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## Bioremediation of oil by marine microbial mats

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**Abstract** Cyanobacterial mats developing in oil-contaminated sabkhas along the African coasts of the Gulf of Suez and in the pristine Solar Lake, Sinai, were collected for laboratory studies. Samples of both mats showed efficient degradation of crude oil in the light, followed by development of an intense bloom of *Phormidium* spp. and *Oscillatoria* spp. Isolated cyanobacterial strains, however, did not degrade crude oil in axenic cultures. Strains of sulfate-reducing bacteria and aerobic heterotrophs were capable of degrading model compounds of aliphatic and aromatic hydrocarbons. Results indicate that degradation of oil was done primarily by aerobic heterotrophic bacteria. The oxygenic photosynthesis of oil-insensitive cyanobacteria supplied the molecular oxygen for the efficient aerobic metabolism of organisms, such as *Marinobacter* sp. The diurnal shifts in environmental conditions at the mat surface, from highly oxic conditions in the light to anaerobic sulfide-rich habitat in the dark, may allow the combined aerobic and anaerobic degradation of crude oil at the mat surface. Hence, coastal cyanobacterial mats may be used for the degradation of coastline oil spills. Oxygen microelectrodes detected a significant inhibition of photosynthetic activity subsequent to oil addition. This prevailed for a few hours and then rapidly recovered. In addition, shifts in bacterial community structure following exposure to oil were determined by denaturing gradient gel electrophoresis of PCR-amplified fractions of 16S rRNA from eubacteria, cyanobacteria and sulfate-reducing bacteria. Since the mats used for the present study were obtained from oil-contaminated environments, they were believed to be preequilibrated for petroleum remediation. The mesocosm system at

Eilat provided a unique opportunity to study petroleum degradation by mats formed under different salinities (up to 21%). These mats, dominated by cyanobacteria, can serve as close analogues to the sabkhas contaminated during the Gulf War in Kuwait and Saudi Arabia.

**Keywords** Bioremediation · Oil spill · Microbial mats · Cyanobacteria

### Introduction

Given favorable environmental conditions, all natural organic compounds degrade. If any organic compound produced in the ecosphere were inherently resistant to recycling, huge deposits of this material would have accumulated throughout the geological ages. Substantial organic deposits, such as fossil fuels, accumulate only under conditions adverse to biodegradation. Petroleum is, in one sense, a natural product, resulting from the anaerobic conversion of biomass under high temperature and pressure. Most of its components are subject to biodegradation, but at relatively slow rates. Petroleum hydrocarbons can be divided into four classes: saturates, aromatics, asphaltenes (phenols, fatty acids, ketones, esters, porphyrins), and resins (pyridines, quinolines, carbazoles, sulfoxides, amides). Hydrocarbons differ in their susceptibility to microbial attack and generally degrade in the following order of decreasing susceptibility: *n*-alkanes > branched alkanes > low-molecular-weight aromatics > cyclic alkanes [10].

It is difficult to examine biodegradation kinetics and relative rates without basic knowledge of the various microbial species and specific environmental conditions. The rate of biodegradation depends on both physicochemical and biological variables. Churchill et al. [5] coined the term “bioavailability of hydrophobic organic compounds”, which is a function of phase-solubility and solution-transport processes. The ability of hydrophobic organic compounds to be solubilized and transported into the immediate vicinity of bacterial cells capable of

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metabolizing them is potentially the rate-limiting step in bioremediation. Degradation of hydrocarbons in the presence of synthetic surfactants is a delicate issue. Generally, the toxicity of surfactants increases with their hydrophobicity [18]. The use of surfactants of biological origin solves the toxicity problem. Barkay et al. [2] tested the effect of the polymeric biosurfactant, Alasan, on the solubilization and biodegradation of polyaromatic hydrocarbons (PAHs). The presence of Alasan, a high-molecular-weight bioemulsifier complex of anionic polysaccharides and proteins produced by *Acinetobacter radioresistens* KA53, increased significantly the rate of [<sup>14</sup>C]-phenanthrene mineralization by *Sphingomonas paucimobilis* EPA505. This result suggests that Alasan solubilized PAHs by a physical interaction, probably hydrophilic.

Petroleum-based products are the major source of energy for industry and daily life. Petroleum is also the raw material for many chemical products, such as plastics, paints, and cosmetics. The transport of petroleum across the world is frequent and the amounts of petroleum stocks in developed countries are enormous. Consequently, the potential for oil spills is significant. The volume of spills usually exceeds the inherent remediation capacity for any given environment, resulting in a significant ecological impact. Type specimens of bacterial strains used in bioremediation exist in various repositories (e.g., ATCC, DSM, etc.), or are commercially available and are usually covered by patent rights. Each of these strains may yield dramatic results in vitro for specific target compounds. However, the overall success of such strains in treating a wide range of contaminants in situ remains limited. The reintroduction of indigenous microorganisms isolated from a contaminated site after culturing seems to be a highly effective bioremediation method, especially when microbial growth is supplemented by oxygen and fertilizers [7, 9]. Thus, bioremediation is normally achieved by stimulating the indigenous microbiota (naturally occurring microorganisms). Stimulation is achieved by the addition of growth substrates, nutrients, terminal electron acceptor, electron donors, or some combination therein, resulting in an increase in contaminant biodegradation and biotransformation. This notion was confirmed in the large-scale operation for bioremediation after the oil spill from the Exxon *Valdez* in Alaska, with the addition of nitrogen and phosphorus fertilizers [3].

This study focused on littoral bioremediation of crude oil spills by microbial mats at Saudi Arabian sabkhas. We investigated the impact of oil on microbial mats and the potential degradation of oil by them. Marine microbial mats develop mostly in sheltered and shallow coastal areas and intertidal zones (e.g., Ebro delta, Spain, Rhône delta, France) and they are composed of different functional groups of microorganisms distributed in layers as a result of physicochemical gradients. Microbial mats have a potential to immobilize and probably also degrade pollutants. This capacity of microbial mats may partly decrease the damaging

impact of pollutant spills on coastal ecosystems. Development and survival of microbial mats after oil spills was reported in coastal areas of Kuwait after the Gulf War [8, 17].

The specific aims of this research were: (1) to find a pattern in the degradation system, (2) to define which of the microorganisms were involved in this degradation, (3) to understand the mechanism behind this degradation, (4) to develop molecular tools to look into the changes of the population following exposure to crude oil, (5) to look into the expression of genes in this environment, and (6) to develop a system to treat highly polluted coastal water in a manner analogous to what is done in activated sludge in an industrial waste (i.e., pumping highly polluted seawater over microbial mats, utilizing their degradation capacity).

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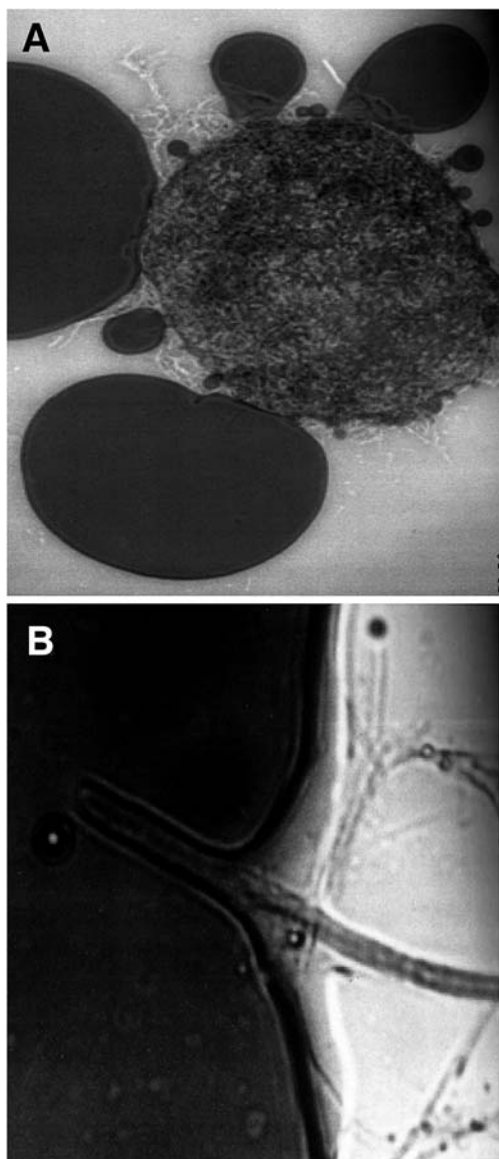
### Microorganisms involved in the laboratory experiment

Identification of the key organisms that play a role in pollutant degradation processes is relevant to the development of optimal in situ bioremediation strategies. Indeed, efforts have been made to characterize bacterial communities and their responses to pollutants [12], to isolate potential petroleum degraders, and to identify the functional genes involved in particular degradation processes [11, 13].

Cyanobacterial mats developing in oil-contaminated sabkhas in the African coasts of the Gulf of Suez and in the pristine Solar Lake, Sinai, were collected for laboratory studies. Both mat samples showed efficient degradation of crude oil in the light, followed by an intense bloom of *Phormidium* spp. and *Oscillatoria* spp. This result agrees with a previous report [1], in which benthic cyanobacterial mats inhabiting a heavily polluted site in a coastal stream were dominated by *Phormidium*- and *Oscillatoria*-like cyanobacterial morphotypes.

In the course of the past 4 years, our group has engaged in the study of biodegradation and bioremediation of petroleum and petroleum-derived compounds, using cyanobacterial mats. At the early stages of these experiments, it was obvious that the major controlling factor for biodegradation was the hydrophilic/hydrophobic interface between the brines and the benthic bacterial mat. We observed that oil droplets attached to a cluster of the cyanobacterial species of *Phormidium*, while many heterotrophs migrated to the oil/water interface. As the degradation process takes place at the oil/water interface, emulsification is the first step in the process. Indeed, cyanobacterial polysaccharides play a major role in the emulsification of the oil, actually breaking the oil into small droplets that are subsequently attacked by the heterotrophs (Fig. 1). Initial experiments with axenic cyanobacterial cultures did not show oil degradation.

Recent reports have demonstrated that photosynthetic microorganisms, particularly cyanobacteria, may play a direct or indirect role in the metabolism and



**Fig. 1a, b** Photomicrographs showing the emulsification of the water/oil interface. **a** Penetration of bacteria into the oil phase (50 $\times$ ). **b** A detail of this physical phenomenon (1000 $\times$ )

degradation of hydrocarbons. Cyanobacteria such as *Anabaena cylindrica*, *P. faveolarum*, *Oscillatoria* sp. strain JCM, and *Agmenellum quadruplicatum* can degrade different aromatic compounds [4]. Cyanobacteria are present in association with oil-degrading bacteria and prevent them from being washed out, by immobilizing them in their extracellular polysaccharide. In addition, cyanobacteria also supply these bacteria with oxygen and fixed nitrogen. This indirect role of cyanobacteria can be important to the overall success of the biodegradation process [1].

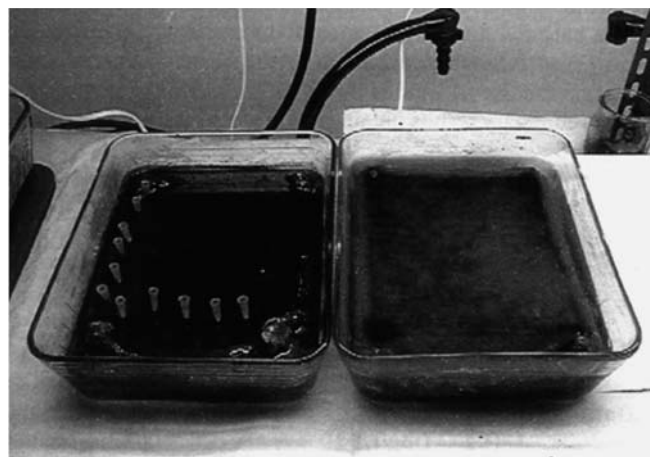
Other petroleum-biodegrading activities were compared with axenic cultures of microorganisms isolated from the Solar Lake, specifically two planktonic organisms, *Desulfovibrio oxycloinae* (a sulfate-reducing bacterium; SRB) and *Marinobacter* sp., and two benthic

organisms from the mat, *Oscillatoria* sp. and *Phormidium* sp. The artificial mixture of petroleum contained *cis*- and *trans*-decalin, isoprenoid C<sub>19</sub> (pristane), phenanthrene and 9-methyl-phenanthrene, dibenzothiophene and *n*-octadecane. The results obtained showed that the model mixture, when exposed to benthic cyanobacteria, lost only the decalin, which also evaporated from the control sample during a 2.5-month incubation. In the case of the planktonic microorganisms, under anaerobic conditions *D. oxycloinae* biodegraded mostly the aromatic compounds, whereas *Marinobacter* sp. under oxic conditions biodegraded the normal hydrocarbon faster than the aromatics, leaving the pristane undisturbed. Strains of SRB and aerobic heterotrophs were capable of degrading model compounds of both aliphatic and aromatic hydrocarbons. These experiments indicated that the aromatic compounds were more water-soluble and hence available to the *D. oxycloinae* bacteria whereas, in the *Marinobacter* sp. experiment, it is possible under these conditions that the bacteria released extracellular bioemulsifiers, which made the normal hydrocarbon more available to biodegradation.

#### The small-scale laboratory experiment: microcosms

Pieces of mat were flooded with water-soluble oil compounds and these mats were compared with an oil-free control microbial mat (Fig. 2). Microelectrodes measured the oxygen fluxes in the system as a function of exposure to oil. The system slowly acclimated with successive addition of oil. Changes in the rates of oxygen production/consumption in the system and changes in the community structure were a major point of interest observed.

Once the oil was introduced for the first time, a drastic inhibition of photosynthesis occurred. However, 2 days later, not only had photosynthesis rebounded, but the rate was actually higher than that at the begin-



**Fig. 2** Microbial mats contained in a microcosm. *Left* Mat contaminated with crude oil. *Right* Control mat

ning of the experiment. Over the duration of the experiment, more oxygen was being produced on the oil-treated mats than in the control. Therefore, the long-term effect turned out to be an enhancement of both oxygen production and photosynthesis, as compared with the oil-free control.

Community analysis was performed using denaturing gradient gel electrophoresis (DGGE) of PCR-amplified 16S rRNA gene fragments [14] obtained from DNA from thin sections (200  $\mu\text{m}$  each) of mat samples, to study the influence of oil pollution on bacterial diversity and community dynamics. No major changes were detected in community composition, but there was a clear vertical migration of SRB in the system exposed to oil. Facultative SRB apparently changed from using oxygen to using sulfate as the terminal electron acceptor after perturbation of the system [15, 16]. Surprisingly, there were no significant changes in the cyanobacterial community. The pattern remained reasonably constant throughout. Thus, treatment of oil did not result in a major succession of the population, but rather a vertical migration by the SRB population and probably a different physiological expression within the system.

In order to minimize the problems of transportation and solubility of both crude oil and topped-off oils, we planned another incubation procedure using flat-bottomed Pyrex flasks with constant shaking. Six different systems were prepared: two with Egyptian crude oil, two with oil topped on it and two controls that were not treated with oil. All flasks contained Solar Lake microbial mats (inoculum) and artificial seawater. One set of containers was covered with aluminum foil and kept in the dark for the incubation period, while the other was kept under continuous artificial light. After 4 weeks, the two sets of vessels were visually different. Those exposed to light were dominated by cyanobacteria, while those kept in the dark had become brownish due to bacterial growth. The biodegradation was slower in the dark than under light conditions. It is postulated that oxygenic photosynthesis by the oil-insensitive cyanobacteria supplied the molecular oxygen for the efficient aerobic metabolism of organisms, such as *Marinobacter* sp. The diurnal shifts in environmental conditions at the mat surface, from highly oxic conditions in the light to an anaerobic sulfide-rich habitat in the dark, may allow the combined aerobic and anaerobic degradation of crude oil at the mat surface.

### Bioremediation in mesocosms

The mesocosm experiments were done outdoors at the marine station of Eilat, Israel. This experimental system allows the controlled development of marine cyanobacterial mats under varying salinity, nutrient load, and addition of hydrocarbon pollutants. There is 1 m<sup>2</sup> of microbial mat in each pond. These mats are used for various in situ measurements of macroscale influx and outflux of CO<sub>2</sub> and O<sub>2</sub> and microscale activity mea-

surements using microelectrodes. An elevator raises the mats twice daily (simulating low tide), allowing the mat surface to cross the oil/water interface (Fig. 3).

Oil was added to the system (about 0.5l of crude oil each time) with successive additions being provided over long periods (e.g., months). Starting with this major contamination by oil, after 1 week, a new layer had overgrown the oil. While it was evident that oil was still oozing out from inside the mat, much of the oil had been degraded. However, the higher molecular fraction was retained. In situ measurements using microelectrodes showed enhancement of both aerobic respiration and photosynthesis, compared with the oil-free control.

We have now started to investigate key enzymes involved in degrading oil, by including the determination of the occurrence of phenanthrodi-oxygenates, as we expect it to be pronounced in such a degradation system. However, these systems are really very complex, involving a complex community and mixed substrates. The “key players” still need to be identified. The new technology of microelectronic chip array may simplify the process of bacterial identification. Combining anchored in situ amplification on a microelectronic chip array with discrimination and detection on the same platform, it may be possible to identify bacterial species through reporter-specific discrimination and allele-specific amplification. Anchored strand displacement amplification allows multiplex amplification and complex genotype discrimination on the same platform. This assay simplifies the bacterial identification process greatly, allowing molecular biological techniques to be performed with minimal processing of samples and practical experience [6]. We have started a project with the Technical University in Munich to develop tools for identifying SRB. The data obtained from chip technology should be comparable with the DGGE profiles we currently have. We have started to develop a “match-



**Fig. 3** Experiments in mesocosms with microbial mats contaminated with crude oil, at the marine station of Eilat, Israel, in the northern tip of the Gulf of Aqaba

chip”, putting signature sequences (both 16S rDNA and functional genes) from known microbial mat species onto a single chip.

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### Concluding remarks

The study of microbial ecology at the mesocosm level requires microscale geochemistry. Specific activities (i.e., photosynthesis, respiration, degradation) identified through microscale geochemistry may be correlated with changes in community structure, helping to define the actual degradation pattern. While working with complex communities presents significant challenges, these systems more closely represent the real situation, as opposed to working with axenic cultures in test tubes. An interdisciplinary approach involving a team of scientists with diverse expertise is required to ensure proper interpretation of the results from this muddy, oily research.

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