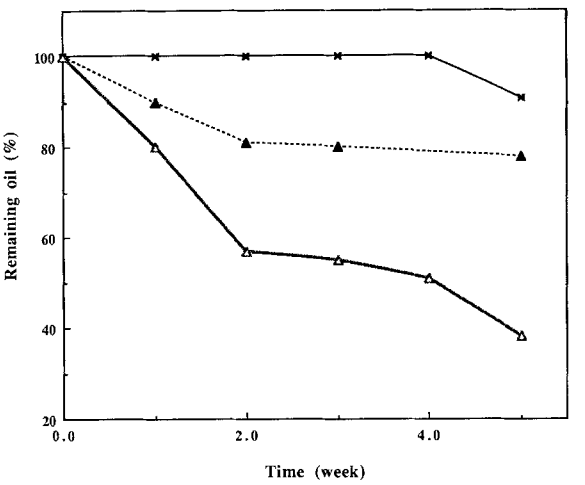

Fig. 11. Trial for bioremediation technology and application to the Haifa contaminated beach sand. One kilogram samples of hydrocarbon-contaminated sand (4.7 mg/g sand) from Haifa beach in 100 cm^2 plastic containers were treated as follows: no treatment (×), plowed and kept moist (▲) and addition of fertilizer F-1 (5 mg/g sand) and 2 ml bacterial 'mixed culture' 5×10^8 cells/ml (△). The amount of residual hydrocarbon remaining in the sand was determined by dry weight following pentane extraction (as described in Materials and methods) from removed samples of 50 g at various times. The containers were incubated for 5.5 weeks outdoors in temperatures varying from 20–35 °C. The sand was plowed and kept moist by addition of sea water daily.

turbed. For each time point, triplicate 50 g samples were extracted and the results presented are the average of the three values. Each 50 g sample was placed in a 500 ml bottle that contained 50 ml n-pentane. After shaking vigorously for 5 min, 25 ml of the pentane extract were transferred to a 100 ml flask containing anhydrous $CaCl_2$. After standing for 5 min with occasional stirring, the dried pentane extract was filtered through a Whatman 2V paper and then evaporated in vacuo (water aspirator) at 30 °C. The residual hydrocarbon was dissolved in 3 ml n-pentane and placed in small glass vials in a chemical hood. The weight of the pentane-extractable hydrocarbon was determined by weighing to constant weight at room temperature on an analytical balance. A control of n-pentane taken through the same procedure contained 0.54 mg residues per 100 ml pentane. Thus, the contribution of the nonvolatiles in the pentane to the values reported, was 0.006 mg/g sand, which is negligible. The values reported were, however, corrected for the moisture content of the specific sand sample.

The experimental data are presented in Tables 9

Table 8. Biodegradation of oil-contaminated beach sand.[a]

Sand source	Addition (per g sand)		Time (days)	Degradation[b] (%)
	F-1 (mg/g)	Bacteria		
Ashdod	6.7	0	10	8
"	6.7	+	10	48
Tel Baruch	0	+	12	5
"	3.3	+	12	35
"	6.7	+	12	51
"	10	+	12	54
"	20	+	12	46

[a] To 500 g of sand from the beach in 100 cm^2 plastic containers were added 2 ml of heavy crude oil (6.9 mg/g sand) that were applied over the surface with a pasteur pipet. The containers were then allowed to remain at room temperature (20–32 °C) for 1 day (to let the volatile fractions evaporate). Fertilizer F-1 was then spread on the surface, followed by addition of 2 ml of the 'mixed culture' (8.2×10^8 cells/ml). The sand was kept moist by addition of sea water daily.

[b] The amount of hydrocarbon remaining was determined by dry weight following pentane extraction.

and 10. On day 0 (September 1, 1991), the core samples were taken prior to any treatment. At the beginning of the experiment, the experimental plot contained significantly more hydrocarbon (3.80 mg/g sand) than the control plot (2.30 mg/g sand). There was only a small decrease in the first day. However, by the fourth day, 30% of the hydrocarbon had been degraded. The biodegradation continued, reaching 50% on day 9 and 84.5% on day 25, when the experiment was concluded. The control plot showed a 18% degradation by day 9 which remained relatively constant until day 25.

The relatively small variations from day to day were not due to the pentane extraction procedure, because the average standard deviations were very low (0.03 mg/g sand, corresponding to less than 2%). Therefore, we assume that the core sampling was probably the source of the day to day variation.

After the first day, the experimental plot was watered every afternoon with 1.5 m^2 sea water and then raked to a depth of 5 cm. Ten random core samples (5 cm diameter × 10 cm depth) were taken, prior to the daily watering, from both the experimental and control plots. The samples were mixed thoroughly in the field and brought to the laboratory for analysis.

Table 9. Enhanced biodegradation of hydrocarbon-contaminated sand at Haifa beach during September 1991.[a]

Day	Pentane-extractables[b] (mg/g sand)	Degradation (%)
0	3.80	0
1	3.70	2.6
4	2.76	30
9	1.89	50
14	0.88	77
21	1.40	63
25	0.59	84.5

[a] An experimental plot of 50 m^2 was selected on the Haifa contaminated beach. The initial concentration of hydrocarbon-contamination in the sand was 3.8 mg/g sand in the upper 10 cm. The plot was treated with 38 kg (~ 15 mg/g sand) fertilizer F-1 in the form of a fine powder and inoculated with 20 l of the 'mixed culture' (5×10^8 cells/ml). The plot was watered with sea water and tilled by hand with a rake to the depth of about 5 cm daily.
[b] For each determination, ten random core samples were taken and mixed thoroughly in the field. For each time point triplicates of 50 g samples were extracted in pentane and determined by dry weight. The results presented are the average of three values (the average standard deviation was 0.1 mg/g sand).

Table 10. Natural biodegradation (no treatment) of hydrocarbon-contaminated sand at the Haifa beach, September 1991.[a]

Day	Pentane-extractable[b] (mg/g sand)	Degradation (%)
0	2.30	0
4	2.53	0
9	1.88	18
14	1.70	26
21	1.94	15.6
25	1.95	15.6

[a] A 50 m^2 control plot was selected on the Haifa contaminated beach. The initial concentration of hydrocarbon-contamination in the sand was 2.3 mg/g sand in the upper 10 cm. The plot was left undisturbed.
[b] See Table 9; the average standard deviation was 0.15 mg/g sand.

Gas chromatographic analyses of the alkane fraction of the extracted sand from the experimental plot at day 0 and at day 25 are summarized in Table 11. Extractions with pentane and CCl_4 gave similar results, i.e. approximately 85% of the hydrocarbon was removed from the sand in 25 days. The C_{20}–C_{32} fraction was the most abundant at day 0, 221 mg per kg sand and fell to 13 mg/kg sand by day 25, a 94% removal. The C_{14}–C_{18} and C_{36}–C_{40}

Table 11. Gas chromatographic analysis of the extracted sand from experimental plot.[a]

	Extracted hydrocarbon (mg/kg sand)		Residual hydrocarbon (%)
	Day-0	Day-25	
Total CCl_4 extractable[a]	3138	474	15.1
Total pentane extractable	3800	590	15.5
C.G. analysis[b]			
C_{14}–C_{18}	23.7	4.7	19.8
C_{20}–C_{32}	221	13	5.9
C_{36}–C_{40}	132	35	26.5
C_{14}–C_{40}	377	53	14.1

[a] See Table 9.
[b] Samples of pentane extracted residual hydrocarbon (day-0 and day-25) were analyzed by gas chromatography at the Israel Institute of Petroleum and Energy.

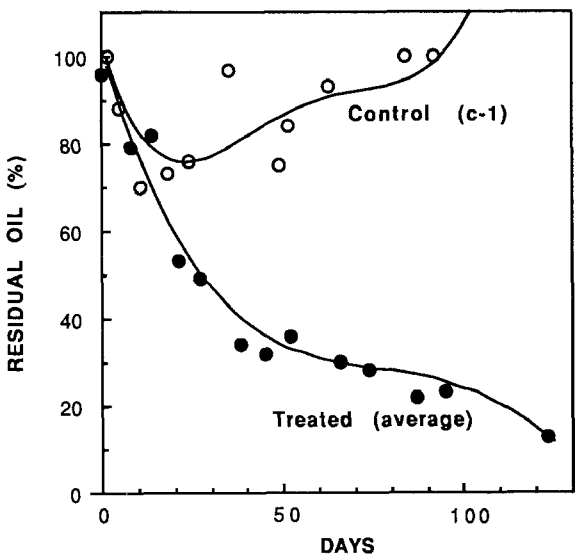

Fig. 12. Microbial clean-up of the Zvulon Beach. The Zvulon Beach in Haifa was treated with bacteria and fertilizer (●) or untreated (○). The treatment and analyses was carried out essentially as described for Fig. 11 and in the text.

fractions were degraded to lesser degrees, 80% and 74%, respectively. Overall, there was an 86% degradation of the alkanes, similar to the data obtained by dry weight analyses.

Visual examination of the beach sand following the treatment, in addition to the analytical data described above, indicated that the F-1/bacteria technology was applicable for bioremediation of the remaining sand.

Microbial clean-up of the Zvulon Beach

The clean-up was of 30,000 m^2 which were treated essentially as in the field trial. As the clean-up was in winter time, it was not necessary to water. It should be noted that the winter of 1992 was unusually hard, and temperatures were around 5–10 °C for a couple of months. The results of the experiment (a brief summary presented in Fig. 12) indicated that even under these conditions 88% of the oil was degraded after 4 months.

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