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HYDROCARBON DEGRADATION UNDER HYPERSALINE CONDITIONS

Some Facts, Some Experiments and Many Open Questions

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1. Introduction

The 1991 Gulf War oil spill – the largest in history – severely impacted not only Kuwait but also several hundred kilometers of the coast lines of Saudi Arabia, Bahrain and Qatar in an unprecedented manner (Fowler et al., 1993). It dramatically reminded the public and the scientific community of the still largely unsolved problem of hydrocarbon degradation under saline and/or arid conditions. The decrease of the total hydrocarbon concentration over the following years was mainly due to evaporation and weathering (Saeed et al., 1998; Sauer et al., 1998) and only slightly alleviated the severe environmental and socioeconomic consequences for the Arabian Gulf (Ahmed et al., 1998). As was shown recently, natural remediation appears to be extremely slow, especially in the coastal salt marshes. Reddy and coworkers found resilient hydrocarbons in the coastal sediments around West Falmouth, MA, USA, more than thirty years after a comparatively small spill of some 700,000 liters of fuel oil into the waters of Buzzards Bay (Reddy et al., 2002).

Since the first reviews on saline biodegradation (Oren et al., 1992) our understanding of the processes and organisms involved has considerably improved. It is, however, still far from comprehensive. Van Hamme et al. (2003) have provided an excellent summary of the latest developments in petroleum microbiology, Margesin and Schinner (2001a, 2001b) have discussed the biotechnological potential of halophilic microorganisms, and Berte-Corti and Fetzner (2002), as well as Ayala and Torres (2004) have concentrated on the mechanisms of hydrocarbon degradation. The status quo of anaerobic hydrocarbon metabolism was also presented recently (Boll et al., 2002; Widdel and Rabus, 2001).

This article now is focused on the aerobic degradation of aliphatic hydrocarbons under hypersaline conditions. Peyton and colleagues have provided a discussion of the degradation of aromatic hydrocarbons at high salt concentrations (Peyton et al., 2004). The present chapter first gives a general overview of the chemistry of aerobic microbial alkane degradations, followed by recent remediation studies under saline or hypersaline conditions, and finally an example of a wet hypersaline bioremediation is discussed in more detail: the clean-up of a former petroleum produced water pit in the Sultanate of Oman.

2. The Biological Chemistry of Aliphatic Hydrocarbon Degradation

Presumably the most common – but possibly only the most thoroughly studied – route for the microbial degradation of aliphatic hydrocarbons is the so-called α-hydroxylation pathway (= "terminal oxidation pathway", Fig. 1). The alkane is hydroxylated at one terminal carbon by a monooxygenase. An alcohol dehydrogenase is thought to produce an aldehyde, which is oxidized to the corresponding long-chain carboxylic acid (= "fatty acid") by an aldehyde dehydrogenase. This acid is then converted into its coenzyme A-thioester and degraded into acetyl-CoA *via* the β-oxidation spiral before the carbon atoms are channeled into the primary metabolism through the tricarboxylic acid cycle.

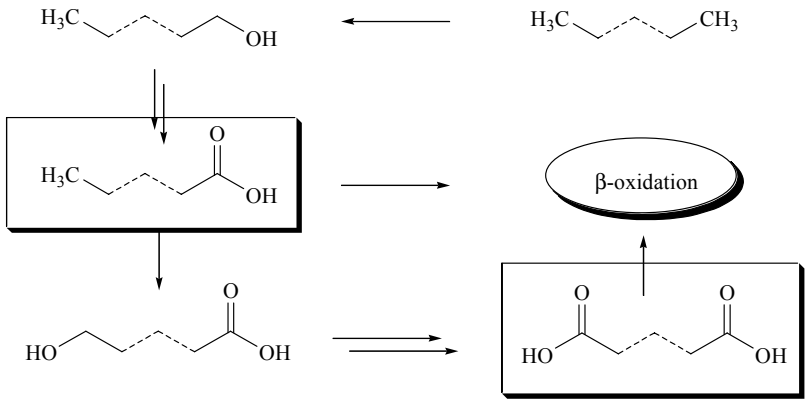

Figure 1. "Classical" pathways for the microbial degradation of aliphatic hydrocarbons: After an initial terminal hydroxylation a long-chain carboxylic acid is formed, which is either directly submitted to the β-oxidation spiral or undergoes prior bi-terminal oxygenation.

Alternatively, the fatty acid may be oxygenated at the other terminal carbon forming an ω-hydroxyalkanoic acid. This alcohol can be analogously oxidized through the corresponding ω-oxoalkanoic acid to an alkanedioic acid, which – after CoA ester formation – is submitted to degradation by β-oxidation. This modification is often referred to as "bi-terminal" or α,ω-oxidation pathway.

Since none of the non-polar substrates is freely water-soluble, all of the catabolic enzymes mentioned should be membrane proteins or at least membrane-associated proteins. For *Pseudomonas putida* Gpo1, this has recently been demonstrated (van Beilen et al., 2001). Two operons containing the genes for the oxidizing enzymes, as well as for rubredoxins, several regulatory proteins and a possible transport protein, were characterized and allowed to model the following scenario. The long-chain hydrocarbon diffuses through the outer membrane or is imported by a transport protein. The alkane hydroxylase is an integral membrane protein that scavenges the alkanes

from the inner membrane and hydroxylates them at the cytosolic side of the membrane. A soluble rubredoxin and rubredoxin reductase regenerate the monooxygenase. The fatty alcohols are then further oxidized by an alcohol and an aldehyde dehydrogenase, which are possibly also at least membrane associated (Van Hamme et al., 2003). The hydrophobic tail of the alkane would then only leave the membrane after its CoA thioester was formed (Fig. 2).

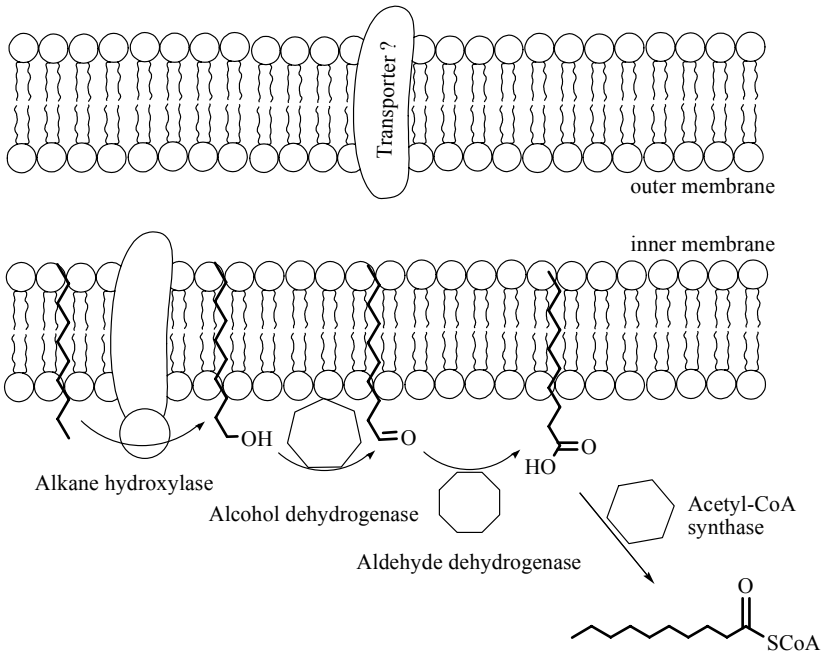

Figure 2. Possible scenario for the hydrocarbon activation in and at the inner membrane of *Pseudomonas pudida* Gpo1 (modified from Van Hamme et al., 2003).

Similar genes are being analyzed for a number of further organisms, such as an additional *P. putida* strain, *Burkholderia*, *Nocardioides* and *Acinetobacter*, and will soon shed more light on the mechanistic details of this pathway (e.g. Hamamura et al., 2001; Ishige et al., 2000; Marín et al., 2001; Tani et al., 2001). It must be emphasized again that the α-oxidation route is by no means the only pathway that has been described for the aerobic degradation of aliphatic hydrocarbons. Interestingly, different genes for multiple distinct alkane hydroxylases were even found in the same organisms (Van Hamme et al., 2003).

Relatively few mechanistic investigations have been reported for the subterminal (= ”internal”) oxidation pathway. Again, a monooxygenase is required to functionalize the alkane, but in this case the initial hydroxylation takes place at a methylene group - usually but not always - at carbon number two (Watkinson and Morgan, 1990) (Fig. 3).

The alcohol is oxidized to a ketone, which is transformed to an ester *via* an enzymatic Baeyer-Villiger reaction. Some of the enzymes involved in this pathway should be very promising for the biotechnological production of fine chemicals since chiral secondary alcohols are produced as intermediates (Bühler and Schmid, 2004; Bühler et al., 2003) and since the feasibility of scale-up and commercial exploitation of Baeyer-Villiger oxygenases has been recently demonstrated (Alphand et al., 2003).

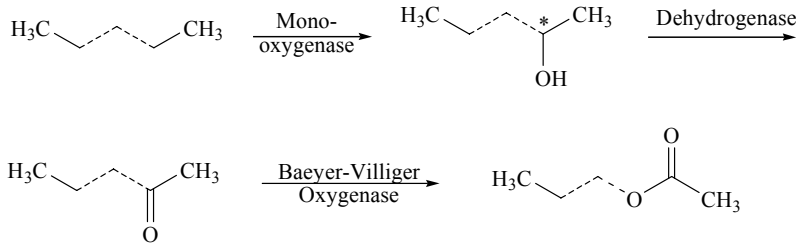

Figure 3. Internal (subterminal) oxidation of alkanes. Chiral secondary alcohols (*) are formed as intermediates during the process.

In 1988 Finnerty discovered that in certain *Acinetobacter* strains alkanes are converted to aldehydes *via* hydroperoxide instead of alcohol intermediates (Finnerty 1988). The dioxygenase, a homodimeric Cu-containing flavoprotein, utilizes molecular oxygen without the formation of radical intermediates (Maeng et al., 1996b). From the observation that peracids also occur in the cell extracts, it was suggested that the hydroperoxide produced is further oxidized, then reduced to an aldehyde and finally disproportionated to an alcohol and a carboxylic acid (Fig. 4) (Berthe-Corti and Fetzner, 2002; Finnerty, 1988).

Ample evidence for the existence of the discussed dioxygenases has accumulated (e.g. Maeng et al., 1996a). It does, however, appear questionable on the basis of the existing data that a further oxidation and reduction step is essential for the pathway. Whilst experimental data are missing, chemical logic suggests that the intermediate hydroperoxide – spontaneously or enzyme-catalyzed – may alternatively undergo an elimination to form the aldehyde directly without further intermediates (Fig. 5). This would also explain why stoichiometric investigations on the isolated dioxygenase have so far failed (Maeng et al., 1996b).

Finally, a conceptually different way of alkane functionalization was observed in a mutated *Rhodococcus* strain, designated KSM-B-3M – a desaturation instead of an oxygenation. Koike and coworkers isolated alkenes after administration of hexadecane, 1-chlorohexadecane and further 1-substituted alkanes (Koike et al., 1999). The double bond was always *cis* configurated and was found preferentially after the ninth carbon from the terminal methyl group, even if the substrate molecule contained further functionalities on the other end of the chain (Fig. 6). The authors hypothesize that the normal further degradation of the hydrocarbon would involve an oxidative cleavage of

the double bond, but that the alkenes accumulated because the genes for the oxygenating enzyme were destroyed by mutagenesis (Koike et al., 2000).

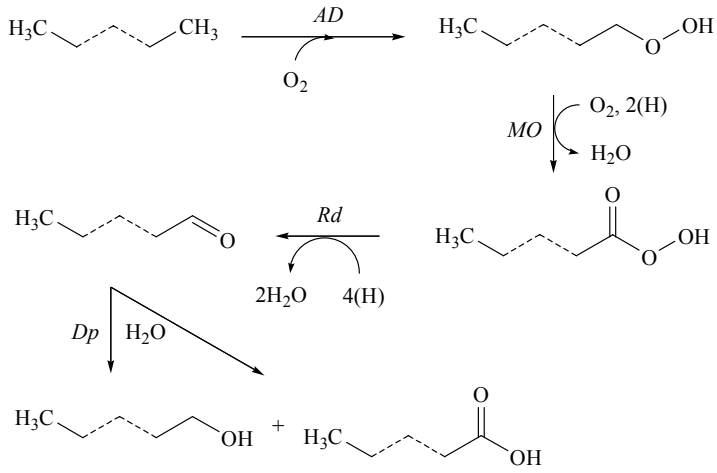

Figure 4. The so-called Finnerty Pathway for alkane degradation (modified from Berthe-Corti and Fetzner, 2002).

Apart from the α-oxygenation pathway all routes suggested leave many questions open. A number of genetic studies have recently shone light on gene clustering and regulation (Hara et al., 2004; Sei et al., 2003; van Beilen et al., 2004), but direct approaches to overproducing strains or isolated enzymes are still not available to a sufficient extent. Enzymatic hydrocarbon functionalization has an enormous industrial potential, both for remediation and for the conversion of petroleum compounds to higher value chemicals (Ayala and Torres, 2004). Thus further insight especially into non-conventional pathways could be invaluable.

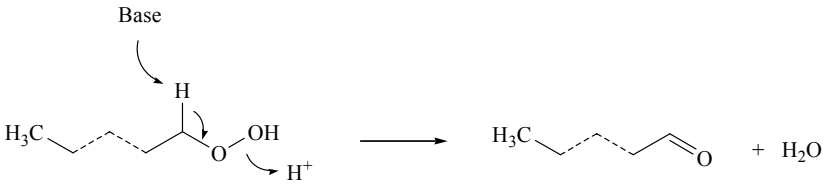

Figure 5. Suggested mechanistic short-cut for the so-called Finnerty pathway.

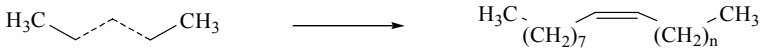

Figure 6. Central desaturation of alkanes according to Koike et al. (1999).

3. Hydrocarbon Metabolism in Hypersaline Environments

The first investigations on hydrocarbon degradation in sea water appeared in the 1970s (e.g. Soli and Bens, 1972), but until the beginning of the 1990s, only very few reports had described a possible hydrocarbon metabolism at hypersaline conditions. The general assumption that with rising salt concentrations – and thus reduced oxygen availability - the catabolic activity and the potential for hydrocarbon degradation decreased (Ward and Brock, 1978) led to a rather pessimistic assessment: "… attempts to demonstrate the degradation of otherwise relatively easily biodegradable pollutants in hypersaline lakes often yielded disappointing results (Oren et al., 1992)." Since then, however, a number of halophilic or at least halotolerant microorganisms have been characterized from hydrocarbon-contaminated saline ecosystems, and ecological or application studies have followed. However, most of this work is concentrated on marine or benthic ecosystems and was recently reviewed (Harayama et al., 1999, 2004).

The first two independent descriptions of a microbial alkane metabolism under truly hypersaline conditions appeared in 1990 (Bertrand et al., 1990) and 1991 (Kulichevskaya et al., 1991). Archaea isolated from a salt marsh in France and a saline oil field in Russia utilized petroleum hydrocarbons at salinities up to 32%. Two further extremely halophilic Archaea were found in association with petroleum hydrocarbons in former Soviet Republics: a *Haloferax* strain on a saline oil field in Kazakhstan (Zvyagintseva et al., 1995) and a *Halorubrum* on an oil field in Tatarstan (Zvyagintseva et al., 1998). Biodegradation experiments with these strains, however, were not reported.

Some further examples exist, where Bacteria were connected to alkane metabolism at high salt concentrations. Again from an oil field an extremely halotolerant streptomycete, *Streptomyces albiaxialis*, was isolated and shown to degrade petroleum hydrocarbons up to a salinity of 30% (Kuznetsov et al., 1992). A 15% salt concentration was tolerated by *Bacillus* and *Staphylococcus* strains that were employed in Japan in 1 m^3 batch fermentations to clean industrial waste waters contaminated with aliphatic hydrocarbons in a broad mixture of aromatics and phenols (Kubo et al., 2001). Presumably not aerobic but sulfur-reducing was the bacterial consortium that Bechtel et al. described from the Bahloul formation in Tunisia (Bechtel et al., 1996). Since it was isolated from hot hypersaline and sulfate-rich effluents of a salt dome it appears to degrade hydrocarbons close to salt saturation.

Finally, three recent publications describe the use of consortial halophiles for oil hydrocarbon degradation. The metabolism of diesel fuel by three bacterial mixed populations from contaminated saline Argentinean soils was monitored indirectly through the O_2 consumption. Although the cells remained viable up to 25% salt, hydrocarbon metabolism ceased at 17.5% and was maximal at below 10% (Riis et al., 2003). Catabolic activity up to 22% was described in Colombia for a bacterial consortium, which was immobilized on a solid support (Diaz et al., 2003). Four of the

strains from the consortium were reportedly able to grow in submersed cultures over a range of 0-32% salt. Finally, a rather unusual application for halophiles was suggested by Sakhno et al. (2003), who added halophilic Archaea to saline contaminated soil to increase the biological activity of the soil and stimulate the hydrocarbon degradation by *Pseudomonas* and *Rhodococcus* strains.

This short compilation may not be comprehensive. It is obvious, however, that detailed investigations on the mechanism of aerobic halophilic hydrocarbon degradation and on the biochemistry of the involved organisms are still not available. On the other hand, the increasing number of novel extremely halophilic or at least halotolerant oil-degrading microorganisms and the first applied investigations clearly show promise for further larger biotechnological applications. In the following section, one such application from the south of the Arabian Peninsula is described.

4. Remediation of a Former Produced Water Pit in Oman

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 deserts in the interior and salt marshes ("sabkhat") along the 2000 km 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 usually high salinity on oil fields can only be successfully attempted using indigenous extremophilic microorganisms.

On several visits to the Safah concession site of Occidental of Oman, Inc. (North-West Oman, 20 km from the border to the United Arab Emirates, Wilayat Ibri, 23.19° N, 55.45° E) between June 1999 and December 2000, soil, water and sand samples were collected from oil-contaminated sites in and around flare, drilling rig and produced water pits on the oil field, and from the "Modern Salt" solar salterns, which are associated with the oil field to produce drilling salt from the saline oil production water. Inoculation in halophilic standard media led to the isolation of 37 extremely halophilic microbial strains, both Bacteria and Archaea, from which 10 were virtually insensitive towards the presence of Oman Crude Oil.

On the Occidental concession site a former unlined produce water pit, the so-called "Halliburton Pit", had already been scheduled for clean-up and closure by the company prior to this study. The pit measures some 60 by 60 m, with a depth of some 10 m. The bottom consisted of a thick layer of salt and oil sludge, covered by fine dust and sand from the desert (Fig. 7). The hydrocarbon concentration of the first meter sediment varied between 10 and 40% (w/w). The infiltration was probably deeper, but no analytical data were available. Some of the isolated oil-tolerant halophilic microbes originated from the pit or its surroundings.

Figure 7. The "Halliburton Pit" in Safah before the start of the field experiment (top total, bottom details). The ground was covered with a thick layer of salt and oil sludge.

The pit was filled to an average depth of 1 m 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 larger scale in the laboratory and were used to inoculate the water – creating a 3600 m^3 open-air bioreactor for the degradation of oil-contaminated sand and soil. Water loss due to evaporation was compensated by monthly refills (Fig. 8).

Figure 8. The water-filled "Halliburton Pit" in June 2001.

During regular visits to the Occidental site the physico-chemical and 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. Furthermore, temperature, pH and salt concentration were recorded weekly by Occidental's laboratory personnel.

4.1. GENERAL APPEARANCE OF THE PIT

The entire ground surface of the pit was covered with water with a depth of about 10-30 cm close to the edges and close to 2 m in the middle. After *ca* 3 months the water became greenish, indicating the presence of photosynthetically active green algae. Later, when the salinity rose, the greenish color disappeared, and the pit water appeared colorless and relatively clear.

During 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. 9, left panel). After few months, however, no significant signs of hydrocarbon contamination could be observed any more (Fig. 9, right panel). About 20-30 cm 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 sulfate-reducing microbes had formed in the microaerobic strata of the water body.

Figure 9. 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).

4.2. 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 (Fig. 10). As anticipated, the temperature curve followed – 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.

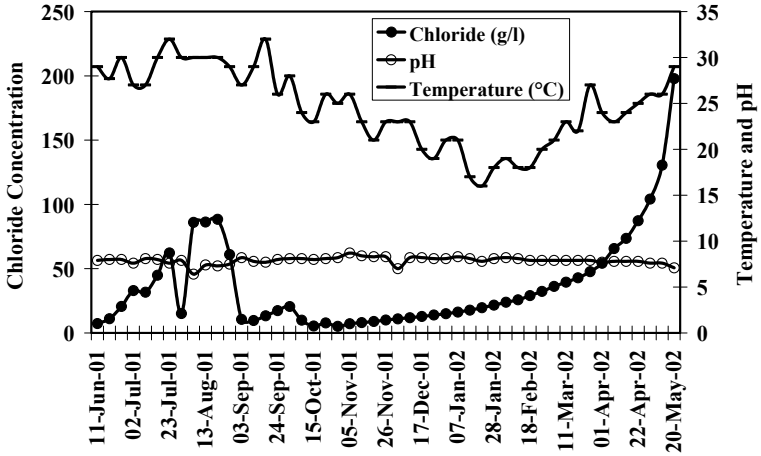

Figure 10. Physico-chemical monitoring of the pit from June 2001 to May 2002. Shown are the data for pH (open circles), water surface temperature (horizontal bars, both on the right ordinate), and chloride concentration (full circles, left ordinate).

The curve for the chloride concentration showed a remarkable behavior. Instead of the anticipated constant rise due to the dissolution of the salt from 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 6-8 g l^{-1} chloride (corresponding to 10-14 g l^{-1} NaCl) for a period of several months. Obviously, stratification had occurred and, since for technical reasons the salinity had to be recorded close to the surface, relatively low values were obtained. 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.

On November 15, 2001, temperature, pH, oxygen concentration and conductivity at different water depths in the north-western corner of the pit were 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 the predictable extreme increase of the chloride concentration toward the bottom and toward the center of the pit. In addition, the temperature rose considerably with increasing depth. Both

observations strongly support the hypothesis of stratification and thus the formation of a solar salt pond.

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. The readings for $c(O_2)$ at 1 and 2 m may be incorrect due to the extremely high conductivity.

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
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

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 leveled close to the saturation concentration at slightly above 200 g l^{-1}.

4.3. MICROBIOLOGICAL MONITORING

Viable cell counts (colony forming units CFU) in the pit waters were performed by direct aseptic spreading of the surface pit waters on plates (triplicates) at intervals of 2-3 months. Five different common halophilic media with salinities ranging from 10-25% were employed. The number of CFUs on the individual plates was not very high, but the microbial diversity in the pit must have been substantial since totally different organisms were isolated in any one analysis on the different media. Results such as those from 15 November 2001 (Table 2) were typical for the entire first year of the experiment. Interestingly, the number of CFUs from the water at various locations around the pit varied strongly between each other and for each location from visit to visit. It appeared to be much stronger influenced by the climatic conditions, such as the prevailing wind direction, than by the selection medium or the recorded salinity.

TABLE 2. Number of viable halophilic cells (colony-forming units) per ml in the surface water of the Halliburton experimental pit on 15 November 2001, plated on Yeast Seawater (YSW) and Halobacterial Standard Medium (HSM).

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

The relatively low number of cells isolated from the water may well have been due to the limited number of selection media. A number of scanning electron micrographs, recorded of the surface of submersed pieces of metal or rock from the pit, always showed a dense coverage with cells and were optically free of hydrocarbon (Fig. 11). In agreement with earlier observations (Obuekwe and Alzarban, 1998) adherent microbes may have played an important role in the remediation.

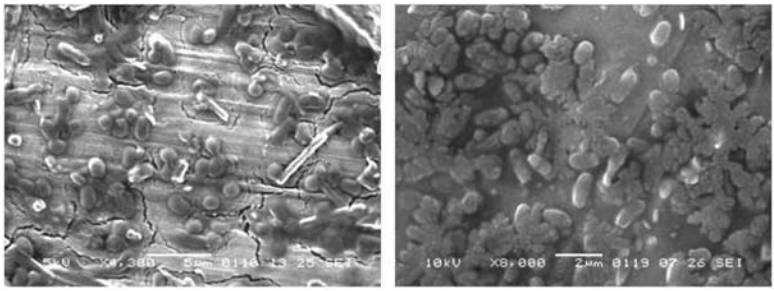

Figure 11. Scanning electron micrographs (gold-coated) of two small pieces of metal recovered from the pit.

Randomly chosen strains were submitted to biotransformation experiments in pure culture using pentadecane and eicosane. In most cases the mass spectrometric analyses of the metabolites suggested an α-degradation pathway (Fig. 1). Several strains produced small quantities of metabolites that could not yet be identified with the available instrumentation. In the medium extracts of one orange archaeon, however, aldehydes and carboxylic acids as well as long-chain esters were found. Since Archaea do not produce significant amounts of fatty acids (*cf. e.g.* Pugh and Kates, 1994, for examples), this can only be interpreted as an indication of a split pathway for hydrocarbon degradation: α-oxidation and subterminal routes proceed in parallel in the same organism at a salt concentration of 20%. Such split pathways are not frequently observed, but interestingly in another archaeon, *Haloarcula hispanica*, isoleucine biosynthesis appears to also proceed through parallel routes (Hochuli et al., 1999).

4.4. CLOSE-OUT ANALYSIS

In summer 2002 the hydrocarbon concentration close to the banks had fallen to below 1% (w/w), the legal limit for disposal in the Sultanate of Oman. Water refilling was halted, and the pit was allowed to dry out over the following months. Further analyses of the deeper sediments in the middle confirmed the drastic reduction of the hydrocarbon concentration in the pit. In January 2004, the entire sediments were excavated to a depth of about 5 m, where a layer of impermeable bedrock was reached (Fig. 12).

Figure 12. In January 2004 the sediments of the experimental pit were excavated, and the pit was closed starting from the northern side. The slope on the left used to be the southern bank.

Some very small pockets - totaling around 5 m^3 – were found, where the hydrocarbon concentration still ranged between 1 and 5%. The several thousand tons of the gravel, salt and sand mixture that remained contained only trace amounts of hydrocarbon. Due to the poor accuracy of the analytical data at the onset of the experiment a quantitative estimation of the total hydrocarbon degradation must remain tentative. From various but unsystematic analyses Occidental assumed a hydrocarbon contamination of the pit sediments between 10 and 40%, down to a depth of 1 m. No data existed from deeper layers. A very cautions calculation using the lowest values and equaling 1 m^3 to 1 t, gives for the 60x60 m surface area and 1 m depth a hydrocarbon content of 360 t. Presumably, however, the infiltration reached much deeper, and the average concentration was higher than 10%. After the end of the experiment the remaining contaminated material contained (5 m^3, now the higher concentration is used for the calculation) a maximum of 250 kg. Even if the trace amounts of the bulk material are taken into account, more than 300 t of weathered hydrocarbon were removed during the 18-months experimental period at extremely low costs.

5. Summary

In a so far unique field experiment, a former produce water pit in northern Oman was converted into an open-air 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. It clearly demonstrates the

feasibility of large-scale halophilic environmental biotechnology. A more detailed characterization of the involved microorganisms will follow.

This example, however, and the others presented above are still few in number. Much work remains to be done before halophilic microorganisms can be predictably employed in commercial environmental biotechnology or even in the production of fine chemicals. A number of further applications have been proposed (Margesin and Schinner, 2001b; Patzelt et al., 1998), but sometimes even the most basic biochemical knowledge, such as on the central metabolism of the organisms (Hochuli et al., 1999), is incomplete. Overall, only small additions have to be made to a conclusion that was drawn previously: "The above survey shows that our knowledge on the degradation of organic pollutants at high salt concentrations is still extremely limited (Oren et al., 1992)".

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