CLEANING UP OIL CONTAMINATIONS IN PRODUCTION WATER USING NOVEL HALOPHILIC ARCHAEA (MICROORGANISMS) FROM OMAN

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Abstract. Some years ago it was shown that Halophilic Archaea can be adapted to the presence of high concentrations of halogenated hydrocarbons, and that these selected strains are capable to efficiently degrade such toxic compounds as DDT and hexachlorocyclohexane. Here we report on the isolation of novel strains of Halophilic Archaea from Oman, and on the first results of a screening of these strains for the potential to oxygenate non-functional hydrocarbons from spilled mineral oil. It could be shown that the tolerance towards the presence of high concentrations of hydrocarbons is widespread amongst Halophilic Microorganisms. Some of the novel isolates grow well in a 1:1 mixture of halophilic medium and crude mineral oil, and show great promise for the remediation of oil spills in arid areas. In a first field experiment in collaboration with Occidental of Oman, a produce water discharge pit on an oil field was inoculated with a selection of these strains, with the aim to reduce the hydrocarbon content of the water to an environmentally benign level.

Introduction

The Sultanate of Oman is located on the south-eastern tip of the Arabian Peninsula, between 17–20°N and 52-60°E. The dominant landscapes of the country are arid gravel and sand desserts in the interior and salt marshes (“sabkhat”) along the 2000km long coastline to the Indian Ocean. The temperatures in summer may exceed 50°C.

Oil production is the major source of income in the country, and, although the modern oil-producing industry has gone a long way to prevent contaminations, small hydrocarbon spills can never be completely excluded. Bioremediation of contaminated soil and sand under the harsh climatic conditions of the Arabian dessert and the usual high salinity on oil fields can only be successfully attempted using indigenous extremophilic microorganisms. No introduced, mesophilic, organism would survive in this “inhospitable” environment. Only locally adapted - extremely halophilic - microbes can thrive in such places by compensating the extreme osmotic pressure by the accumulation in the cytoplasm of high concentrations of salt, usually KCl (Archaea), or by the production of organic osmolytes ("compatible solutes", usually Bacteria)[1)].
In contrast to the two more common phylogenetic domains (“kingdoms”) of microorganisms, Bacteria and Eukarya, the biotechnological potential of Archaea has, so far, been widely neglected. Although this situation is rapidly changing for the thermophiles, the halophilic branch of the domain has still not received much attention. One single protein, bacteriorhodopsin from *Halobacterium salinarum*, is used on an industrial scale[2)]. Investigations in the area of biotransformations and enzyme chemistry, however, did not pass an initial test stadium[3)]. Although halophilic Archaea live under growth conditions normally considered "unphysiological"[4)], reports on xenobiotic metabolism are rare[5)].

Some years ago it was shown that Halophilic Archaea can be adapted to the presence of high concentrations of halogenated hydrocarbons, and that these selected strains are capable to efficiently degrade such toxic compounds as DDT and hexachlorocyclohexane[6)]. Zvyagintseva and coworkers isolated halophilic microorganisms from oil fields in a former Soviet Republic[7)], and halophilic microbes may also have played a role in the land-farm operations in post-war Kuwait[8)]. Here we report on the isolation of novel strains of Halophilic Archaea from oil-contaminated sites in the Sultanate of Oman, and on the results of a screening of these strains for the potential to oxygenate non-functional hydrocarbons from spilled mineral oil. In a first field experiment in collaboration with Occidental of Oman, a produce water discharge pit on an oil field was inoculated with a selection of these strains, with the aim to reduce the hydrocarbon content of the water to an environmentally benign level.

Sampling and Enrichment of Oil-tolerant Halophilic Microorganisms From Sites Around the Safah Oil Field of Occidental of Oman

On several visits to the Safah concession site of Occidental of Oman, Inc., between June 1999 and December 2000 soil, water and sand samples were collected from oil-contaminated sites in and around flare, drilling rig and produce water pits on the oil field, and from the "Modern Salt" solar salterns, which are associated to the oil field and use the saline produce water to generate drilling salt (fig. 1). Liquid samples were spread out on microbial growth plates (Petri dishes) on site and cooled during transportation to Sultan Qaboos University. Solid samples were collected in sterile containers and transported in cool boxes to the laboratory; before being used to inoculate halophilic standard media and incubation on rotary shakers at 40°C for 7 to 14 days. If cultures showed signs of microbial growth, inoculates were transferred into fresh media. After stable growth over three such "generations" the cultures were purified by dilution and plating on Agar. A total of 37 different halophilic microbial strains were isolated.

The optimum growth conditions of these strains and their tolerance towards high temperature and xenobiotic stress were examined in the laboratory, using standard adaptation tests. Growth experiments in the presence of crude oil and model compounds, such as long-chain alkanes, provided 10 microbial strains that were virtually insensitive towards the presence of Oman Crude Oil.

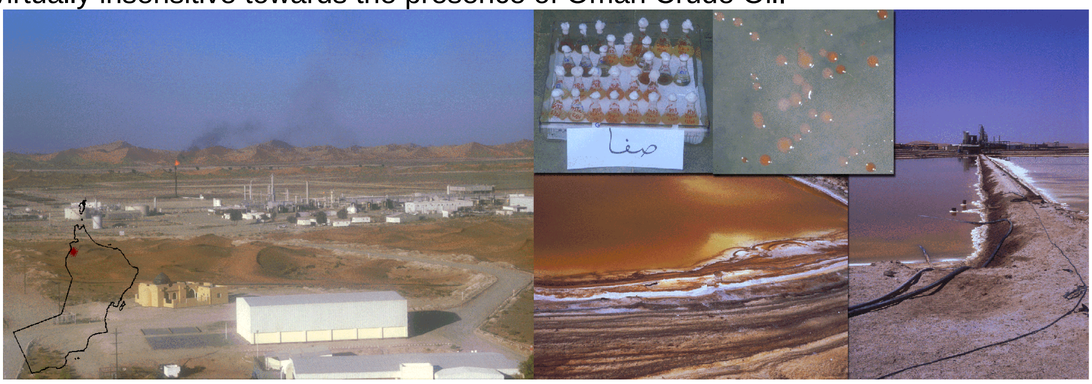


Figure 1 – The Occidental of Oman oil field (left) and the Modern Salt salterns (right) in Safah, Ibri, Oman. The inlets show examples of isolated microbial cultures and colonies on agar plates.

A "3600m³ Open-Air Bioreactor" in the Arabian Dessert

Clear evidence for microbial growth – or at least for the abundant presence of microorganisms - could be detected in all examined contaminated sites on the Safah oil field. This finding was especially interesting, since hydrocarbons from crude oil appear to be the only available carbon source in most of the sites and strong hydrocarbon-degrading activities were to be expected. It also prompted for an employ of these adapted microorganisms for a field biodegradation experiment.

On the Occidental of Oman oil field in Safah, a former unlined produce water pit, the so-called "Halliburton Pit", had already been scheduled for clean-up and closure by Occidental prior to this study. The pit measures some 60 by 60m, with a depth of some 10m. The bottom consisted of a thick layer of salt and oil sludge, covered by fine dust and sand from the desert. The pit and its surroundings were also the source of some of the isolated oil-tolerant halophilic microbes (fig 2).

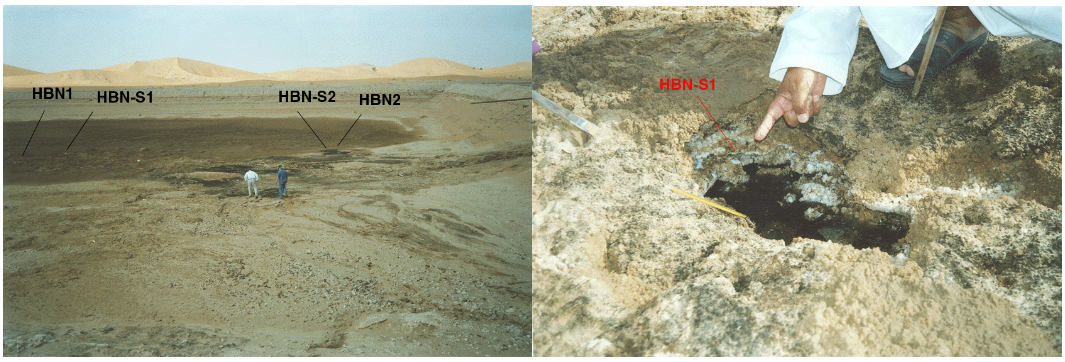


Figure 2 – The "Halliburton Pit" in Safah before the start of the field experiment (left total, right detail). The bottom was covered with a thick layer of salt and oil sludge. The captions indicate positions from where halophilic microbes were isolated.

For the present study, the pit was filled about 1m high with slightly brackish water from a nearby well and fertilized with sludge from the camp's sewage plant. The ten selected "sturdy" microbial cultures were grown on a large scale in the laboratory and were used to inoculate the water – creating a 3600 m³ open-air bioreactor for the degradation of oil-contaminated sand and soil. Water losses due to evaporation were compensated by monthly refills.

Regular Monitoring Proves Degradation of Hydrocarbons and Provides Further Novel Halophilic Microbes

During regular visits to the Occidental camp the physico-chemical and especially microbiological conditions in the Halliburton experimental pit were monitored. From all eight sides and corners of the pit, water was used to inoculate standard halophilic microbial growth plates on site, and sediment samples were collected for later analysis in the SQU laboratory. Furthermore, temperature, pH and salt concentration were weekly recorded by Occidental's chemical laboratory personnel.

General appearance of the pit

The entire surface of the pit was filled with water. The depth was about 10-30cm close to the edges and more than 1m in the middle. After ca 3 months the water became greenish, indicating the presence of photosynthetically active green algae.

Later on, when the salinity rose, the greenish color disappeared, and the pit water appeared colorless and relatively clear (fig. 3).

Figure 3 – The water-filled "Halliburton Pit"

In the weeks after the first filling, the salty sediments from the ground dissolved and, as expected, black viscous oil tar floated up to the surface, forming dense layers on the water surface along the banks of the pit (fig 4 left). After few months, however, no significant signs of hydrocarbon contamination could be observed any more (fig 4 right). About 20-30cm below the water line, the sediments appeared slightly blackish. The color was not tightly absorbed but rather loosely mixed with the sediment since it was easily released into the water, when the sediments were poked or stirred with a rod. This gave rise to the concern that the tar was simply adsorbed by the sand. However, chemical analyses showed that these pigments did not contain significant amounts of hydrocarbons but consisted mainly of iron sulfide. Apparently, a consortium of sulfat-reducing microbes had formed in the microaerobic strata of the water body.

Figure 4 – Details from the "Halliburton Pit": Tar floating on the water surface some six weeks after filling and inoculation (left) and the clean banks after ca six months (right).

Physico-chemical characterization of the pit water

Temperature, pH and chloride concentration of the water were recorded in weekly intervals over one year close to the surface at two opposing corners of the pit. As anticipated, the temperature curve follows – with the expected short delay – the seasonal changes of the air temperature. The pH remained very stable and neutral throughout the observation period, indicating the presence of a "healthy", stable microbial consortium. Any significant acidification would have suggested that aerobically metabolizing cells die and anaerobic fermentation processes take over (fig 5).

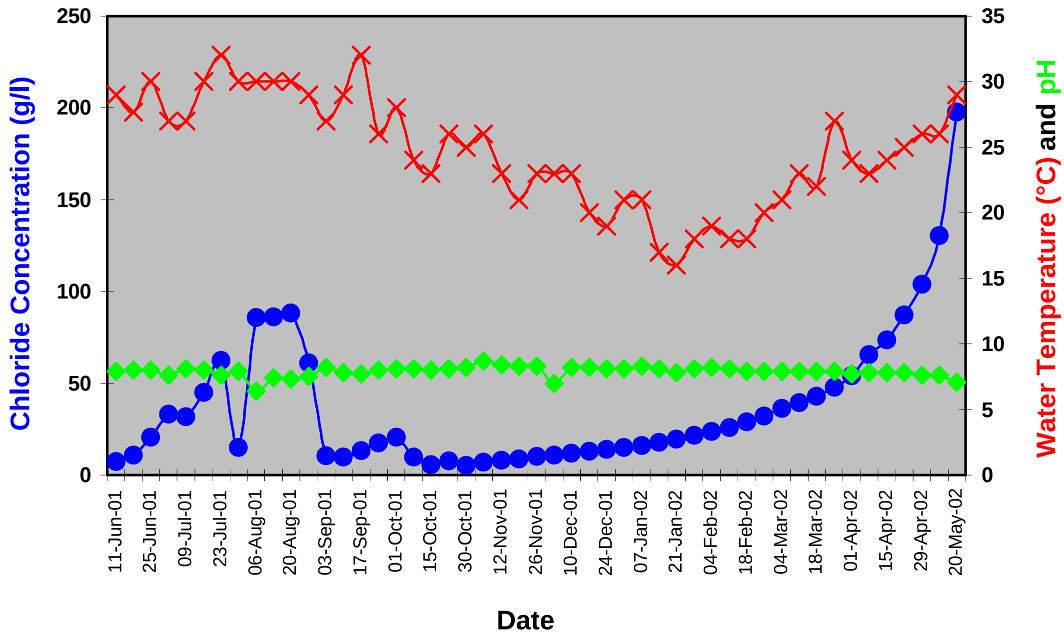


Figure 5 – Physico-chemical monitoring of the pit from June 2001 to May 2002. Shown are the data for pH (green), water surface temperature (red, both on scale on the right), and chloride concentration (blue, scale on the left).

The curve for the chloride concentration showed a remarkable behavior (fig 5). In lieu of the anticipated constant rise due to dissolution of the salt in the ground sediments, a sudden drop of the salinity was observed following a refill some three months after the start of the experiment. It remained between 6-8g/l chloride (corresponding to 10-14g/l NaCl) for a period of several months. Obviously, the salt still had to be present in the pond, and since all data were recorded close to the surface of the water, the only conceivable explanation for this observation was stratification. In line with the principle of a solar pond, a layer of comparatively fresh or brackish water floated on a downward increasing concentration gradient of brine.

Temperature, pH, oxygen concentration and conductivity at different water depths in the north-western corner of the pit were then recorded, using a tetrafunctional probe with a long cable. The probe was thrown into the water using varying cable lengths, and the reading was performed after the probe had reached the ground and the values had become stable (table 1).

The data disclosed an extreme increase of the chloride concentration toward the bottom and toward the center of the pit. Also, the temperature rose considerably with increasing depth. Both observations strongly support the hypothesis of a stratification and thus the formation of a solar salt pond.

Depth (m)	Temperature (°C)	PH	$c(O_2)$ (ppm)‡	Conductivity (µS)
“0”	24.6	8.1	9.2	30.9
ca. 0.25	28	8.36	13.8	30.6

‡ The readings at 1 and 2m may be incorrect due to a possible disturbance of the instrument by the increased conductivity (salt contents) recording.

ca. 0.5	31.9	7.2	6.7	70.3
ca. 1	41	6.4	>50	>999
ca. 2	42	6.4	>50	>999

Table 1 - Temperature, pH, oxygen concentration and conductivity of the water in a transect starting in the north-western corner, facing towards the center of the Halliburton experimental pit on 15 November 2001.

Since a more homogeneous salt distribution was desired to facilitate the establishment of a stable microbial consortium, the construction of an aeration or agitation device was considered. However, after a period of strong winds, the gradient collapsed, and the salinity at the surface resumed its rise and is now expected to plateau close to saturation at slightly above 200g/l.

Viable cell count on microbial growth plates

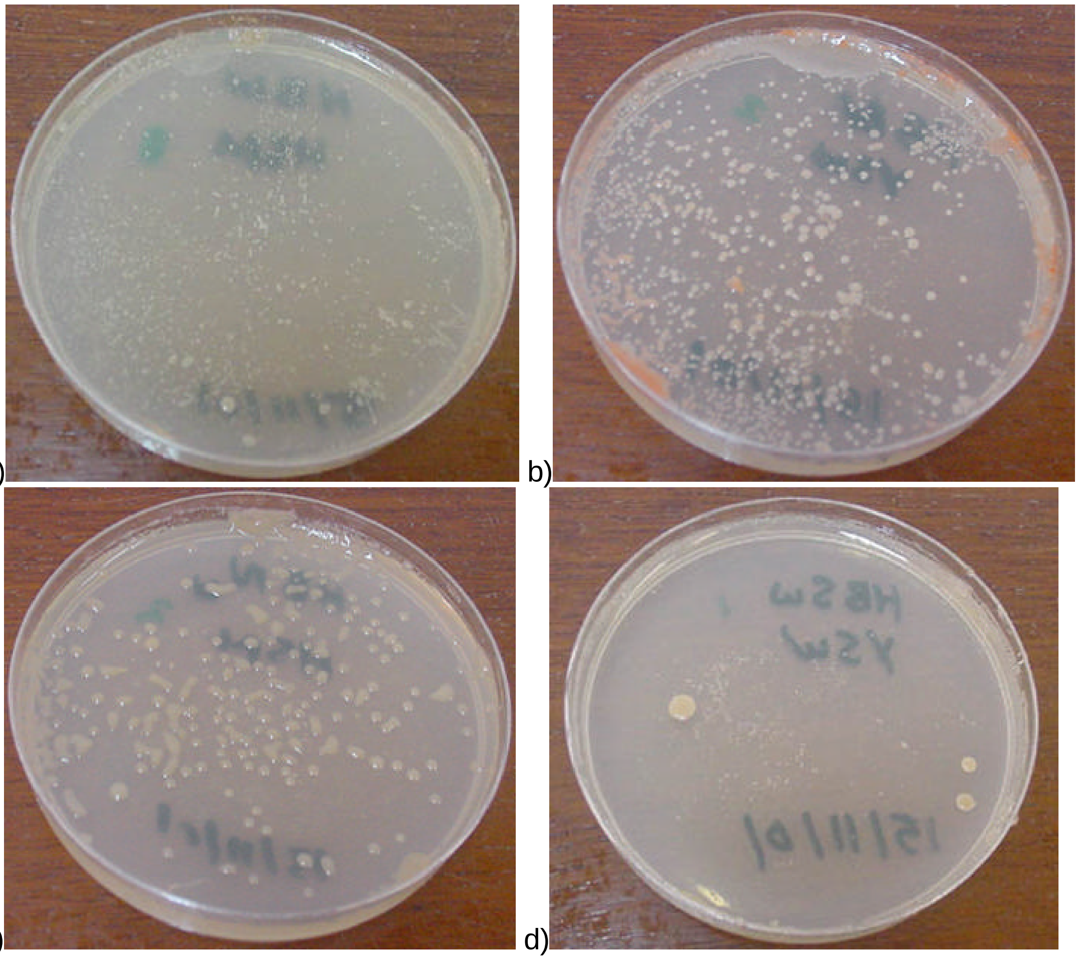
Viable cell counts in the pit waters were performed by direct aseptic spreading of the surface pit waters (samples of 400µl each) on microbial growth plates in intervals of 4-6 months. In expectation of a very high salinity in the pit two extremely halophilic growth media were chosen: YSW ("Yeast Sea Water") on the basis of yeast extracts, containing 190g/l salt, and HSM ("Halobacterial Standard Medium") on the basis of meat extract, containing 250g/l salt. Again, the plates were cooled to 4°C, transported to SQU and incubated for two weeks at 40°C. Although the salinity at the surface turned out to be considerably lower than that of the selection media, a high number of halophilic colony-forming units (CFU) were detected in the pit water. Results such as those from 15 November 2001 (table 2) were typical for the entire first year of the experiment.

Location	Temperature (°C)	pH	CFU (YSW)	CFU (HSM)
East	23.3	8.4	5	0
Northeast	23.6	8.4	0	65
North	23.6	8.4	>100	>100
Northwest	23.8	8.4	>100	>100
West	24.2	8.4	18	3
Southwest	23.9	8.4	8	5
South	24.0	8.3	8	60
Southeast	23.9	8.0	0	5

Table 2 - Number of viable halophilic cells ("colony-forming units" (CFU)) per ml in the surface water of the Halliburton experimental pit on 15 November 2001, plated on two different media.

Interestingly, water from the various corners (NE, NW, SW, SE) and sides (E, N, W, S) around the pit gave strongly varying cell counts. Although the locations of high and low cell density changed from time to time, presumably influenced by the direction of the prevailing winds, the overall heterogeneity of the distribution remained invariable.

The population of extremophilic microorganisms in the pit was not only heterogeneously distributed, but also very divers. For all sample sites, HSM plates selected apparently different species than the YSW plates. Generally the observed species diversity was higher on YSW. Some differences in colony number, size and color are exemplified in figure 6. Pictures a) and b) show agar plates with HSM (a) and YSW (b) media, inoculated on the north side of the pit. The plates in figure 6c) and d) demonstrate the pronounced quantitative heterogeneity of the microbial population, here between the two western corners. The plate on picture c) was inoculated in the north-western, that in d) in the south-western corner, all in November 2001.



a) b) c) d)

Figure 6 – Examples of halophilic microbial growth plates, inoculated on two different media with water from the Halliburton Experimental Pit on 15 November 2001; a) HSM, inoculated on the north side, b) YSW north side, c) HSM north-west corner, and d) YSW south-west corner.

Summary and Outlook

In a so far unique field experiment, a former produce water pit in northern Oman was converted into a large-scale bioreactor with the aim to device an economic and environmentally benign method for the bioremediation of oil contaminations in arid areas using a “wet” (=submerged culture) system, and to identify the responsible extremophilic microorganisms.

All relevant physical (e.g. salinity, pH, temperature) data in the pit are regularly monitored, and spot-checks provide insight into the microbial population dynamics. As soon as the data become stationary, the pit will slowly be filled with contaminated drilling sand, and the kinetics of the hydrocarbon degradation will be followed by mass-selective gas chromatography. Eventually– after the hydrocarbon contents will have sunk under an environmentally acceptable level – the pit can be closed.

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