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Degradation of petroleum model compounds immobilized on clay by a hypersaline microbial mat

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Abstract

In this study the degradation of hydrophobic petroleum model compounds (phenanthrene, pristane, octadecane and dibenzothiophene) added to a submersed hypersaline microbial mat was investigated. Montmorillonite with an artificially altered, hydrophobic surface was used as carrier material, forming an organo-clay complex (OCC) with the attached mixture of petroleum model compounds. 6 mg/cm^2 OCC were applied to cyanobacterial mat pieces, containing ~33.3 μg/mg OCC of each compound. The degradation experiment was performed under controlled laboratory conditions and accompanied by chemical analyses by GC/GC-MS, molecular analyses by PCR and DGGE as well as functional analyses by microsensor measurements of oxygen, photosynthesis, sulfide, pH and light. All applied model compounds were degraded, but residues were still present after 18 weeks. The aromatic compounds phenanthrene (5.1 μg/mg OCC) and dibenzothiophene (4.3 μg/mg OCC) were preferentially degraded compared to the alkanes pristane (12.4 μg/mg OCC) and n-octadecane (13.4 μg/mg OCC). Metabolic changes during the degradation process could not be detected by microsensor measurements. The molecular population analyses did not reveal any significant community changes concomitant with the decrease of the petroleum model compounds. We conclude, that the pristine mats represent an intact, robust ecosystem in which the enzymatic requirements for the degradation of the applied pollutants exist. The slow degradation process did not affect the usual high internal turnover rates and did not favor a certain population in the community of the mats.

Introduction

Pollution of marine environments with oil and oil products became a world wide problem on the tide of industrialization. The sources of marine hydrocarbon pollution are mainly runoff from land and municipal/industrial wastes (13.74 $\times$ 10^8 l/a), routine ship maintenance like bilge cleaning (5.19 $\times$ 10^8 l/a), air pollution from cars and industry (3.48 $\times$ 10^8 l/a), natural seeps (2.35 $\times$ 10^8 l/a), tanker accidents (1.4 $\times$ 10^8 l/a) and offshore oil production (0.57 $\times$ 10^8 l/a) (NRC 1985, US Coast Guard 1990). At seawater salinity, microbial degradation can be a successful remediation approach even at low temperatures and after major catastrophes (Pritchard & Costa 1991). However, under hypersaline conditions microbial degradation of otherwise easily biodegradable contaminants is often more difficult, because salt stress is superimposed on pollution stress (Oren et al. 1992). For example, at a salinity of 20% in Great Salt Lake brines (Utah/USA) no degradation of long chain alkanes was observed (Ward & Brook 1978). Nevertheless, when biofilms are formed, tolerance to hydrocarbons or their degradation products is possible, even in hypersaline environments. After the 1991 Gulf War tar-covered beaches and hypersaline enclosures ("Sabkhas") were recolonized by cyanobacterial mats at salinities higher than 5% (Hoffmann 1996; Höpner et al. 1996). Since it is essential for successful biodegradation that the involved microorganisms come in direct contact with the pollutants (Rosenberg et al.

1992), the situation of immobilized cells in biofilms may have been advantageous in that particular case.

In hypersaline microbial mats species of different physiological groups form a compact community, where oxic regions with oxygen oversaturation and anoxic regions are in close contact. Such mats are often dominated by cyanobacteria in terms of biomass, which provide oxygen and photosynthates produced during photosynthesis (Grötzschel & de Beer, unpublished). In addition, many cyanobacteria have the ability to fix N_2 and supply the mat with nitrogen. The availability of oxygen and a nitrogen source are prerequisites for successful aerobic degradation (Rosenberg et al. 1992). For these reasons hypersaline cyanobacterial mats represent a promising ecosystem for biodegradation purposes.

In our laboratory experiment we used five substances as representative petroleum components for straight-chain, branched and cyclic alkanes, as well as aromatic and sulfur-aromatic compounds. To establish direct contact between these model compounds and benthic cyanobacterial mats, a modified clay mineral was used as carrier material. Over a period of four months changes in oxygen and sulfide concentration, pH, photosynthetic rates and light fields were followed with microsensors. The population structure was analyzed with PCR and DGGE, degradation of the model compounds was followed with GC/GC-MS.

Materials and methods

Collection and pre-incubation of the mat samples

Eight cyanobacterial mat samples (15 × 15 × 4 cm) were collected from a hypersaline experimental pond with a salinity of 7.5% at the Interuniversity Institute IUI, Eilat/Israel and transferred to the Max Planck Institute for Marine Microbiology in Bremen/Germany, within 24 h. Upon arrival, the mats were incubated in a glass aquarium filled with aerated artificial seawater (ASW, Wiegandt, Germany). The salinity was maintained at 7.5%, the temperature at 27 °C and the pH at 8.2. The mats were incubated at a light regime of 12 h light/12 h dark with an intensity of 600 μmol photons/m^2s.

Preparation of organo-clay complexes (OCC)

n-octadecane, pristane, phenanthrene, perhydrofluorene, and dibenzothiophene were used as model compounds for petroleum constituents representing straight-chain alkanes, branched alkanes, aromatic hydrocarbons and organo-sulfur compounds, respectively. In order to overcome the poor accessibility of the hydrophobic pollutants for the benthic mats, a clay mineral was used as a carrier substance. By previous adsorption of organic cations the clay surface was changed to hydrophobic characteristics. A 2% aqueous suspension of montmorillonite KSF (Aldrich) was slowly mixed with a 10 mM solution of benzyl-trimethylammoniumchloride (BTMA, 0.8 mmol/g clay) and stirred for 24 h (modified after El-Nahhal 1998). Subsequently, the mixture was washed three times with deionized water to remove excess BTMA and then freeze-dried.

The petroleum model compounds in n-hexane solution were slowly added under continuous stirring to a n-hexane suspension of the hydrophobic BTMA-clay (20 mg model compounds per 100 mg of hydrophobic clay). The resulting organo-clay complex slurry (OCC) was dried in a vacuum rotary evaporator, which yielded homogenous powder of hydrophobic clay loaded with 16.67 wt-% of petroleum model compounds. To verify the amount of loaded model compounds, the OCC was re-extracted with dichloromethane (DCM) and analyzed by gas chromatography.

Contamination experiment

After five days of pre-incubation, 10 × 10 × 4 cm mat pieces were transferred to small glass aquaria (16 × 16 × 8 cm) and fixed in agar (1.5%, ASW). Each aquarium was filled with 1 l ASW and put into a basin containing water for temperature regulation. After additional five days of incubation, the contamination experiment was started:

1. Two mat samples were contaminated by direct contact with the OCC: 0.6 g OCC (containing 0.12 g petroleum model compound mixture) were suspended in a small amount of the aquarium water. The suspension was poured under gentle stirring into a high plasticized glass frame that confined the OCC cover to the surface of the mats only. Within minutes the OCC settled to the mat surface and the frame was removed.
2. Two mat samples were covered in the same manner with 0.48 g of the hydrophobic clay each as controls for the effect of the carrier material.
3. The hydrophobic petroleum model compounds were directly applied to the water phase of two aquaria with mat samples: 0.12 g of a mixture of the petroleum model compounds was dissolved in

1 ml dichloromethane and transferred to a glass slide. After evaporation of the solvent one slide was placed in each of the two aquaria.

4. One aquarium did not contain a mat sample but only agar and ASW. 0.6 g OCC were applied to the surface of the agar to serve as a control for physical removal of the petroleum model compounds from the OCC.
5. Two mat samples were left undisturbed as an untreated control.

Microsensor analyses

Oxygen concentrations were determined with fast responding Clark-type O_2 sensors with guard cathodes (Revsbech 1989). Gross photosynthetic rates were determined with the same sensors by the light-dark-shift method (Revsbech & Jorgensen 1983). The O_2 sensors had tip diameters of less than 6 μm, a stirring sensitivity $< 2\%$, and a 90% response time of $t_{90} < 0.5$ s. Measurements of pH values were performed with potentiometric glass microelectrodes (Revsbech et al. 1983), which had a tip diameter of less than 10 μm with the pH sensitive glass at the very tip not longer than 100 μm. The electrodes showed a 90% response time of $t_{90} < 10$ s. The H_2S sensors (Kühl et al. 1998) had a tip diameter of less than 20 μm. All electrodes were calibrated as described elsewhere (Revsbech & Jørgensen 1986; Revsbech 1989; Santegoeds et al. 1998). Fiberoptic microprobes for field radiance and scalar irradiance (Kühl et al. 1994) were applied at the beginning and at the end of the experiment after 18 weeks to compare the light fields of the mats.

For each set of measurements (pH, sulfide, oxygen) the aquaria were taken out of the incubation setup and fixed in a measuring setup for one day under the same incubation conditions. The microsensors penetrated the mats always at the same spots marked with small glass rods with an angle of 37° to avoid shading effects. The exact positioning of the sensors at the mat surface was achieved by means of a dissection microscope. At the end of the dark period in the morning, pH, sulfide and oxygen concentration profiles were determined. Several hours after switching on the light when oxygen concentration profiles had reached steady state, photosynthetic rates, pH and sulfide concentrations were measured.

We defined the photic zone as the layer in which gross photosynthetic activity could be detected by use of a one second dark period (Glud et al. 1992). All directly measured data were plotted as an average of three measurements.

Chemical analyses

Mat samples collected in week 0, 5, 9, 13 and 18 (cores of ~7 mm in diameter, ~2.5 g wet weight) were extracted ultrasonically with a 1:0.5:0.4 (v/v/v) mixture of methanol (MeOH), dichloromethane (DCM), and water (modified after Bligh & Dyer 1959). After centrifugation, the supernatant was collected in a separatory funnel. This procedure was repeated four times. DCM and water were added to the combined supernatant to give a MeOH:DCM:H_2O ratio of 1:1:0.9 (v/v/v) resulting in phase separation. The DCM layer was collected and the methanol-water phase was washed three times with DCM. The solvent of the combined DCM phase was removed in a rotary evaporator and the extract was diluted to concentrations appropriate for gas chromatography analyses.

The extracts were analyzed using a Hewlett Packard 6890 gas chromatograph equipped with a Gerstel KAS3 temperature-programmable injector, a flame ionization detector and a fused silica column (J & W DB-5HT, 30 × 0.25 mm, film thickness 0.15 μm). After an isothermal phase at 60 °C (2 min) the oven was heated at a rate of 20 °C/min to 150 °C, and then at a rate of 3 °C/min to 310 °C which was held for 15 minutes. For gas chromatography-mass spectrometry (GC-MS) a HP 5890 gas chromatograph (GC conditions as above) with a DB-5 HT (J & W) fused silica column was connected to a Finnigan SSQ 710B mass spectrometer as detector (operated at 70 eV, cycle time 1 scan/s). Model compounds were quantified by integration of the FID signals and comparison with that of an internal standard (squalane) which was added directly after extraction. The calculated initial amount of each model compound in 2 ml sample was 66.67 μg. Data on the abundance of the model compounds are presented relative to this value.

Molecular analyses

Regularly collected mat cores (see chemical analyses) were subjected to nucleic acid extraction, polymerase chain reaction (PCR) and denaturing gradient gel electrophoresis (DGGE) as described previously (Abed and Garcia-Pichel 2001). The PCR was carried out for the amplification of 16S rRNA using two sets of oligonucleotide primers. CYA359F (with a 40 nucleotide GC clamp at the 5′ end) and CYA781R were used as specific primers for cyanobacteria (Nübel et

al. 1997) and GM5F with a GC-clamp in combination with the general primer 907R for all bacteria (Muyzer et al. 1993). A hot start program was performed for the cyanobacteria-specific primers as described by Nübel et al. (1997). In case of bacterial general primers (GM5 and 907R), a hot-start touch-down program was used to minimize nonspecific amplifications (Santegoeds et al. 1998). The DGGE was performed at 60 °C and a constant voltage of 200 V for 3.5 hours.

Results and discussion

At the beginning of the experiment the appearance of the mat surfaces differed significantly. The control mats and the mats with the petroleum model compounds added directly to the water phase had a soft, gelatinous, dark green surface, whereas the mats with the hydrophobic clay or the organo-clay complex (OCC) added showed the beige color of the clay material. At the end of the experiment in all of the treatments the mats showed the same green-beige surface color.

Chemical analyses

In the contamination treatment with the organo-clay complex applied to the surface of the mats, four out of five model compounds were present at the start of the experiment at nearly equal concentrations of ~33.3 (±0.1) mg/g organo-clay complex (OCC) each (Figure 1). Only perhydrofluorene could not be detected in reproducible amounts and turned out to be too volatile for the application on the hydrophobic clay. The amounts of the model compounds that had been applied to the mats decreased continuously. The aromatic substances phenanthrene and dibenzothiophene decreased faster than the aliphatic compounds pristane and n-octadecane. The decrease proceeded continuously for all the four compounds (linear decline, r^2: 0.81–0.89) but in no case a complete degradation could be detected. At week 18 the amounts of the aliphatic compounds were 2.5–3 fold higher than the ones of the aromatic compounds (Table 1).

At the end of the degradation experiment, pieces of the mats contaminated with OCC and the reference mats were sectioned into layers of 1 or 2 mm thickness in order to analyze the vertical distribution of the petroleum model compounds and the lipid composition in individual layers. In the OCC-treated mats the major portion of the petroleum model compounds was present in the top layer (Figure 2). This layer was characterized by filamentous cyanobacteria which penetrated the applied organo-clay complex covering. Here the aromatic compounds were strongly depleted relative to the saturated hydrocarbons. The concentrations of the petroleum model compounds decreased sharply with increasing depth concomitant with a strong change in the relative composition of the model compounds. Being at nearly equal amounts in 1–2 mm depth, the aromatic compounds prevailed against the aliphatic compounds below that layer.

In the aquaria with the petroleum model compounds added directly to the water phase, none of the compounds could be detected in the mats by GC analyses. The substances re-solidified after addition and floated as white particles on the water surface.

In the aquaria where the OCC was applied to the agar surface without any mat material the relative compositions of the petroleum model compounds did not change significantly.

Microsensor analyses

Oxygen profiles in the light as well as profiles of gross photosynthetic rates and respiration rates in the light are presented in Figure 3. Microprofiles measured in the light at different locations showed only small variations (data not shown).

At the beginning of the experiment (Figure 3A 0–D 0), the thickness of the photic zone varied from 1.3 mm in the control mat (A 0) and the mat with the hydrophobic clay (B 0) to 2 mm in the OCC mat (D 0) with similar maximum gross photosynthetic rates. Oxygen penetration depth varied between 4.1 mm (A 0) and 2.4 mm (C 0). After 5 weeks, the oxygen penetration depth decreased in all of the mats. In week 18 the thickness of the photic zone increased in all of the mats, but the oxygen penetration depth was further reduced, except for the OCC mat (Figure 3D 18), in which it increased by 0.8 mm. This was the only treatment, in which an increase of gross photosynthetic rates and respiration rates in the photic zone could be observed after 13 weeks (Figure 4D). After a decrease of areal gross photosynthetic rates and respiration rates in this mat in the first 9 weeks of the experiment, the rates more than doubled in the following months. On average 73% of the areal respiration took place in the photic zone (data not shown). This indicates a concentration of the respiratory activity in the photic zone. In all other mats areal photosynthetic rates and respiration rates decreased

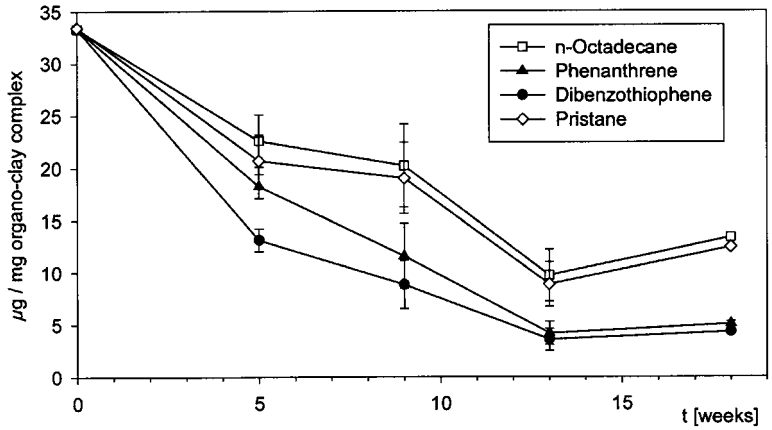

Figure 1. Concentrations of petroleum model compounds (means ± standard deviations, n = 3) in the OCC-contaminated microbial mats during 18 weeks.

Table 1. Degradation characteristics of four petroleum model compounds 18 weeks after addition to a hypersaline microbial mat

Compound	Final concentration [μg/mg OCC]	Decrease [μg/mg OCC]	Average removal rate [μg/mg OCC*d]	Half life [d]	Removal [%]
Pristane	12.4 ± 0.08	21.0 ± 0.08	0.17 ± 0.001	100.3 ± 0.4	62.8 ± 0.2
n-Octadecane	13.3 ± 0.25	19.9 ± 0.25	0.16 ± 0.002	105.2 ± 1.3	59.9 ± 0.8
Phenanthrene	5.1 ± 0.25	28.3 ± 0.25	0.22 ± 0.002	74.3 ± 0.7	84.8 ± 0.8
Dibenzothiophene	4.3 ± 0.20	29.0 ± 0.20	0.23 ± 0.002	72.4 ± 0.5	87.1 ± 0.6

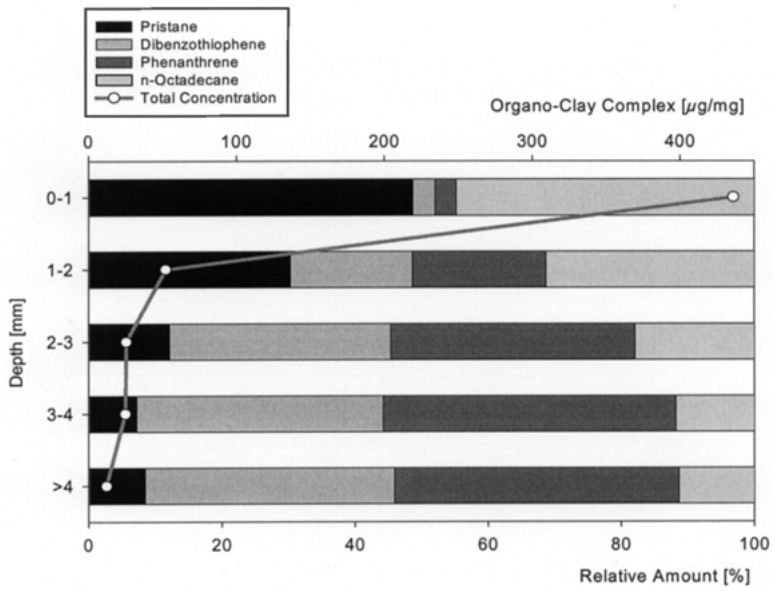

Figure 2. Relative composition of the petroleum model compounds present in different layers of the OCC-contaminated microbial mats at the end of the experiment (normalized to 100%, bottom horizontal axis) and the total concentrations (top horizontal axis).

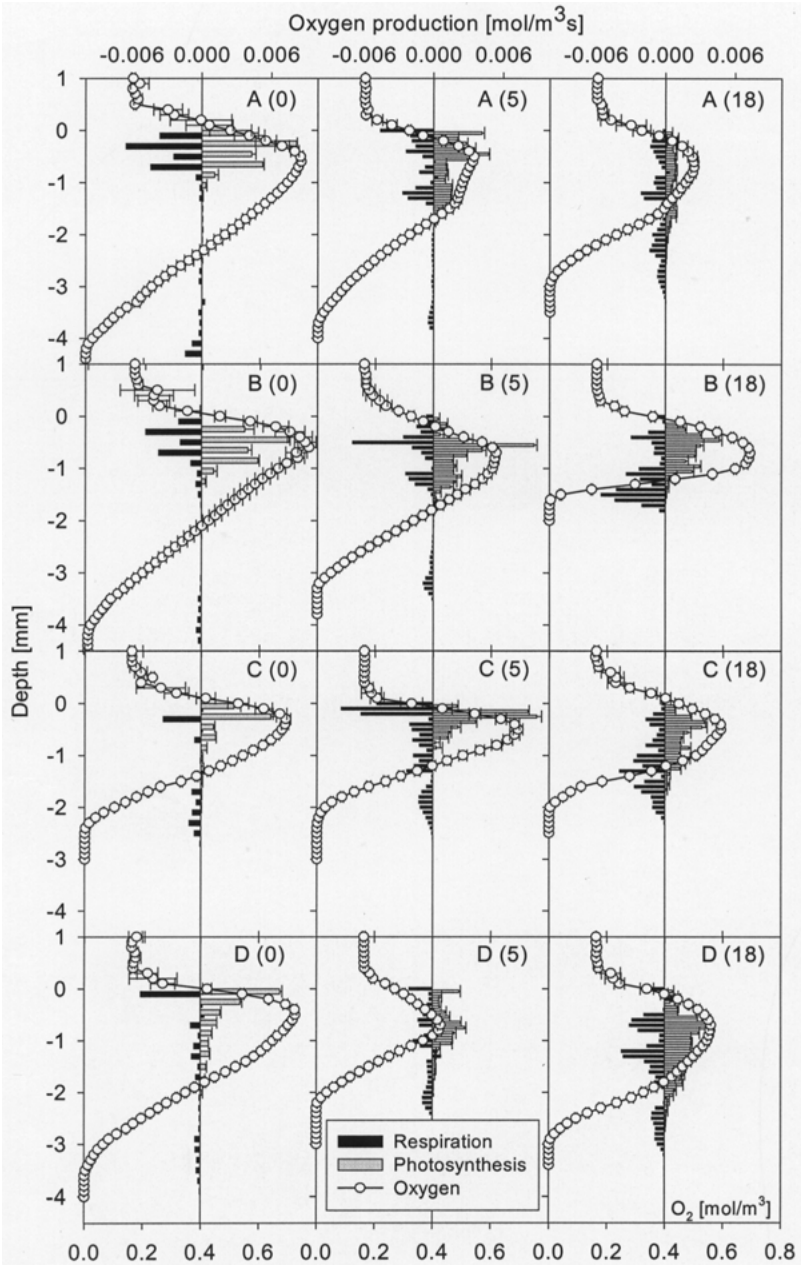

Figure 3. Profiles of oxygen concentrations (means ± standard deviations, n = 3), volumetric gross photosynthetic rates (means ± standard deviations, n = 3) and calculated volumetric respiration rates in microbial mats with different additions in week 0, 5 and 18. A: control; B: hydrophobic clay on the surface of the mats; C: petroleum model compounds added to the water phase; D: OCC on the surface of the mats.

continuously (Figure 4A) or remained largely constant after an initial increase (Figure 4C) or decrease (Figure 4B) in the first week. Sulfide concentration profiles and pH profiles showed equal trends in the different mats. In the beginning the light intensities as measured with fiberoptic microprobes in the OCC and the hydrophobic clay treatments were higher as in the other treatments; at the end of the experiment the light fields in all mats were equal (data not shown).

Molecular analyses

The community response of both cyanobacteria and bacteria to the incubation with the four model com-

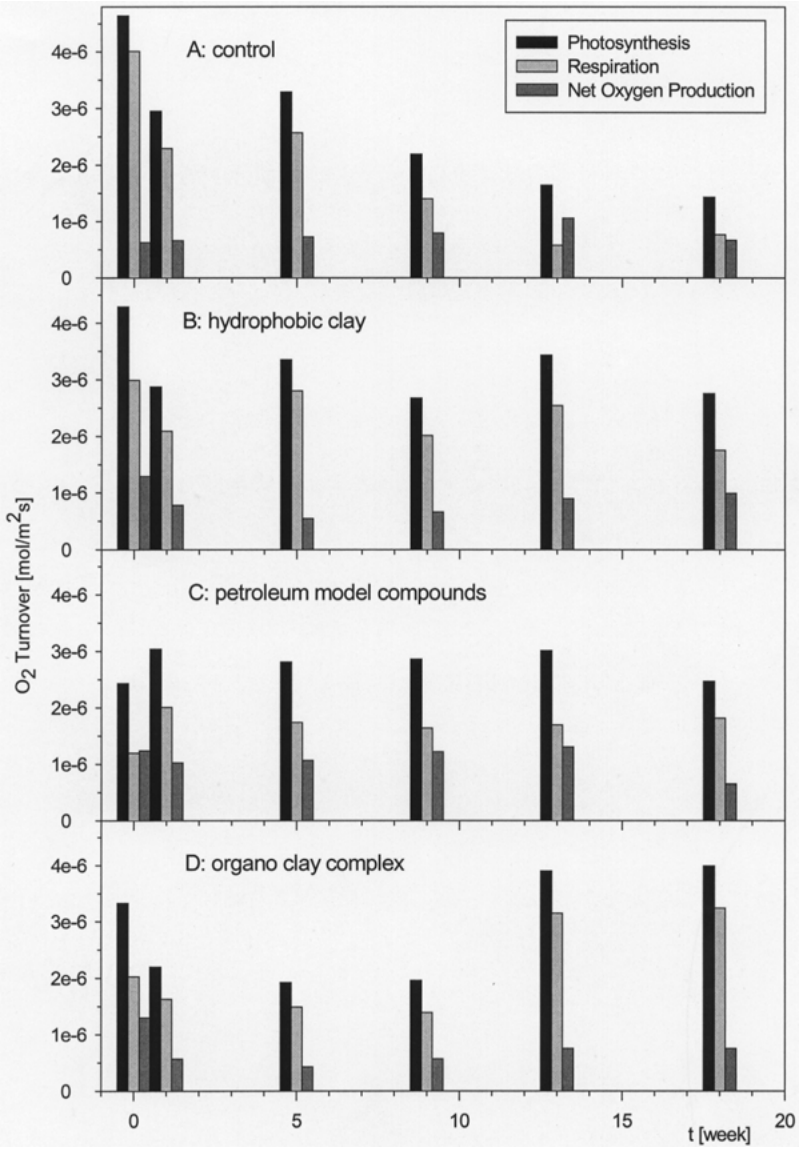

Figure 4. Areal photosynthetic rates, areal respiration rates and areal net oxygen production rates in microbial mats during 18 weeks calculated from oxygen and photosynthesis profiles. A: control; B: hydrophobic clay on the surface of the mats; C: petroleum model compounds added to the water phase; D: OCC on the surface of the mats.

pounds is shown in Figure 5. In case of the cyanobacteria, the community composition at the beginning of the experiment was identical in all mat pieces, as assessed by DGGE patterns. A detailed description of the cyanobacteria inhabiting these mats is given elsewhere (Abed & Garcia-Pichel 2001). The community was dominated by two cyanobacterial populations; *Microcoleus chthonoplastes* and a small unicellular cyanobacterium (*Picobenthos*), as revealed by subsequent sequencing and phylogenetic analyses of the two bands displayed on the gel. At the end of the experiment, the same dominant community members persisted with the development of one extra population represented by band D. Phylogenetic analyses of this band revealed that its 16S rRNA sequence belonged to a diatom plastid. In the treatment in which the model compounds were applied directly to the water phase without a carrier, the development of an extra population represented by band O1 was observed. As the chemical analyses showed that the model compounds

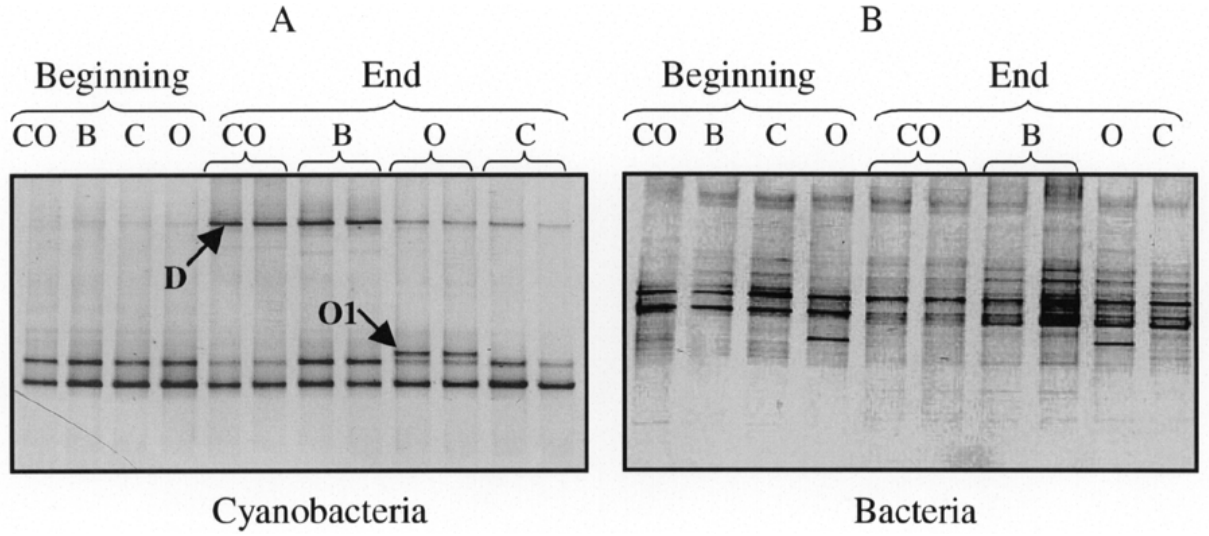

Figure 5. DGGE banding patterns of PCR-amplified 16S rRNA fragments obtained from microbial mats with different additions at the beginning and at the end of the experiment. The amplification was carried out using A) cyanobacterial specific primers (CYA359F and CYA781R) and B) bacterial primers (GM5F and 907R). Additions: "CO": organo-clay complex on the surface of the mat; "B": control; "C": hydrophobic clay on the surface of the mat; "O": petroleum model compounds added to the water phase.

did not dissolve in the water phase and did not reach the mats, this change in the community is unlikely to be attributed to degradation activities.

Using the general bacterial primers, the banding pattern was more complex compared to that with cyanobacterial primers. This is not surprising as this primer set amplifies 16S rRNA of all bacteria. The banding pattern, at the beginning of the experiment, suggested a slight difference in the community composition in case of the mats subjected directly to the petroleum model compounds. At the end of the experiment, as in the case of cyanobacteria, the bacterial community did not show any significant response attributable to the incubation with the model compounds. The total number of bands at the end of the experiment was relatively higher than their number at the beginning of the experiment. The mats exposed to the organo-clay complexes exhibited a lower number of bands in comparison to other mats.

The decreasing concentrations of the petroleum model compounds (Figure 1) as well as the changes in their relative amounts indicated a slow biodegradation in the course of the experiment (Figure 2, Table 1). Other major losses which might explain a depletion to the observed degree are unlikely. Major photooxidation, which might account for the selective disappearance of aromatic compounds, is excluded since the agar control treatment without mat material did not show the compositional changes that were observed in the treatment with OCC. Furthermore, losses due to water solubility are not likely since regular water analyses of the treatments with petroleum model compounds or OCC did not show significant concentrations of the compounds dissolved in the water phase. The freshwater solubilities (at 20 °C) of phenanthrene and dibenzothiophene are 1.3 mg/l and 1.47 mg/l, respectively (Hassett et al. 1980; Pearlman et al. 1984). Own solubility tests using organo-clay complex and water with 7% salinity yielded values < 0.5 mg/l for all four model compounds. Therefore, significant losses from the organo-clay complex, as well as a transport of model compounds to the mats in case of the direct addition to the water phase are excluded.

The biodegradation of the petroleum model compounds in the present study is considered to be slow and incomplete since after 18 weeks a residue of 13–40% was left, depending on the type of the compounds (Table 1). A comparison of the microbial degradation quality in our experiment with literature data is possible only to a limited extent since there are principal differences between slurry or culture approaches and the investigation of intact mats. *Rhodococcus erythropolis* was reported to desulfurize alkylated dibenzothiophenes at a much faster rate of 0.115–0.547 μmol/d $\times$ mg (dry cell weight) (Folsom et al. 1999), *Rhodococcus sp* 5F degraded dibenzothiophene at a rate of 240 μg/d $\times$ mg dry biomass and phenanthrene

at a rate of 288 μg/d $\times$ mg dry biomass (Surovtseva et al. 1997). The phenanthrene half life in river sediments varied from 1.8 days to 9.1 days (Li et al. 2001) or from 0.12 days to 5.78 days (Yuan et al. 2001). Pristane is widely used as a recalcitrant biomarker in geochemistry and its bioremediation is strongly related to the degradation conditions: a pure culture of *Rhodococcus equi* P1 degraded 10 g/l pristane in 24 h (Ko & Lebeault 1999), whilst a 85% degradation of 470 μmol/l pristane in anaerobic sediment slurries took six months (Grossi et al. 2000). Since in our experiment a comparable pristane removal of 63% in four months was determined (Table 1), we conclude, that the degradation rates of the model compounds in our experiment were low, except for pristane. All in all, the aromatic compounds were degraded faster and to a larger extent than the alkanes. In subsequent degradation experiments with organo-clay complexes in batch cultures of different origin it was observed that ~25% n-octadecane and ~30% of pristane were not degraded, whereas the aromatics were removed completely (Abed, unpubl.). This is in agreement with the higher concentrations of alkanes at the end of the experiment. After the 1991 Gulf War oil spill it has been observed, that aromatic compounds in Gulf seawater were degraded at much higher rates than aliphatic compounds (Fayad & Overton 1995). In the present study, the association of the petroleum model compounds to the hydrophobic clay could have influenced the bioavailability of the different substances. It has been shown previously that bacteria have different abilities to degrade hydrophobic xenobiotic compounds associated with solid material (Guerin & Boyd 1997). Differences in the accessibility of sorbed compounds may depend on whether microorganisms are able to degrade these compounds directly from the surface of the carrier material or if they are dependent on the spontaneous desorption of the substances (Calvillo & Alexander 1996; Tang et al. 1998). No accumulation of possible degradation products or metabolites has been observed in our study which suggests, that the degradation of the petroleum model compounds have led to a complete mineralization favored by a complex, intact microbial community.

It was expected to find changes in the physiology of the mat as measured with microsensors since in previous experiments pronounced changes were detected after additions of glucose, glycolate or acetate (Grötzschel et al., unpublished). The apparently accelerated disappearance of aliphatic model compounds between week 9 and 13 (Figure 1) coincided with enhanced photosynthetic rates and respiration rates as measured by microsensors (Figure 4D). After 13 weeks, no further degradation was observed. The concentrations of the petroleum model compounds as well as the photosynthetic and respiratory activity remained approximately constant. This agreement in the chemical and the microsensor data seem to indicate an apparent connection between higher activity rates in the mats and increased alkane degradation. The total amount of C added to the 10 $\times$ 10 cm mats in the form of the four petroleum model compounds was 1.9 mmol. Calculating with a net oxygen production rate of 5×10^{-7} mol/m^{2}s (Figure 4D, first week), a total stoichiometric oxidation of the added C to CO_2 would have taken 4.4 days, if all the oxygen produced by the mats would have been used for that purpose. For the total oxidation of the added C during the treatment only 3.5% of the net oxygen production would have been needed. Since even after 18 weeks a considerable amount of the petroleum model compounds was detected, we conclude that the model compounds did not significantly affect the internal carbon cycle in the mats and that their degradation was rather a side effect of the usual high metabolic activity. The shifts in metabolic activity in the OCC-treated mats as revealed by the microsensor measurements did not reflect the degradation activity since also in the OCC-untreated mats major shifts in the metabolic activity could be measured (Figures 3 and 4).

The bacterial and cyanobacterial community structure of the mat, revealed by DGGE, did not exhibit any new development of specific populations in the OCC-treated samples (Figure 5) in response to the degradation of the petroleum model compounds. This indicates that the addition of petroleum compounds to these mats did not favor the growth of certain microorganisms over others. This is supported by only small changes in the lipid composition of the mats. At the end of the experiment, the control mat and the mat exposed to the organo-clay complex showed very similar profiles of their major lipid components (data not shown). The only community changes detected before and after the experiment could be attributed to experimental settings. Such changes have been observed earlier when mats were maintained under laboratory conditions (Abed and Garcia-Pichel 2001). In the presence of organo-clay complexes, we observed a decrease in the intensity (Figure 5, cyanobacteria, panel A) or in the number (Figure 5, bacteria, panel B) of some bands. This may indicate that the petroleum compounds have a toxic effect on the growth of certain

microorganisms. Many studies have demonstrated that crude oil contains constituents that are inhibitory to cyanobacteria even at low concentrations (Radwan & Al-Hasan 2000 and references therein).

The chemical analyses of the mats at different depths showed that the major portion of the petroleum model compounds remained in the surface layer of the mat (Figure 2). In the different treatments the same light fields were measured at the end of the experiment, which indicates, that the top layer consisting of OCC or hydrophobic clay was penetrated by cyanobacteria and diatoms, which was observed also under the microscope. The depletion of aromatic compounds in the top layer corresponds to the observation made in the time series (Figure 1). However, a minor transport of model compounds into the mats to at least 6 mm was found. The change in the relative composition implies that this transport occurs after desorption but not by mixing of organo-clay complex with the microbial mat. Whether composition changes are due to different desorption, diffusion or transport rates of aromatic vs. aliphatic compounds or the preferential degradation of the alkanes may play a role needs further investigation.

Although the mats showed the highest activity in the photic zone (Figure 3), a degradation in the anaerobic parts can not be ruled out. Anaerobic degradation of aliphatic and aromatic compounds is a well studied process (Cerniglia 1993; Cunningham et al. 2000; Zwolinski et al. 2000). However, in this experiment with intact microbial mats it can be expected that degradation rates are limited by the much lower availability of the contaminants in comparison to the surface of the mat (Figure 2). It has been observed before that the available surface area of the pollutants is a major limiting factor for bioremediation (Fayad & Overton 1995). The carrier material used in this study (hydrophobic clay) which provided access of the microorganisms inhabiting the mats to the petroleum compounds in the first place may have prevented a faster degradation. The small contact area between the OCC and the mats and low desorption rates could be responsible for the slow removal of the petroleum model compounds. In a study similar to the presented one, 20 mg of the herbicide 2,4-dichlorophenoxyacetic acid (2,4-D) dissolved in 1l of artificial salt water were degraded in 13 days by a pristine hypersaline microbial (S. Grötzschel, unpublished) which indicates the important role of the physical contact between pollutant and microorganism.

Conclusions

Hydrophobic petroleum compounds with a limited biodegradation potential due to their low water solubility have been made available for microbial degradation by a hypersaline cyanobacterial mat. The bioavailability of the compounds was successfully increased by making use of an artificially modified clay with a hydrophobic surface as a carrier material. The pristine microbial mats were able to degrade the petroleum components, albeit at low rates. The degradation rates depended on the type of compound, aromatics were degraded faster than aliphatics. The internal carbon cycling in the mats was not affected by the pollutants and only slight indications for toxic effects to some members of the microbial community were found. We conclude that the enzymatic requirements for the degradation of organic pollutants exist in the investigated pristine microbial mats but on top of the usual high turnover rates of the intact mats. The degradation activity did not appear to favor a certain population in the community or affect the physiology of the mat.

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References

Abed RMM & Garcia-Pichel F (2001) Long-term compositional changes after transplant in a microbial mat cyanobacterial community revealed using a polyphasic approach. Environ. Microb. 3(1): 53–62

Bligh EG & Dyer WJ (1959) A rapid method of total lipid extraction and purification. Can. J. Biochem. Physiol. 37: 911–917

Calvillo YM & Alexander M (1996) Mechanism of microbial utilization of biphenyl sorbed to polyacrylic beads. Appl. Microb. Biotechn. 45(3): 383–390

Cerniglia, CE (1993) Biodegradation of polycyclic aromatic hydrocarbons. In: Rosenberg E (Ed) Microorganism to Combat Pollution (pp 227–244). Kluwer Academic Press, Dordrecht

Cunningham JA, Hopkins GD, Lebron CA & Reinhard M (2000) Enhanced anaerobic bioremediation of groundwater contaminated by fuel hydrocarbons at Seal Beach, California. Biodegradation 11(2–3): 159–170

El-Nahhal Y (1998) Development of stabilized and ecologically acceptable herbicide formulations for the use in hot and sunny regions. Ph.D. Thesis, The Hebrew University of Jerusalem, Jerusalem, Israel

Fayad NM & Overton E (1995) A unique biodegradation pattern of the oil spilled during the 1991 Gulf-War. Mar. Pollut. Bull. 30(4): 239–246

Folsom BR, Schieche DR, DiGrazia PM, Werner J & Palmer S (1999) Microbial desulfurization of alkylated dibenzothiophenes from a hydrodesulfurized middle distillate by *Rhodococcus erythropolis* I-19. Appl. Environ. Microb. 65(11): 4967–4972

Glud RN, Ramsing NB & Revsbech NP (1992) Photosynthesis and photosynthesis-coupled respiration in natural biofilms quantified with oxygen microsensors. J. Phycol. 28: 51–60

Grötzschel S, Abed, RMM & de Beer D (accepted) Metabolic shifts in hypersaline microbial mats upon addition of organic substrates. Environ. Microb.

Grötzschel S & de Beer D (in print) Effect of oxygen concentration on photosynthesis and respiration in two hypersaline microbial mats. Microbial Ecol.

Grossi V, Raphel D, Hirschler-Rea A, Gilewicz M, Mouzdahir A, Bertrand JC & Rontani JF (2000) Anaerobic biodegradation of pristane by a marine sedimentary bacterial and/or archaeal community. Organic Geochemistry 31(7–8): 769–772

Guerin WF & Boyd SA (1997) Bioavailability of naphthalene associated with natural and synthetic sorbents. Wat. Res. 31(6): 1504–1512

Hassett JJ, Means JC & Banwart WL (1980) Sorption of benzidine by sediments and soils. J. Environ. Qual. 9(2): 184–186

Hoffmann L (1996) Recolonisation of the intertidal flats by microbial mats after the Gulf war oil spill. In: Krupp F, Abuzinada AH, Nader I (Eds) A Marine Wildlife Sanctuary for the Arabian Gulf (pp 96–114). NCWCD, Riyadh and Senckenberg Research Institute, Frankfurt a. M.

Höpner T, Yousef M, Berthe-Corti L, Felzmann H, Struck H & Al-Thukair A (1996) Cyanobacterial mats on oil-polluted sediments- start of a promising self-remediation process? In: Krupp F, Abuzinada AH, Nader I (Eds) A Marine Wildlife Sanctuary for the Arabian Gulf (pp 85–95). NCWCD, Riyadh and Senckenberg Research Institute, Frankfurt a. M.

Ko SH & Lebeault JM (1999) Effect of a mixed culture on co-oxidation during the degradation of saturated hydrocarbon mixture. J. Appl. Microb. 87(1): 72–79

Kühl M, Lassen C & Jørgensen BB (1994) Optical properties of microbial mats: light measurements with fiber-optic microprobes. In: Stal LJ, Caumette P (Eds) Microbial Mats: Structure, Development and Environmental Significance (pp 149–167). Springer, Berlin

Kühl M, Steuckart C, Eickert G & Jeroschewski P (1998) A H_2S microsensor for profiling biofilms and sediments: application in an acid lake sediment. Aquatic Microbial Ecology 15: 201–209

Li K, Christensen ER, Van Camp RP & Imamoglu I (2001) PAHs in dated sediments of Ashtabula River, Ohio, USA. Environ. Sci. Technol. 35(14): 2896–2902

Muyzer G, de Waal EC & Uitterlinden AG (1993) Profiling of complex microbial populations by denaturant gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes encoding for 16S rRNA. Appl. Environ. Microb. 59: 695–700

NRC (1985) Oil in the sea. National Academy Press, National Research Council, Washington, D.C.

Nübel U, Garcia-Pichel F & Muyzer G (1997) PCR primers to amplify 16SrRNA genes from cyanobacteria. Appl. Environ. Microb. 63: 3327–3332

Oren A, Gurevich P, Azachi M & Henis Y (1992) Microbial degradation of pollutants at high salt concentrations. Biodegradation 3: 387–398

Pearlman RS, Yalkowsky SH & Banerjee S (1984) Water solubility of polynuclear aromatic and heteroaromatic compounds. J. Phys. Chem. Ref. Data 13: 555–562

Pritchard PH & Costa CF (1991) EPAS Alaska oil-spill bioremediation project. Environ. Sci. Technol. 25(3): 372–383

Radwan SS & Al-Hasan RH (2000) Oil pollution and cyanobacteria. In: Whitton BA, Potts M (Eds) The Ecology of Cyanobacteria (pp 307–319). Kluwer Academic Publishers

Revsbech NP (1989) An oxygen microsensor with a guard cathode. Limnol. Oceanogr. 34(2): 474–478

Revsbech NP & Jørgensen BB (1983) Photosynthesis of benthic microflora measured with high spatial resolution by the oxygen microprofile method: capabilities and limitations to the method. Limnol. Oceanogr. 28(4): 749–756

Revsbech NP & Jørgensen BB (1986) Microelectrodes: Their use in microbial ecology. In: Marshall KC (Ed) Advances in Microbial Ecology (pp 293–352). Plenum Publishing Corporation

Revsbech NP, Jørgensen BB, Blackburn TH & Cohen Y (1983) Microelectrode studies of the photosynthesis and O_2, H_2S, and pH profiles of a microbial mat. Limnol. Oceanogr. 28(6): 1062–1074

Rosenberg E, Legmann R, Kushmaro A, Taube R, Adler E & Ron EZ (1992) Petroleum bioremediation – a multiphase problem. Biodegradation 3: 337–350

Santegoeds CM, Ferdelman TG, Muyzer G & de Beer D (1998) Structural and functional dynamics of sulfate-reducing populations in bacterial biofilms. Appl. Environ. Microb. 64: 3731–3739

Surovtseva EG, Ivoilov VS & Belyaev SS (1997) Degradation of the aromatic fraction of oil by an association of gram-positive and gram-negative bacteria. Microbiology 66(1): 65–69

Tang WC, White JC & Alexander M (1998) Utilization of sorbed compounds by microorganisms specifically isolated for that purpose. Appl. Microb. Biotechn. 49(1): 117–121

US Coast Guard (1990) Update of inputs of petroleum hydrocarbons into the oceans due to marine transportation activities. National Academy Press, Washington, D.C.

Ward DM & Brook TD (1978) Hydrocarbon biodegradation in hypersaline environments. Appl. Environ. Microb. 35: 353–359

Yuan SY, Chang JS, Yen JH & Chang BV (2001) Biodegradation of phenanthrene in river sediment. Chemosphere 43(3): 273–278

Zwolinski MD, Harris RF & Hickey WJ (2000) Microbial consortia involved in the anaerobic degradation of hydrocarbons. Biodegradation 11(2-3): 141–158