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Variations in Community Composition of Cyanobacterial Mats in Response to Environmental Disturbance with Emphasis on the Biodegradation of Petroleum Derivatives

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Front page:
A cartoonist's view of laboratory-grown microbes returning to their original environment. Source. Tibtech 1993 11:344-352.

For my parents
&
For Manal, Amal and Feras

This thesis would not have been possible without the help of many people, which I would like to thank all. I am indebted to Ferran Garcia-Pichel for his continuous support and encouragement during these years. Ferran, who supervised this work, was always available for advice and motivating discussions.

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Summary

The main objective of this work was to study the microbial diversity, particularly cyanobacteria inhabiting the upper layer of microbial mats and their community changes in response to incubation with petroleum compounds. For this purpose, pristine microbial mats originating form Solar Lake, Egypt but transferred to an experimental pond were used. In the first study (chapter 2), the cyanobacterial community changes induced by transplanting the mat from its original site to artificial settings; either in the laboratory or the experimental pond were investigated. The study aimed at distinguishing between community changes caused by modifications of environmental parameters and those in response to incubation with petroleum compounds. It also provided us with the basic knowledge of cyanobacterial diversity within the system so that community changes in later experiments could be easily detected. Molecular analysis showed significant community changes following transplantation of the mats (chapter 2). The phylogenetic analysis of 16S rRNA cyanobacterial sequences suggested the appearance of two unidentified novel populations in the transplanted mats, which were subsequently characterized. The first population was isolated in culture and found to have extremely thin filamentous morphology (0.75-1 µm width). The taxonomy of such cyanobacteria is not clear in both the botanical as well as the bacteriological systems. Therefore, a new genus *Halomicronema* gen. nov., to describe benthic, moderately halophilic, moderately themophilic, cyanobacteria with very narrow trichomes was proposed (chapter 3). The second population could neither be identified by direct microscopy nor could be isolated in culture in spite of repeated efforts. Therefore, *in situ* hybridization was carried out in order to reveal the corresponding morphology of this population. It was demonstrated that this population consisted of very small (ca. 1µm) colony-forming unicellular cyanobacteria, which was termed "picobenthos" (chapter 4). After identifying all major cyanobacteria, mat pieces were incubated with four model compounds in order to find out their degradation capability and changes in bacterial and cyanobacterial communities were monitored (chapter 5). This study showed that these pristine mats degraded the petroleum compounds only at a very slow rate. The microbial communities of those mats were very robust and did not show any detectable changes in response to the added compounds. We reasoned that microbial mats already growing in the presence of petroleum pollution could serve as better models for degradation studies. Therefore, microbial mats growing in the heavily polluted site of Wadi Gaza, Palestine were investigated for their degradation potential to the same petroleum compounds (chapter 6). These mats degraded the compounds within five days with detectable community changes in bacteria as well as cyanobacteria. This system could be further optimized for use in bioremediation purposes.

Zusammenfassung

Gegenstand der vorliegenden Arbeit war die Untersuchung der mikrobiellen Diversität, insbesondere der der Cyanobakterien, in der oberen Schicht mikrobieller Matten und Änderungen in der Zusammensetzung der Gemeinschaft nach Inkubation mit verschiedenen Erdölbestandteilen. Zu diesem Zweck wurden unbelastete mikrobielle Matten aus dem Solar Lake (Ägypten) verwendet, die zuvor in experimentelle Teiche transferiert worden waren. In der ersten Studie (Kapitel 2) wurde untersucht, welche Änderungen in der Gemeinschaft der Cyanobakterien durch die Verbringung vom ursprünglichen Standort in künstliche Umgebungen –in Laborsysteme oder in experimentelle Teiche– induziert werden. Die Studie zielte darauf ab, durch Modifikation von Umweltparametern hervorgerufene Veränderungen in der mikrobiellen Gemeinschaft abzugrenzen von solchen, die durch die Inkubation mit Erdölbestandteilen verursacht wurden. Zugleich erhielten wir daraus grundlegende Erkenntnisse zur cyanobakteriellen Diversität in dem System, auf deren Basis die Veränderungen der mikrobiellen Gemeinschaft in späteren Experimenten leicht erkannt werden konnten. Die molekulare Analyse zeigte, daß die Verpflanzung der Matten signifikante Veränderungen in der mikrobiellen Gemeinschaft nach sich zog (Kapitel 2). Die phylogenetische Analyse der 16S rRNS-Sequenzen von Cyanobakterien ergab Hinweise auf das Auftreten zweier neuer nicht identifizierter Populationen in den verpflanzten Matten, die nachfolgend charakterisiert wurden. Die erste Population konnte isoliert und in Kultur gebracht werden. Diese Organismen zeichneten sich durch eine extrem dünne filamentöse Morphlogie aus (0,75-1 µm Durchmesser). Die Taxonomie solcher Cyanobakterien-Morphotypen is sowohl in der botanischen als auch in der bakteriologischen Systematik nicht eindeutig. Daher wurde für diesen Typus von benthischen, moderat halo- und thermophilen sowie in feinen Trichomen auftretenden Cyanobakterien eine neue Gattung, *Halomicronema* gen. nov., vorgeschlagen (Kapitel 3). Die zweite Population konnte weder durch direkte Mikroskopie identifiziert noch durch wiederholte Versuche isoliert und kultiviert werden. Aus diesem Grund wurde die Methode der In situ-Hybridisierung verwendet, um der (bis dahin nur molekular charakterisierten) Population einen Morphotyp zuordnen zu können. Es gelang zu zeigen, daß diese Population aus sehr kleinen (ca. 1 µm), koloniebildenden, unizellulären Cyanobakterien bestand, die in der Folge als "Picobenthos" bezeichnet wurden (Kapitel 4). Nach der Identifikation aller wesentlichen Cyanobakterien-Populationen wurden

Stücke der Matte mit 4 Modellsubstanzen inkubiert, um das Abbauvermögen und einhergehende Veränderungen in der bakteriellen und cyanobakteriellen Gemeinschaft zu verfolgen (Kapitel 5). In dieser Studie konnte nachgewiesen werden, daß die zuvor unbelasteten Matten die zugesetzten Substanzen nur sehr langsam umsetzen konnten. Die mikrobiellen Gemeinschaften in diesen Matten erwiesen sich als sehr stabil und zeigten keine detektierbaren Veränderungen als Folge des Zusatzes der Modellsubstanzen. Diese Ergebnisse führten zu der Überlegung, daß mikrobielle Matten, die in bereits mit Erdöl belasteten Bereichen gewachsen sind, als Modelle für Abbauuntersuchungen besser geeignet sein könnten. Deshalb wurden Matten aus dem hochbelasteten Wadi Gaza (Palästina) auf ihr Abbaupotential in bezug auf dieselben Modellsubstanzen hin unersucht (Kapitel 6). Dabei zeigte sich, daß der Abbau in diesen Matten innerhalb von fünf Tagen erfolgte und mit erkennbaren Veränderungen in der Gemeinschaft der Bakterien wie auch der Cyanobakterien einherging. Dieses System könnte nach Optimiertung ein Potential für den Einsatz in zukünftigen biologischen Sanierungsvorhaben besitzen.

بسم الله الرحمن الرحيم

خلاصة

إن الهدف الرئيسي لهذا العمل هو دراسة التنوع البكتيري وخصوصا البكتيريا الخضراء المزرقة في الطبقة العليا من أغطية الأحياء المجهرية ، ودراسة التغيرات التي تطرأ على التركيبة البكتيرية المكونة لها عند تعريضها للمركبات البترولية .

لذلك تم دراسة أغطية الأحياء المجهرية التي نشأت أصلا من بحيرة طبيعية في سيناء مصر تعرف ب Solar Lake، وتم زرع أجزاء منها في بحيرة صناعية لهذا الغرض (الفصل الثاني).تم دراسة التغيرات في تركيبه البكتيريا الخضراء المزرقة لهذه الأغطية خلال نقلها من مكانها الأصلي وزراعتها إما في مختبر وفي بحيرة صناعية ، وتهدف هذه الدراسة الى التمييز بين هذه التغيرات التي قد تطرأ نتيجة للعوامل الطبيعية مقارنة بتلك الناجمة عن معالجتها بالبترول ، بالإضافة فأنها تساعد على التعرف على أنواع هذه البكتيريا التي تعيش الآن في هذه الأغطية بحيث يمكن الكشف عن أي تغير في هذه الأنواع في التجارب اللاحقة .

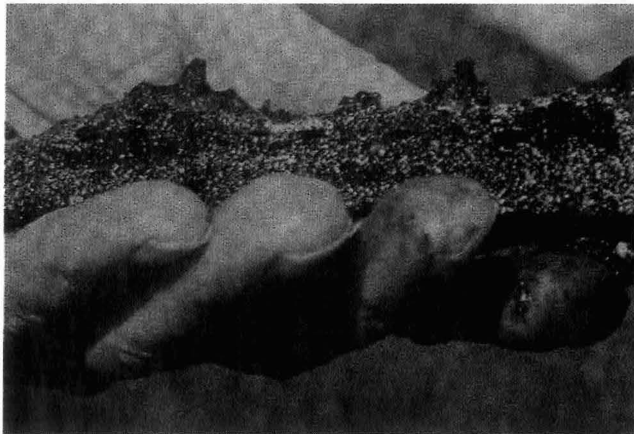
باستخدام تقنيات البيولوجيا الجزيئية تبين أن التركيبة البكتيرية قد تغيرت عند نقلها من منشأها مما أدى الى ظهور نوعين فريدين من أنواع هذه البكتيريا لم تكتشف من قبل . تم عزل النوع الأول في مزرعة وتبين أنها بكتيريا ذات خيوط دقيقة جدا(0.75 - 1 ميكرمتر) ، وحيث أن تصنيف هذه الأنواع في نظامي التصنيف النباتي والبكتيري غير واضح وقد تم اقتراح اسم Halomicronema لوصف البكتيريا من هذا النوع ذات خيوط دقيقة والتي تعيش في القاع، ولها القدرة على تحمل درجات الحرارة والملوحة المرتفعة (الفصل الثالث). أما النوع الثاني فلم يكن من الممكن التعرف عليه باستخدام الميكروسكوب أو حتى عزله في مزرعة ، ولكن باستخدام تقنية *In Situ* Hybridization ، فقد تم الكشف عن هذه البكتيريا الخضراء المزرقة وتبين أنها تتكون من خلايا وحيدة يصل قطرها الى (1 ميكرومتر)، وتكون مستعمرات وقد أطلق عليها اسم (Picobenthos) (الفصل الرابع). بعد التعرف على جميع أنواع البكتيريا الخضراء المزرقة في هذه الأغطية تم تعريضها الى أربع مركبات بترولية للتعرف على قدرتها لتحليل هذه المركبات وأي تغيرات في تركيبتها البكتيرية نتيجة ذلك .

هذه الدراسة بينت أن هذه الأغطية التي لم تتعرض من قبل الى التلوث بمثل هذه المركبات لها قدرة بطيئة على تحليل المركبات البترولية من دون تغيرات بتركيبتها البكتيرية ، لذلك قد تم استخدام أغطية أحياء مجهرية تنمو في منطقة ملوثة جدا في وادي غزة – قطاع غزة – فلسطين لدراسة قدرتها على تحليل نفس المركبات ، هذه الأغطية حللت المركبات خلال خمسة أيام مع تغيرات واضحة في تركيبتها البكتيرية لصالح تلك التي تتغذى على هذه المركبات . لذلك فان هذه الأغطية يمكن استخدامها مستقبلا للتخلص من التلوث بهذه المركبات البترولية بيولوجيا .

Chapter 1

INTRODUCTION

Cyanobacterial mats and oil pollution



Front page:
General view of cyanobacterial mats on top of oil pollution in the intertidal zone of the Arabian Gulf. (Photos: S. S. Radwan & R. H. Al-Hasan, plate 18). The Ecology of Cyanobacteria book by Whitton and Potts 2000.

A. INTRODUCTION

1. Microbial mats

1.1 General description

Microbial mats are accretionary, cohesive, macroscopic accumulations of microbial communities, which are often laminated and grow at the sediment-water (occasionally sediment-air) interface (Pierson 1992). They develop in time as a result of microbial growth, sediment trapping and binding in the organic matrix, and sedimentation (Margulis et al. 1980). These benthic, organo-sedimentary structures stabilize unconsolidated sediment and develop in stationary or flowing systems, intertidal areas and covering rocks or within them as endolithic communities. Their extension ranges from a few square centimeters (as in small thermal springs) to several square kilometers (hypersaline mats, intertidal flats). They vary in thickness from massive accumulations measured in meters, such as those in the Persian Gulf and Red Sea region, to thin biofilms less than a few millimeters. Different mat systems have distinct morphologies, community compositions and biogeochemistry. Microbial mats are considered to be one of the oldest and one of the smallest ecosystems on earth, sharing very close morphological similarities to their alleged fossil Precambrian analog, the stromatolites, which date back to 3.5 billion years (Walter et al. 1992, Van Gemerden 1993). They are distributed world wide in a surprisingly wide range of environments including hypersaline ponds and lakes, hot springs, alkaline lakes, marine intertidal flats, fresh water lakes, hot and dry deserts and ice-covered Antarctic lakes. (Pierson 1992, Stahl 1995, D'Amelio et al. 1989). Most mats develop under conditions that have limited abundance and activity of grazing organisms (Cohen 1989, Farmer 1992, Javor and Castenholz 1984).

Cyanobacterial mats are composed of physiologically different groups of microorganisms such as cyanobacteria and diatoms as oxygenic phototrophs, chemolithotrophic colorless sulfur bacteria, anaerobic phototrophic purple sulfur bacteria, anaerobic sulfate-reducing bacteria as well as aerobic heterotrophs. Other numerically less important groups in mats are nitrifying and denitrifying bacteria and methanogenic bacteria. Due to the spatial separation (lamination) of diverse metabolism of mat-inhabiting groups of microorganisms, steep physico-chemical gradients of light, oxygen and sulfide are developed (Revsbech et al. 1983, DesMarais 1995, Stahl 1995)

(Fig. 1). Cyanobacteria dominate the upper few millimeters of most mats, where high primary production occurs, providing physical support to the sediment. Their photosynthetic activity drives the rest of the microbial community. The organic matter produced by excretion, lysis and decomposition of cyanobacteria is respired by aerobic heterotrophs leading to oxygen depletion and regeneration of CO_2, which is used by autotrophic organisms. The same organic compounds as well as low molecular weight compounds produced by fermentative bacteria can also be used by sulfate-reducing bacteria under anaerobic conditions producing sulfide. The sulfide is inhibitory to cyanobacteria but can be re-oxidized to sulfate by both colorless and purple sulfur bacteria. During the daytime the photosynthetically active surface layer of the mat is supersaturated with oxygen while CO_2 concentration is low (Fig. 1) (Canfield and DesMarais 1994, Wieland and Kühl 2000). In contrast, during the night, anoxic conditions tend to prevail in mats while, if sulfate is available, sulfide accumulates.

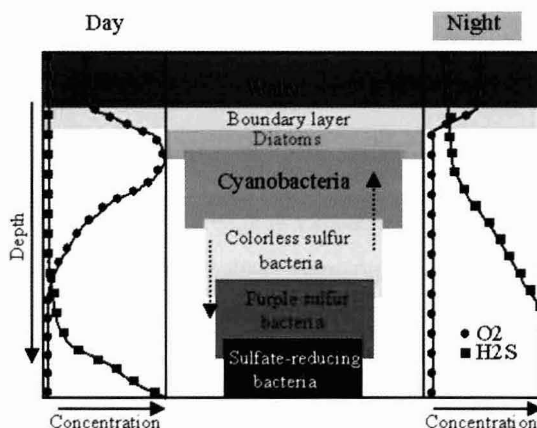


Fig 1. Simplified scheme of a laminated microbial mat showing the diel fluctuations of oxygen and sulfide concentrations in relation to the vertical distribution of functional groups. (After Van Gemerden 1993, modified)

Fermentation of intracellular storage compounds, like glycogen, by cyanobacteria can result in the excretion of acetate, ethanol, propionate and lactate (Nold and Ward 1996, Stahl 1995). Diurnal variations of light intensity bring about the formation of steep, opposing gradients of oxygen and sulfide, which move up and down in the microbial mat. Hence, the mats naturally contain physiologically flexible and tolerant microorganisms to these fluctuating conditions. For example, some cyanobacterial species carry out sulfide-dependent anoxygenic photosynthesis while others have higher rates of oxygenic photosynthesis in the presence of sulfide, a substrate that is toxic to

most cyanobacteria (Cohen et al. 1986, Stahl 1995). On the other hand, some sulfate-reducing bacteria, traditionally regarded to be strictly anaerobic organisms, may occur in the upper oxic layer of the mats (Teske et al. 1998).

1.2 Cyanobacteria: morphology, physiology, phylogeny and taxonomy

Cyanobacteria constitute a group of prokaryotic microorganisms able to carry out oxygenic photosynthesis (Garcia-Pichel 2000). They are the dominant primary producers in many environments including microbial mats (Van Gemerden 1993, Stahl 1995) and they are believed to contribute largely to the global primary production in oceans (Schmidt et al. 1991). All cyanobacteria are able to synthesize chlorophyll a as photosynthetic pigment while most of them synthesize phycobiliproteins as light-harvesting pigments. They exist in different morphologies including unicellular and filamentous forms (Castenholz and Waterbury 1989). Unicellular cyanobacteria may exist as single cells, suspended or benthic, or aggregates. Filamentous morphotypes may be thin or thick, single trichome or bundles either with or without a sheath. Some cyanobacteria form heterocysts, specialized cells unable to perform oxygenic photosynthesis, which are the sites of nitrogen fixation. Cyanobacteria have different modes of metabolism with the capacity to switch from one mode to another (Stahl 1995). Several cyanobacterial species can switch to the typical bacterial anoxygenic photosynthesis using sulfide as electron donor while others are able to carry out fermentation under anoxic conditions. Phylogenetic analysis of cyanobacteria based on 16S rRNA genes showed that they are a diverse, monophyletic phylum of organisms within the bacterial radiation. A phylogenetic tree of cyanobacteria is presented in (Fig.2)

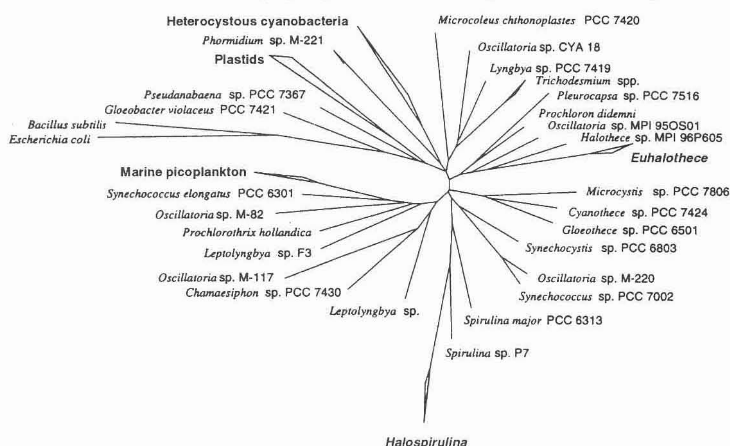


Fig. 2. Phylogenetic tree of the cyanobacteria and plastids. The 16S rRNA of *E. coli* and *B. subtilis* were used as outgroups (from Nübel 1999)

There are two different taxonomic systems for the classification of cyanobacteria; the botanical and the bacteriological systems. The botanical system is based mainly on overwhelmingly morphological descriptions whereas the bacteriological system integrates some genetic and physiological traits. The Geitlerian system has been the most widely accepted botanical system (Geitler 1932). According to the most recent bacteriological treatment, cyanobacteria have been classified under five different subsections: Chroococcales, Pleurocapsales, Oscillatoriales, Nostocales, and Stigonemales (Castenholz and Waterbury 1989) (Table 1).

Table 1. Subsections of the cyanobacteria according to the Bergey's Manual of Determinative Bacteriology (from Garcia-Pichel 2000).

Subsection	*Definatory criteria*
Subsection I (order Chroococcales)	Unicellular and nonfilamentous; cells occur singly or in aggregates; cell division by binary fission in one to three planes, symmetric or assymetric, or by budding
Subsection II (order Pleurocapsales)	Unicellular and nonfilamentous; cells occur singly or in aggregates; reproduction by multiple fission without growth yielding baeocytes (cells smaller than the parent cells) or by binary and multiple fission
Subsection III (order Oscillatoriales)	Filamentous; binary fission in one plane yielding uniseriate trichomes without true branching; no heterocysts or akinetes formed
Subsection IV (order Nostocales)	Filamentous; division occuring only in one plane to yield uniseriate trichomes without true branching; heterocysts formed when combined nitrogen is low
Subsection V (order Stigonematales)	Filamentous; division periodically or commonly in more than one plane yielding multiseriate trichomes, truly branched trichomes, or both

2. Chemistry of crude oil and problems of oil spills

2.1 Composition of crude oil

Crude oil consists predominantly of carbon and hydrogen elements in the ratio of about 1.85 hydrogen atoms to one carbon atom (Hunt 1979). Other elements such as oxygen, sulfur and nitrogen are present in minor proportions (less than 3%). Traces of phosphorous and heavy metals such as vanadium and nickel are also usually present (Radwan et al. 1999, Hunt 1979). Petroleum compounds range in molecular size from very small molecules such as methane to large ones like asphaltenes. As the molecular size increases, the individual members go from gases to liquids to solids. Crude oils, irrespective of their origin, can be split into a number of fundamental chemical classes on the basis of their structures; saturated hydrocarbons, aromatic hydrocarbons, asphaltenes and resins (Fig. 3). In the following a brief description of the different classes is given. More details can be found in Colwell and Walker 1977, Hunt 1979, and Tissot and Welte 1984.

1) Saturated hydrocarbons: This group of hydrocarbons is usually the most important constituent. It includes normal and branched alkanes (paraffins) and cycloalkanes (naphthenes). They have the general formula C_nH_{2n+2} where n is any number from 1 to about 60. These compounds are gases when n equals 1 to 4 and liquids when n is between 5 to 16 whereas above 16 they are viscous to solid waxes. Straight-chain alkanes are more dominant over branched ones. Cycloalkanes have the general formula C_nH_{2n} and their cyclic structure is formed by joining the carbon atoms in a ring. Cycloalkane rings generally contain 5 or 6 carbon atoms and the compounds are liquid at room temperature.

2) Aromatic hydrocarbons: These compounds are usually the second most important group of constituents. They range from benzene to multi-ring polycyclic aromatic hydrocarbons (PAHs). Compounds belonging to this group comprise benzene (one ring), naphthalene (2 rings), phenanthrene and anthracene (three rings), pyrene, benzanthracene, and chrysene (4 rings). Another group of compounds has one five-membered ring in addition to six-membered rings such as fluorene, benzofluorene and fluoranthene and benzofluoranthene. The aromatics are unsaturated hydrocarbons that will react to add hydrogen or other elements to the ring. They rarely amount to more than 15% of total crude oil and tend to be more concentrated in the heavy fraction of petroleum. Some of these compounds exhibit toxic, mutagenic and/or carcinogenic properties.

3) Asphaltenes and resins: This group is composed of high molecular weight compounds consisting primarily of crossed-linked NSO units. Their content is usually high in shallow immature petroleum and decreases with increasing depth. Asphaltenes include phenols, fatty acids, ketones, esters and porphyrins while resins include constituents like pyridines, quinoliness, carbazoles, sulfoxides and amides. Most of the resins are rich in heteroatoms, particularly oxygen. They are not stable especially when exposed to air and sunlight, and they probably evolve further and go on to form asphaltenes. These heterocompounds represent the minor constituents of all crude oils whereas alkanes and aromatics are the major constituents.

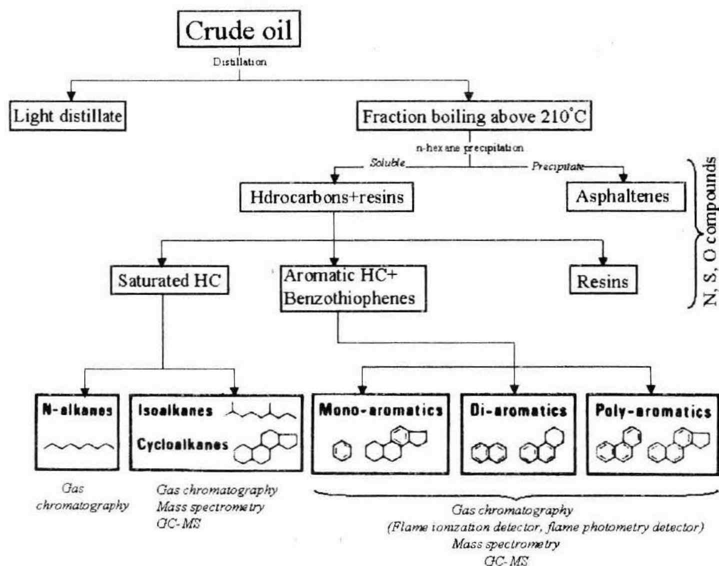


Fig. 3. Flow chart showing crude oil composition, separation of main structural types of molecules, and subsequent quantitative analysis of fractions by gas chromatography and mass spectrometry. This figure was modified from Tissot and Welte 1984)

For our experiments we selected four model compounds; n-octadecane, pristane, phenanthrene and dibenzothiophene. The compounds were selected to represent the petroleum constituents, straight-chain alkane, branched alkane, aromatic hydrocarbon and organo-sulfur compounds, respectively. The molecular structure of these compounds is shown in Fig. 4.

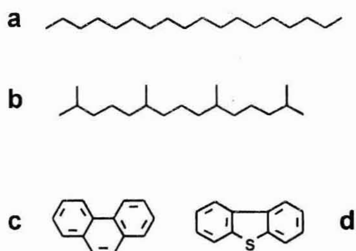


Fig. 4. Carbon skeletons of the petroleum compounds: a) n-octadecane, b) pristane, c) phenanthrene and d) dibenzothiophene

2.2 Oil spills and environmental pollution

Oil spills pose a serious threat to the environment especially when they occur over large areas. Several large accidental oil spills have resulted in a significant contamination of marine and shoreline environments (Swannell et al. 1996). For example, during the notorious 1989 oil spill of the "Exxon Valdez", $4x10^7$ tons were discharged into the Prince William Sound, Alaska, contaminating a substantial length of the coastline. In Kuwait, at the time of the Gulf war in 1991, $82x10^7$ tons of oil were released, threatening the desalination plants and coastal ecosystem of the Gulf (Sorkhoh et al. 1992). In oil spills, volatile hydrocarbon fractions evaporate quickly, leaving longer-chain aliphatic and aromatic components (Madigan et al. 2000). These compounds are destructive to various forms of marine life. Pollution of oil may also lead to generalized killing of birds, fish, shellfish and other invertebrates (Atlas and Bartha 1998). Even at low concentrations, dissolved aromatic components of oil disrupt the chemoreception of some marine organisms. As feeding and mating responses largely depend on chemoreception, such disruption can lead to elimination of many species even if the concentration of pollutants is below lethal (Atlas and Bartha 1998). Some polynuclear components of petroleum that are relatively resistant to biodegradation and are carcinogenic may move up marine food chains and taint fish or shellfish. Compounds like gasoline and low-viscosity distillation products may seep into soils leading to pollution of groundwater. Oil spills are destructive to vegetation not only because of contact toxicity, but because hydrocarbon biodegradation in the soil renders plant root zones anoxic. The lack of oxygen and accompanying H_2S evolution kills the roots of most plants including large trees (Bossert and Bartha 1984).

These dramatic effects of oil spills reflect the demanding need for the development of techniques that deal with oil pollution both at the sea and on shorelines. There are different physical and chemical methods, which are fairly well established. It is technically difficult to collect oil floating on water and its cleanup costs are very high (Swannell et al. 1996). Observations in the field have shown that many indigenous microorganisms have the potential to effectively degrade hydrocarbons (Höpner et al. 1996, Morgan and Watkinson 1994). This opened a new prospective for using such organisms in cleanup of polluted sites and elimination of environmental pollutants known as bioremediation.

2.3 Definitions and Bioremediation strategies (after Madsen 1998).

Biodegradation is the partial simplification or complete destruction of the molecular structure of environmental pollutants by physiological reactions catalyzed by microorganisms. It is routinely measured by applying chemical and physiological assays to laboratory incubations of flasks containing pure cultures of microorganisms, mixed cultures, or environmental samples (soil, water, or sediment). Bioremediation is the intentional use of biodegradation processes to eliminate environmental pollutants from sites where they have been released either intentionally or inadvertently. Bioremediation technologies use the physiological potential of microorganisms, as documented readily in laboratory assays, to eliminate or reduce the concentration of environmental pollutant in field sites. Microbial processes may destroy environmental contaminants in situ, where they are found or ex situ, which requires that contaminants be mobilized out of the landscape into some type of containment vessel (a bioreactor) for treatment. Intrinsic bioremediation is passive, it relies on the innate capacity of microorganisms present in field sites to respond to and metabolize the contaminants. Engineered bioremediation takes an active role in modifying a site to encourage and enhance the biodegradative capabilities of microorganisms. Selection of the most effective bioremediation strategy is based on characteristics of the contaminants (toxicity, molecular structure, solubility, volatility, and susceptibility to microbial attack, the contaminated site (ecology, hydrology, soil type, and climate) and the microbial process that will be exploited, such as pure culture, mixed cultures, and their respective growth conditions and supplements.

3. Microbial mats and pollution with petroleum compounds

The significance of cyanobacterial mats in bioremediation studies was realized with the interesting observations after the Gulf war (August 1990 until February 1991). During this war, a large amount of crude oil was released into the Arabian Gulf. About 500,000 tons of this crude oil drifted from the Kuwait coasts to severely pollute about 770 km of the Saudi Arabian coasts as well (Sorkhoh et al. 1992). The Gulf environment was therefore confronted with this great oil spill and all forms of higher life on the oil-contaminated beaches were seriously affected. Interestingly, after the oil spill, massive microbial mats, dominated by cyanobacteria such as *Microcoleus* sp. and *Phormidium* sp., colonized the oiled sediments (Fig. 5) (Höpner et al. 1996, Hoffmann 1996). The presence of these cyanobacterial mats was remarkably associated with oil pollution, so

that oil-free sediments were free of such mats. In addition, this kind of mats had not been previously reported in the region, except in the unpolluted Abu Dhabi mats (Golubic 1992). The appearance of microbial mats was also correlated with the disappearance of oil compounds, perhaps indicating a self-cleaning activity in these polluted sites. During the present work, we also observed a similar phenomenon in the highly polluted site of Wadi Gaza (Gaza Strip-Palestine) at the Mediterranean coast. This stream receives a variety of pollutant sources such as diesel oil, hydrocarbons, sewage, pesticides, solid waste as well as agricultural and industrial discharges. In spite of this high level and varied sources of pollution, microbial mats also developed there. Since these mats are obviously recalcitrant to high level of pollution, it is assumed that they contain communities that possess oil-degrading ability.

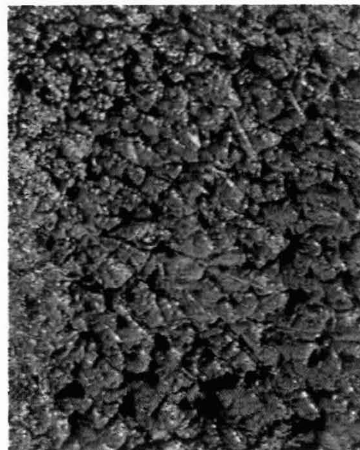


Fig. 5. Cyanobacterial mats covering heavily contaminated sand in the Arabian Gulf

In the Gulf mats, biodegradation experiments performed with photosynthetic microorganism, particularly cyanobacteria demonstrated their ability to degrade both aliphatic and aromatic hydrocarbons. Two non-axenic cyanobacterial isolates, ascribed to *Microcoleus chthonoplastes* and *Phormidium corium*, respectively, obtained from oil-rich sediments of the Arabian Gulf, were shown to degrade n-alkanes (Al-Hasan et al. 1998). It was concluded that the cyanobacteria and not the organotrophic bacteria degraded the compounds as they intracellularly accumulated fatty acids with chain lengths equivalent to those of the alkanes. Kuritz and Wolk (1995) had demonstrated the potential of *Anabaena* sp. and *Nostoc ellipsosporum* for the degradation of the highly chlorinated pesticide lindane. Naphthalene could be metabolized to 1-naphthol by *Oscillatoria* sp. strain JCM and *Agmenellum quadruplicatum* (Cerniglia et al. 1979). Further experiments on naphthalene oxidation by 18 different cyanobacterial and microalgal strains showed that this aromatic hydrocarbon was readily degraded by photosynthetic microorganisms (Cerniglia et al. 1980a). Other studies showed the ability of *Oscillatoria* sp. strain JCM to oxidize biphenyl to 4-hydroxybiphenyl (Cerniglia et al. 1980) and *Agmenellum quadruplicatum* to metabolize phenanthrene to trans-9,10-dihydroxy-9,10-dihydrophenanthrene and 1-methoxy-phenenthrene (Narro 1985). In mat systems, cyanobacteria are present in association with

aerobic oil-degrading heterotrophs. Organotrophic bacteria such as *Acinetobacter calcoaceticus* and *Micrococcus*- sp. were isolated from the oiled sediments of the Arabian Gulf and found to degrade petroleum compounds (Radwan et al. 1999). Cyanobacteria may contribute indirectly to the biodegradation process by immobilizing such bacteria in their mucilage, thus preventing them from being washed out. In addition, cyanobacteria also provide these bacteria with the oxygen produced by photosynthesis and the fixed nitrogen needed for their activity in the degradation processes (Radwan et al. 2000).

It has also been presumed that petroleum compounds may be degraded, at least partially, by anaerobic organisms inhabiting the anoxic bottom part of microbial mats. This was based on the assumption that a large proportion of these compounds penetrates into the mats by diffusion. In some cases when polluted sites are freshly colonized by microbial mats, as in the Arabian Gulf, oil is in direct contact with the bottom of those mats already at the onset. Recently several reports demonstrated the potential of different types of anaerobic microorganisms isolated from polluted sediments to utilize both aliphatic and aromatic compounds. Several strains of sulfate-reducing bacteria isolated from sulfide-rich marine sediments and a North Sea oil tank were able to oxidize anaerobically hydrocarbons in crude oil (Harms et al. 1999, Rabus et al. 1993, Rueter et al. 1994). *Desulfobacula toluolica* was the first sulfate-reducing bacterium in pure culture that oxidizes toluene under strictly anaerobic conditions (Rabus et al. 1993). Other newly isolated strains of iron-reducing and denitrifying bacteria were also able to anaerobically oxidize different hydrocarbons (Lovely and Lonergan 1990, Rabus and Widdel 1995, Rabus and Widdel 1996, Rabus et al. 1999). Compounds like ethylbenzene, toulene, n-alkanes and the aromatic plant hydrocarbon p-cymene were degraded by such microorganisms.

In the light of this introduction it becomes clear that microbial mats, inhabited by physiologically diverse micro-organisms, represent a promising system for biodegradation studies. Most of these biodegradation activities are believed to occur in the upper part of the mats, dominated mainly by cyanobacteria. Therefore, the main objectives of this thesis are:

1. Investigating the microbial diversity in the upper oxic layer of microbial mats with particular emphasis on cyanobacteria. A comprehensive description of the community structure of microbial mats is a base-line requirement for further studies.

2. Following cyanobacterial community changes that result when mat samples are transferred form their original habitats. The awareness of such community changes, owing to experimental settings, is essential in the design and in understanding the results of biodegradation experiments.
3. Investigating the potential of cyanobacteria-dominated microbial communities in the cleanup of contamination with petroleum compounds.
4. Monitoring community responses of microbial mats when exposed to pollution with petroleum compounds. The detection of community changes, attributed to incubation with petroleum compounds, help in the identification of those micro-organisms, which may be involved in the degradation processes.
5. Comparing the behavior of two microbial mat systems, a pristine and a polluted one, in the presence of petroleum compounds. The comparison includes their ability to degrade the provided compounds and changes in their microbial communities. Such comparison emphasis on the significance of prior exposure to pollution with such compounds in the development of a tolerant system with a biodegradative capability.
6. Investigating the potential of cyanobacterial isolates, unialgal and axenic, to degrade petroleum compounds.

B. METHODS FOR COMMUNITY DETERMINATION

1. Traditional techniques

For many years traditional techniques such as microscopy and enrichment cultivation have been used to study microbial diversity and community structure. Cyanobacteria can be easily identified by direct microscopy as they have distinct morphology and conspicuous structure. They can be distinguished from other bacteria by their autofuorescence. However, it has been shown that phylogenetically distant cyanobacteria may have similar morphologies (Wilmotte 1994, Ward et al. 1998). This is especially true for morphologically simple cyanobacteria such as unicellular *Synechococcus* species or filamentous forms like extremely thin filamentous *Phormidium*-like morphotypes. In addition, microscopy may fail to unravel minor morphological differences leading to misidentification of species. On the other hand, enrichment cultivation may succeed in isolating some microorganisms, particularly those which fit the culturing conditions, but fail to recover all community members (Ferris et al. 1996a, Ward et al. 1997). Recently,

it has been demonstrated that enrichment cultivation conceal the real diversity of cyanobacteria within a microbial mat system (Ferris et al. 1996b).

2. Molecular techniques: culture-independent approaches

Microbial communities are complex assemblages of populations (species) with diverse morphology, phylogeny and physiology. Understanding the dynamics of such communities by traditional techniques would be difficult due to the known limitations of these techniques (Ferris et al. 1996a, Ward et al. 1997, Weller et al. 1991). On the other hand, the development of molecular techniques has provided new insights into microbial ecology. Using these techniques, we are now able to study the composition of microbial communities and monitor any changes in their composition as a result of environmental perturbations without the need to isolate individual organisms. Most of these techniques are based on 16S rRNA molecules thus referred to as the rRNA approach (Amann et al. 1995, Pace 1996) (Fig. 6). The 16S rRNA molecule is an excellent genetic tool for community analysis and phylogenetic comparison as it is present in all organisms in high numbers and it possesses both conserved and variable sequences which enables easy design of specific primers and probes for covering different phylogenetic ranges. An overview of the widely employed techniques in community analysis is presented here.

2.1 Molecular fingerprinting techniques

Several fingerprinting techniques have been widely used to study microbial diversity and community changes in time or after disturbances such as Denaturant Gradient Gel Electrophoresis (DGGE) and Terminal Restriction Fragment Length Polymorphism (T-RFLP) (Muyzer et al. 1993, Liu et al. 1997, Muyzer and Smalla 1998, Marsh et al. 1999, Moesender et al. 1999). DGGE has been successfully applied to study the diversity of cyanobacteria particularly within microbial mat systems (Garcia-Pichel et al. 1996, Nübel et al. 1999, Nübel et al. 2000a, Nübel et al. 2000b). This technique is based on the extracted total nucleic acids, DNA and RNA, from environmental samples. The extracts are then subject to amplification by polymerase chain reaction (PCR) using different sets of primers. Recently Nübel et al. (1997) developed primers that amplify specifically cyanobacterial 16S rRNA genes including plastidic 16S rRNA. PCR primers amplifying the variable regions V6 to V8 form oxyphotobacterial strains (cyanobacteria and prochlorophytes) were also described (Rudi et al., 1997). Several other primers, which

amplify the 16S rRNA of other microorganisms of the domain *Eubacteria* (universal primers) or different groups of bacteria are also available.

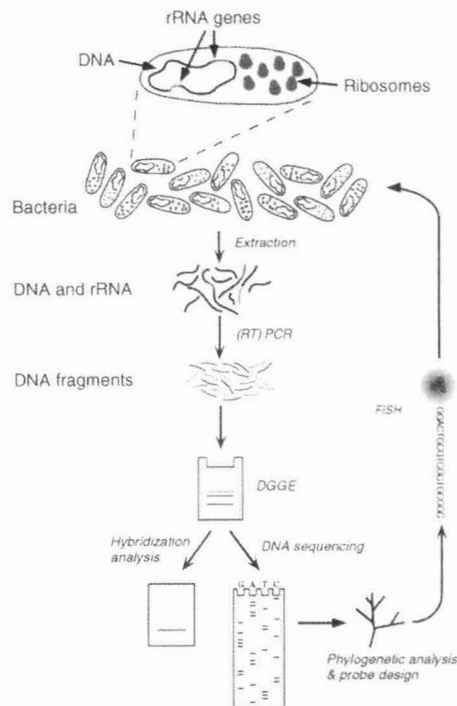


Fig 6. An overview of a microbial community study using molecular techniques. Nucleic acids from an environmental sample are extracted, amplified and analyzed by denaturing gradient gel electrophorsis (DGGE). Sequences are retrieved and probes are designed for detection of specific bacteria with fluorescence in situ hybridization (FISH). (After Muyzer et al. 1995, modified)

The various alleles of the 16S rRNA fragments amplified are subsequently separated on polyacrylamide gels containing increasing denaturant gradients of urea and formamide. The fragments of different species alleles have different melting behaviors due to sequence variations, therefore migrate to different positions on the gels. The banding pattern reflects the composition of the dominant species in the environmental sample. The bands displayed on the gel can then be excised, re-amplified and sequenced. The sequences retrieved can be analyzed phylogenetically by comparison to other sequences available in the existing databases (encompassing about 20,000 sequences) in order to find the closest relatives. These sequences can also be used for further probe design.

Terminal restriction fragment length polymorphism (T-RFLP) measures the size polymorphism of terminal restriction fragments from a PCR amplified marker (Liu et al. 1997, Marsh et al. 1999). The DNA extract is subjected to amplification by PCR using

one of the primers being fluorescently tagged. Subsequently, the product is digested with selected restriction endonucleases producing terminal fragments appropriate for sizing on high resolution sequencing gels. The separation is based on the different sized PCR products. This approach provides a sensitive and rapid technique for assessing amplification product diversity within a single community as well as comparative distribution across communities. Comparison of data obtained by this technique to those obtained by DGGE showed comparable results with slightly higher resolution of T-RFLP than DGGE (Moesender et al. 1999). T-RFLP was used to study the microbial diversity in different sludges (Liu et al. 1997) and in the analysis of marine bacterioplankton communities (Moesender et al. 1999). So far this technique has not been used in the analysis of cyanobacterial communities within microbial mat systems, but it is in principle possible to use it for this purpose.

2.2 Hybridization techniques

Hybridiztaion techniques enable us to link bacterial sequences retrieved directly from the environment to their corresponding populations, with known morphologies and ecological distribution without cultivating them (Amann et al. 1995). In addition, they can also provide information on the abundance, distribution and spatial arrangement of different populations. The techniques are based on individual population detection using labeled probes either with a fluorescent dye, a radioactive label, an enzyme or a marker molecule like digoxigenin. The probes can be designed to target specifically one organism or a group of organisms. Dot-blot and slot-blot hybridizations are PCR independent approaches that are applied directly to nucleic acid extracts (DNA and/or RNA) from environmental samples. The extracted nucleic acids are fixed on a membrane and hybridized with the specific probes selected to identify and quantify the relative abundance of specific rRNA sequences in the original samples. The technique has been used to quantify different sulfate-reducing bacteria inhabiting microbial mats (Risatti et al. 1994) and marine sediments (Sahm et al. 1999). It is also possible to apply this hybridization technique to blots of DGGE gels in which the denatured DNA is hybridized with specific probes to determine the presence of specific populations (Abed et al. unpublished). Fluorescently labeled oligonucleotide probes have been widely used in whole cell hybridizations (Amann et al. 1995). These probes hybridize specifically with the rRNA molecules inside target cells in the environmental sample. Hybridized cells can be visualized by epifluorescence microscope. *In situ* hybridization can be now

coupled to other techniques such as microautoradiography in order to identify *in situ* specific substrate uptake of individual micro-organisms within complex communities (Lee et al., 1999). The fluorescently labeled probes, however, can not be used to detect cyanobacterial populations due to the strong autofluorescence of their cells. Therefore, a new in situ hybridization technique based on horseradish peroxidase (HRP)-labeled probes for the identification of individual cells of cyanobacteria has been developed (Schönhuber et al. 1999). This technique has been successfully used on sections of a microbial mat in order to identify different cyanobacterial morphotypes (Schönhuber et al. 1999, see also chapter 4). In a recent study, the abundance and spatial distribution of two closely related Prochlorococcus genotypes in the North Atlantic Ocean and the Red Sea were investigated using the same technique (West et al., 2001). Another whole-cell hybridization technique applied to marine cyanobacterial picoplankton was also developed. This technique is based on fluorescently labeled-rRNA targeted peptide nucleic acid (PNA) probes (Worden et al., 2000).

2.3 Other techniques

Several other techniques have also been applied to cyanobacteria in different environments. For example, paramagnetic beads have been recently used in cell concentration and DNA purification of cyanobacteria (Rudi et al., 1998; Rudi et al., 2000). Using specific probes immobilized on them, they were also used to identify cyanobacterial genera (Matsunaga et al., 2001). Competitive PCR has been used for quantification purposes. This technique is based on the addition of an internal standard i.e. the competitor to the PCR mixture. The PCR primers can be designed specifically to target a single cyanobacterium or a group of cyanobacteria. The PCR-amplificate of the target cyanobacteria is then quantified by comparison to the standard. This technique was used to quantify genetically tagged cyanobacteria in Baltic Sea sediments (Moller and Jansson, 1997) and to estimate the abundance of toxic cyanobacteria in water bodies (Rudi et al., 1998). Recently, quantification of toxin-producing cyanobacteria by competitive PCR has been modified (Rudi et al., 1998). The new technique includes a competitive PCR step followed by sequence-specific labeling of oligonuclotides probes with fluorescein. The labeled oligonucleotides are then hybridized to immobilized complements. A chromogenic detection of the label is performed, and the relative signal intensities are determined. This technique was successfully used to detect and quantify

toxic cyanobacteria belonging to the genus *Microcystis* (Rudi et al., 1998). DNA arrays have also been used to study the abundance and diversity of cyanobacteria (Rudi et al., 2000). These arrays consist of several different probes spotted on a solid support thus enabling fast and specific detection of the community members.

3. Polyphasic approach

This approach has been proposed in order to circumvent the limitations of different techniques. In this approach the information from different techniques such as microscopy, cultivation and culture-independent molecular tools are combined to complement each other. Research done on cyanobacterial communities using this approach yielded congruent results in most cases (Nübel et al. 2000a, Nübel et al. 1999, Abed and Garcia-Pichel 2001). Polyphasic characterization of certain groups of cyanobacteria lead to the description of new genera (Nübel et al. 2000b). Therefore, this approach seems to have a strong potential in the study of cyanobacterial communities within environmental samples.

C. RESULTS AND DISCUSSION

The main focus of this thesis was to study the microbial diversity present in cyanobacterial mats and to monitor variations in community composition in response to environmental disturbance with particular emphasis on the biodegradation of petroleum compounds. The results of the five chapters of this thesis are summarized in the following section.

1. Cyanobacterial community changes in microbial mat transplants

In this study we compared the cyanobacterial community composition of original Solar Lake mats (Sinai, Egypt) to samples transferred to experimental ponds or laboratory settings for longer periods of time. For this purpose we used a polyphasic approach that included microscopy, cultivation and molecular techniques (i.e. DGGE). The community analysis of the original Solar Lake mat showed that *Microcoleus chthonoplastes* and unicellular cyanobacteria (Halothece *sensu* Garcia-Pichel et al. 1998) were the dominant members of the community while other *Spirulina* species *(or Halospirulina sensu* Nübel et al. 2000b) were minor community components. Transfer of mats form the natural environment to artificial settings resulted in significant changes in their cyanobacterial communities, with the conspicuous loss of dominance by *M. chthonoplastes*. The most dramatic shifts in the community occurred in the mat sample kept in the laboratory for three years. This resulted in the overwhelming dominance of *Oscillatoria*-like morphotype with curved end cells. The experimental pond mats exhibited similar community structure to the original mat after six months of transfer but with the development of additional new cyanobacterium as assessed by DGGE analysis. After two years of transfer, the mats showed a prevalence of two novel populations with a prominent decrease in the viability of *Microcoleus chthonoplastes*. The 16S rRNA sequences of these two populations were not phylogenetically close to any known described strains in the databases. Through cultivation, one population was isolated and shown to correspond to a filamentous, non-heterocystous cyanobacteria with extremely narrow trichomes (0.75-1μm). These cyanobacterial isolates were further used in the description of a new genus (see below). The second population exhibited the most

dominant molecular signature on the DGGE gel, even though it could not be identified by cultivation efforts or correlation with direct microscopy. The morphological identity of this cyanobacterium was further revealed by using *in situ* hybridization technique with horseradish-peroxidase labeled probes designed specifically for its 16S rRNA (see below). The differences observed in the cyanobacterial community structure between the original Solar Lake mat and the transplants into the laboratory or the experimental ponds demonstrate that these experimental systems develop their own unique community structure and cannot be representative of the system operating in the field. The extent of changes is commensurate with the extent of differences in conditions between the original site and the sites of transfers.

2. *Halomicronema*: a proposed new genus

We isolated four strains of extremely thin filamentous cyanobacteria (0.75-1 µm in width) from a hypersaline microbial mat maintained in an experimental pond in Eilat. The 16S rRNA sequences of these strains were closely related to each other (99% sequence similarity) but distant from other cyanobacterial sequences available in the database (10% sequence divergence). The classification of such cyanobacteria by both botanical and bacteriological cyanobacterial taxonomic systems has been problematic. This is because the morphological features that make up the base of all cyanobacterial taxonomic systems in use do not suffice to differentiate morphologically simple cyanobacteria. Furthermore, 16S rRNA analysis of cyanobacteria with *Phormidium*-like morphologies revealed that they are polyphyletic. Physiological analysis of our four strains showed that they were moderately halophilic, moderately halotolerant (salinity optima between 3.2-12 %) and moderately thermophilic (temperature optima between 28-45 °C). Interesting features were shown by electron microscopy. For example, thylakoids were shown to be parallel to the cell wall but were often excentrically arranged within the cytoplasm with respect to the main trichome axis. We proposed the new genus and species *Halomicronema excentricum* to circumscribe these strains.

3. Picobenthic cyanobacteria

From the novel populations developed in the experimental pond microbial mats, one of them was the extremely thin filamentous cyanobacteria used in the description of the new genus *Halomicronema*, whereas the other was cryptic. The DNA molecular

signature of this population on the DGGE gel suggested that it was dominant in this community, nevertheless we could not relate it to any cyanobacterial morphology in the field mat by direct microscopy or bring it into culture. Its 16S rRNA sequence showed 10% or more sequence divergence to other sequences in the databases. To shed light on the morphological identity of this cyanobacteria, we designed two horseradish-peroxidase labeled oligonucleotide probes to target specifically this cyanobacteria in situ. The hybridizations between the probes and the 16S rRNA of this cyanobacteria revealed them as extremely small, unicellular, colony forming cyanobacteria, 0.75 to 1 µm in diameter, which were embedded in abundant mucilagenous investments. Due to their small size, atypical for cyanobacteria but within the range of heterotrophic bacteria, and low levels of autofluorescence, they were difficult to differentiate them microscopically. Unicellular cyanobacteria of this size have not been described before except for free living picoplanktonic cyanobacteria, belonging to the genera *Prochlorococcus* and *Synechococcus*, inhabiting modern oceans. In benthic communities, such cyanobacteria have not been reported before. This cyanobacteria may represent a new ecological niche, which we term Picobenthos.

4. Degradation of model compounds by a pristine hypersaline cyanobacterial mat: a polyphasic study

In this study we investigated the ability of an intact hypersaline microbial mats growing in an experimental pond in Eilat and cyanobacterial strains to degrade four model compounds, *n*-octadecane, pristane, dibenzothiophene and phenanthrene. Changes in the physiology of the mat were followed by microsensor techniques (data not shown in this thesis). Changes in the community structure in response to the incubation with the four compounds were monitored by molecular techniques. The mat was able to degrade aromatic compounds (dibenzothiophene and phenanthrene) better than aliphatic once (*n*-octadecane, pristane) at a very slow rate. Both cyanobacterial and bacterial communities were robust and did not undergo any major changes in the course of the experiment, as assessed by DGGE analysis. None of the cyanobacterial strains degraded any of the compounds except the *Oscillatoria* sp., which degraded the aliphatic compounds partially. This demonstrates that this pristine hypersaline microbial mat has stable community structure and low degradation potential. Furthermore, degradation of petroleum compounds is not common among cyanobacteria.

5. Microbial mats from Wadi Gaza, Palestine: microbial diversity and community changes following degradation of model compounds

Research on Wadi Gaza was initiated with the assumption that microbial systems grown in polluted sites could be better candidates than pristine ones for biodegradation studies. Wadi Gaza is a stream wash that is located very close to the Mediterranean Sea in Gaza, Palestine. The Wadi receives a variety of pollutant sources such as Diesel oil, hydrocarbons, sewage, pesticides, solid waste as well as agricultural and industrial discharges. Microbial biofilms dominated by cyanobacteria were found to develop on the sediment surface in the presence of this high level of pollution. We studied the diversity of cyanobacteria as well as bacteria inhabiting this site using direct microscopy as well as molecular approaches. The site was dominated by different cyanobacteria with *Phormidium* and *Oscillatoria*-like morphotypes. Other bacterial populations belonging to *Cytophaga/Flavobacter/Bacteroides* group as well as β and δ subclasses of the Proteobacteria were also detected. Phylogenetic analysis showed that some of these bacteria were related to environmental sequences retrieved from activated sludge, a finding consistent with the sewage pollution in the site, while others were related to bacteria capable of degrading aromatic compounds. In a slurry experiment, these microbial mats were checked for their potential to degrade the same four model compounds used before, n-octadecane, pristane, dibenzothiophene and phenanthrene. The mats degraded these compounds rapidly within 3 days. The community structure of cyanobacteria showed dramatic shifts with the development of one particular cyanobacterium attributable to the addition of the model compounds. Another cyanobacterium, similar to *Synechocystis* PCC 6803, developed in the controls as well as in the presence of the compounds. The latter cyanobacterium is known to have the dioxygenase enzyme system involved in the aerobic degradation of aromatic compounds, suggesting a possible role of this cyanobacterium in the degradation of these compounds as well. The bacterial community also showed prominent changes in the presence of the model compounds, both in the light and the dark, with the replacement of originally dominant species by others. Among the novel species, four could be attributed to the incubation with the model compounds. Two of them were phylogenetically affiliated to sequences belonging to the newly described phylum *Holophaga/Geothrix*. Some members of this group are able to degrade some aromatic compounds under anaerobic

conditions. A third species, enriched in the light treatments, fell within the green non-sulfur bacteria while a fourth one was related to sequences from activated sludge. The phylogeny of other bacterial sequences suggested that this site is rich in many new, yet undescribed species. This mat system seems to have a strong potential in the degradation of petroleum compounds.

Future perspectives

This research demonstrates the ability of cyanobacteria-dominated microbial mats in the cleanup of petroleum pollution. Microbial mats pre-exposed to pollution exhibit more tolerance and potential to degrade petroleum compounds than pristine ones. Community change studies suggest the involvement of different groups of mat microorganisms in the degradation of petroleum. Whether these microorganisms can degrade the compounds individually or they rely on each other still needs to be investigated. Therefore, to answer this question, it is required to isolate axenic cultures representing different groups of mat-microorganisms. These cultures should be further incubated in the presence of petroleum compounds in order to check for their individual ability to degrade them. The degradation rates among the positive cultures can be compared. The efficiency of the degradation process can also be compared when the petroleum compounds are incubated with different combinations of more than one organism. Furthermore, the direct or the indirect role of cyanobacteria in the degradation processes needs to be examined. This can be achieved by comparing degradation patterns of the compounds in the presence and in the absence of cyanobacteria. Axenic cultures of anaerobic micro-organisms from mats enables the demonstration of anaerobic degradation. The interaction between aerobic and anaerobic degradation processes and whether these processes complement each other or they function independently are among the interesting studies, which need to be expanded.

Using the axenic isolates, it becomes easy to obtain the complete 16S rRNA sequence of the corresponding organisms. This, however, enables the design of specific probes. It is now possible to find these populations back in the field, study their spatial arrangements and estimate how numerically important they are in the field with the help of *in situ* hybridization technique with fluorescently labeled probes (Amann et al., 1995). Recently, this technique has been coupled to another technique namely, microautoradiography in order to determine *in situ* the identity, activity, and specific substrate uptake profiles of individual cells within complex communities. This combined

technique can be applied to thin sections of microbial mats after incubation in the presence of radioactively labeled petroleum compound. This helps in specific identification of the mat micro-organisms, which are able to take up the radioactive label and thus play a role in degrading this compound.

As this system has the ability to degrade petroleum compounds, it is furthermore interesting to explore the different key functional genes involved in the degradation steps. These genes can be subsequently compared to those from other systems such as soil, aquifers etc. Several sets of PCR-primers specific for such functional genes are now available (Stapleton and Sayler, 1998, Lloyd-Jones et al., 1999, Laurie and Lloyd-Jones, 2000, Mesarch et al., 2000). They can be directly applied to DNA extracts form mat samples or from individual organisms. The degradation process can also be quantified using these primers in combination with competitive PCR (Laurie and Lloyd-Jones, 2000, Mesarch et al., 2000).

The different degradation pathways of different compounds can be further illustrated. For this purpose, it is recommended to use radioactively labeled compounds. This helps in demonstrating whether these compounds are completely metabolized to CO_2 or partially degraded. With the stable-isotope techniques intermediate metabolites can also be identified by means of GC-MS (GC chromatography coupled to mass spectrometry).

In the future, further optimizations are necessary to use microbial mats for efficient bioremediation purposes. This includes kinetic studies to find out the optimum temperature, salinity, nutrients concentration and other key parameters at which the maximum biodegradation of petroleum compounds can be achieved.

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Chapter 2

Long-term compositional changes after transplant in a microbial mat cyanobacterial community revealed using a polyphasic approach

Solar Lake, Sinai, Egypt

An Experimental Pond

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Front page:
The hypersaline Solar Lake, located at the Red Sea coast, Sinai, Egypt, from which microbial mats were transplanted to an experimental pond.

Long-term compositional changes after transplant in a microbial mat cyanobacterial community revealed using a polyphasic approach

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Summary

Using a polyphasic approach that included microscopy, cultivation and 16S rRNA based cultivation-independent molecular fingerprinting, we compared the cyanobacterial composition of Solar Lake microbial mats and of samples thereof transplanted and maintained in new settings for extended periods of time. Significant changes in community composition, with clear replacement of the dominant cyanobacterium, *Microcoleus chthonoplastes,* were detected in all cases. The most dramatic shifts occurred in a sample kept in the laboratory for three years, which resulted in dominance by an *Oscillatoria*-like cyanobacterium whose 16S rRNA closely matched that of a morphologically similar isolate from mats in Mexico. Transfer of Solar Lake mat to an artificial experimental pond with incubation under semi-natural conditions resulted in an increase in cyanobacterial diversity. Judging from the molecular signatures, two novel, previously unrecognized, and phylogenetically well-delimited cyanobacterial populations became dominant there. Through cultivation, one population was shown to correspond to a filamentous, non-heterocystous group of cyanobacteria with very narrow trichomes (ca. 0.75-1.5 µm). The most dominant novel molecular signature, however, could not be identified by cultivation efforts or correlation with microscopy and, upon phylogenetic analyses, its 16S rRNA genes showed no particular close association to known cyanobacterial groups.

Introduction

Microbial mats are considered important model ecosystems to study biological dynamics and interactions between microorganisms (Stal and Caumette, 1994). They develop in a wide variety of extreme environments, including hypersaline ecosystems (Javor, 1989) which are distributed worldwide (Bauld, 1981; Pierson, 1992). Hypersaline microbial mats are inhabited by diverse groups of microorganisms including chemolithotrophs, aerobic and anaerobic heterotrophs, and contain cyanobacteria as the dominant phototrophs. Cyanobacteria provide physical support and their activity drives the rest of the microbial community. Functional studies on microbial mats are oftentimes performed on discrete samples transferred to artificial or semi-artificial settings and maintained for various periods of time, with the underlying assumption that results obtained are still applicable to the field conditions of origin. This practice raises the following questions: How sensitive is the community composition of microbial mats to such manipulations? How fast do changes in environmental conditions selectively enhance the dominance of certain microorganisms over others?

Techniques of molecular fingerprinting are appropriate to investigate such questions. Many studies of population dynamics have focused on the detection of microbial community shifts due to short-term (weeks) responses to external disturbance (Ferris et al., 1997; Jürgens et al., 1998; Chin et al., 1999; Netherwood et al., 1999; Macnaughton et al., 1999). Some were performed in order to follow these changes over longer periods of time (years; Fernandez et al., 1999), while others followed seasonal changes (Ferris and Ward, 1997). We used the hypersaline microbial mats of Solar Lake, Sinai peninsula (Egypt), to study changes in cyanobacterial community structure after mat transplants.

The microbial mats of Solar Lake are among the most intensively studied microbial sediment ecosystems (Cohen et al., 1976; Cohen et al 1977; Jorgensen and Cohen, 1977; Krumbein et al., 1977; Campbell and Golubic, 1985; D'Amelio et al., 1989). The cyanobacterial diversity in Solar Lake communities has been comprehensively assessed using traditional microscopy and morphotype description (Campbell and Golubic, 1985). However, more recently it has been shown that phylogenetically distant cyanobacteria may have similar morphologies (Wilmotte and Golubic, 1991; Wilmotte,

1994; Ferris et al., 1996; Ward et al., 1998). This is especially true for morphologically simple unicellular or filamentous forms. On the other hand, morphological traits in structurally more complex cyanobacteria, may allow the identification of phylogenetically coherent groups (Garcia-Pichel et al., 1996, 1998, 2000)

To date no single approach can be deemed an unbiased tool to gauge biodiversity (and its changes) within cyanobacterial communities. For example, enrichment cultures fail to recover all microorganisms by selectively enhancing the growth of those fit for the conditions provided on culturing (Ferris et al., 1996; Weller et al., 1991; Ward et al., 1997). Recently employed molecular techniques, such as DGGE (Denaturant Gradient Gel Electrophoresis; Muyzer et al., 1993) have multiple ecological applications, including the analysis of cyanobacterial communities (Garcia-Pichel et al, 1996; Nübel et al., 1997, 1999, 2000). This technique also has limitations due to biases caused by preferential PCR amplification, which may favor detection of certain populations within a community (Weller et al., 1991;Ward et al., 1998). To minimize the limitations of different methods, a polyphasic approach has been proposed, in which the information from different techniques is combined to complement each other (Nübel et al., 1999, Garcia-Pichel et al., 2000).

We used a polyphasic approach combining information from microscopy, enrichment cultivation and molecular techniques to compare the composition and phylogenetic affinities of cyanobacterial populations growing in i) a natural mat (SL) from Solar Lake, Sinai Peninsula, Egypt, ii) a mat (EP) originating from Solar Lake but maintained for 6 months in an experimental, artificial pond in Eilat iii) samples of Solar Lake mat that had been transplanted and maintained in our laboratory for 3 years (M), and iv) parts of a mat in the Eilat experimental pond that had developed *in situ* from a Solar Lake inoculum over a period of 2 years (FB). Such comparisons were aimed at probing long-term changes in community structure due to different levels of disturbance.

Experimental procedures

Mat samples and their maintenance

Four samples of hypersaline microbial mats derived originally from Solar Lake were used in this study. These samples had the following histories:

SL: Undisturbed mat from Solar Lake, harvested in Nov.1998, air-shipped directly to us in brine-filled containers, and analyzed within 14 days. The Solar Lake mat at this time of the year was exposed to a salinity of 100‰ and a temperature of 27°C. Salinity ranges in Solar Lake at various dates between 1996 and 1998 were 8.3 to 11.4 %, and temperature ranges during daytime between 28 and 33 °C (Wieland, 1999).

EP: Solar Lake mat transplanted to an experimental pond at the Marine Biology Laboratory in Eilat and maintained there for 6 months, subject to natural illumination. A sample was air-shipped along with sample SL and analyzed simultaneously.

M: Solar lake mat collected in 1996 and maintained at MPI, Bremen in an aquarium for 3 years under low light (10-20 μmol photon m^{-2} s^{-1}, PAR) conditions at 30°C. This sample was analyzed along with SL and EP mat samples.

FB: Fresh mat from the experimental pond of Eilat, which developed *in situ* on bare substrate over two years after inoculation of the pond with Solar Lake mats. The pond contains some 48 m^2 of mats that developed from some 10 m^2 of Solar lake inocula. It is fed by oligotrophic Gulf of Aqaba seawater, which is allowed to evaporate to a salinity of 9%. Samples were taken in April, 1999, frozen on site in liquid nitrogen and transported to our laboratory, stored at -80°C and analyzed in June 1999. In April 1999, the temperature range was 22° C in the morning and reached 30° C at noon. Salinity ranged between 8 and 10 %.

Microscopy, Cultivation and morphotype quantification

The depth of the oxygenic photosynthetic mats studied was found to be 2-3 mm (as measured with oxygen microelectrodes; Grötzschel, S., pers. comm.) We used only the photosynthetically active layer of the mats for our study, which was excised under a dissecting microscope with a clean scalpel blade and sterile forceps. This delimited the analyses to populations that are potentially active as oxygenic phototrophs (primary producers) and excluded buried (but possibly still viable) cyanobacteria from deeper layers). Samples were torn apart, mounted in water on a microscope glass slide and observed using transmitted light, phase contrast and fluorescence microscopy. Different morphotypes were identified and photographed. Three cores from each mat sample were observed microscopically to ensure a good overall representation of resident

morphotypes.

Cultivation efforts were done after enrichment in ASN-III defined medium with 70‰ salinity and incubation at 28°C, according to Garcia-Pichel et al. (1998). Isolates were used to relate the cyanobacterial morphotypes to their corresponding 16S rRNA gene signatures.

Morphotype quantification was done as previously described (Nübel et al., 1999) with the following modifications. The upper mat layer was cut with a sterile blade and suspended in sterile medium. An aliquot of this suspension was filtered through 0.2 μm GTTP filter (Millipore), to achieve uniform distribution of the cyanobacterial filaments. Counting and size measurements were performed under a fluorescent and phase contrast microscope considering only the focused autofluorescent cyanobacteria. Three major groups of cyanobacteria were distinguished for the purpose of quantification: unicellular, thick filamentous and thin filamentous cyanobacteria.

Nucleic acid extraction

Three different cores from each mat (ca. 300-500 mg each) were cut with a sterile scalpel blade and homogenized in Dounce tissue homogenizers (Novodirect, Kehl, Germany). Lysis of the bacterial cells was achieved by subjecting the suspensions to freeze (in liquid N_2) and thaw (at 65°C) cycles 3-5 times with subsequent incubation at 50°C for 20 minutes in 5ml TESC buffer (100 mM Tris-HCl at pH 8), 100 mM EDTA, 1.5 M NaCl, 1% (wt/vol) hexadecylmethylammonium bromide, 550 μl of 10% (wt/vol) sodium dodecyl sulphate (Sambrook et al., 1989) and 30 μl proteinase K (20 mg/μl). The DNA was extracted by adding phenol-chloroform-isoamyl alcohol 25:24:1 (vol/vol/vol at pH 5; Sigma Chemical Co., Ltd.) and precipitated by adding 0.6 vol. of isopropanol to the aqueous phase with subsequent spinning at 4500 rpm for 45 minutes (Ausubel et al., 1993). The pellets obtained were washed with 70% (vol/vol) ice cold ethanol, vacuum-dried and resuspended in 100 μl TE buffer (10mM HCl and 1 mM EDTA at pH 8.0).

RNA Extraction and Reverse Transcription

Bulk nucleic acid extracts were obtained as for DNA, but RNA extractions as described by Ward et al. (1995) were performed. Briefly, 10 μl of the extract was

incubated at 37°C for 30 minutes in the presence of DNase buffer (40 mM Tris-HCl at pH 7.9, 10 mM NaCl, 6 mM $MgCl_2$, 10 mM $CaCl_2$)] and 10 µl of RNase-free DNase (Boehringer Mannheim, Germany). RNA was then recovered by the addition of 150 µl sodium acetate (3M) and phenol-chloroform mixture and precipitated by the addition of 0.6 vol. isopropanol. The RNA was resuspended in 30 µl TE buffer and stored at –80°C for further use.

Reverse transcription was performed as described by Muyzer et al., (1998). 10-15 µl RNA (0.5-1 µg) was incubated at 70°C for 10 minutes in the presence of 1.5 µl hexanucleotide (10 pmol; Boehringer-Mannheim, Germany). The mixture was incubated at 37°C for 1 hour after adding 6 µl of RT buffer (250 mM Tris-HCl at pH 8.3, 375 mM KCl, 15mM $MgCl_2$), 6 µl of 2.5mM deoxynucleoside triphosphate and M-MLV Reverse transcriptase (Promega, Germany) followed by incubation at 95°C for 5 minutes to stop the reaction. These products were further amplified by PCR and used for reverse-transcription DGGE (see below).

Polymerase chain reaction (PCR)

The polymerase chain reaction was carried out for amplification of nucleic acid extracts (DNA and RNA). 400 bp fragments were amplified using group-specific oligonucleotide primers CYA359F (with 40 nucleotide GC clamp at the 5′ end) and CYA781R (Nübel et al., 1997). Approximately 10 ng of DNA was used as template for the PCR. Thermocycling was performed using a Mastercycler gradient cycler (Eppendorf, Hamburg, Germany). The denaturation temperature was 95°C for 5 minutes followed by 80°C for 1 minute at which time 1 µl of Super Taq DNA polymerase (HT Biotechnology, Ltd. Cambridge UK) was added. Thirty five cycles were performed at 94°C for 1 minute, 60°C for 1 minute (annealing temperature) and 72°C for 1 minute. The PCR products were stored at 4°C.

Denaturing gradient gel electrophoresis (DGGE)

Polyacrylamide gel was poured between two glass plates separated by 1mm plastic spacers with a 20%-60% urea-formamide gradient. The PCR products were applied directly onto this gel and the DGGE was then performed at 60°C and a constant voltage

of 200V for 3.5 hours. After electrophoresis, the gels were incubated for 30 minutes in aqueous ethidium bromide solution (0.5 µg/l) and photographed on an UV-transilluminator with a Polaroid camera.

The DGGE bands were excised from the gel using a sterile scalpel blade and eluted in 30 µl of the TE buffer. These bands were re-amplified by PCR, and subsequently purified and concentrated using the QIA quick PCR purification kit (Diagen, Düsseldorf, Germany). The amplification products were then commercially sequenced. The sequences obtained have been submitted to the EMBL, their accession numbers are **AF266657 to AF266672**.

Phylogenetic affiliation

Complete cyanobacterial 16S rRNA gene sequences available from GenBank were imported and aligned to the sequences in the database of the ARB software (Ludwig et al., 1998). Phylogenetic trees were constructed based on long 16S rRNA sequences (more than 1300bp) by applying different methods integrated in the ARB software such as maximum likelihood, maximum parsimony and neighbor joining (a maximum likelihood tree is presented here). The 16S rRNA sequences of *Escherichia coli* and *Bacillus subtilis* were included in the calculations as outgroup sequences. Partial sequences (400bp) obtained in this study were aligned to the sequences in the ARB database using the alignment tool of the ARB software package. These sequences were then inserted into the pre-established tree using the parsimony ARB tool and maintaining the overall tree topology without changes.

Results and Discussion

Community differences based on cyanobacterial morphotypes

Microscopic observations, identification and distribution of different cyanobacterial morphotypes within the four microbial mat samples are summarized in Table 1.

Table 1. Dominant cyanobacterial morphotypes encountered and their presence in the different mat samples, determined by light and epifluorescence microscopy (see Fig. 1). Tentative taxonomic epithets have been assigned to those according to our previous studies or to microbiological usage.

Morphotype	Cell width or diameter	Probable taxon	Microbial mat sample			
			SL	EP	M	FB
Filamentous (Fig 1A)	2-6 μm	*Microcoleus chthonoplastes*	+++	+++	++	++
Unicellular (Fig 1B)	3-7 μm	"Halothece"	+++	+++	++	+++
Tightly coiled filaments (Fig 1C)	1.5-2 μm	*Halospirulina spp.*	-	+	+	+
Tightly coiled filaments (Fig 1D)	2.5-4 μm	*Halospirulina tapeticola*	+	-	-	+
Filamentous (Fig 1F)	0.75-1 μm	*Phormidium* -like	-	+++	+	+++
Filamentous (Fig 1E)	4-4.5 μm	*Oscillatoria*-like	-	-	+++	++

+++ Dominant
++ Secondary
+ Observed but very minor
- Not detected

Most of the morphotypes could be assigned to botanical species, but only in some cases has it been shown that a morphotype (and a botanical species or genus) corresponds to a phylogenetically and physiologically coherent group of cyanobacteria. To avoid such bias we refer only to morphotypes, regardless of their systematic validity. The mats harbored a variety of morphologically recognizable cyanobacterial types, differing in their abundance within every mat sample (Fig.1 A-F). Two dominant cyanobacterial morphotypes were the filamentous *Microcoleus chthonoplastes* and large unicellular *Cyanothece*-like cells. The latter typically correspond to the Halothece cluster of unicellular, extremely halotolerant cyanobacteria in hypersaline mats (see below). *M. chthonoplastes* is characterized by closely bundled trichomes (2-6 μm diameter) in a common sheath, and by bullet-shaped apical cells (Fig.1A). This cosmopolitan mat-forming cyanobacterium is known to be a dominant constituent in many hypersaline environments worldwide (Garcia-Pichel et al., 1996). Its role in the formation and stabilization of hypersaline microbial mats is well known (Prufert-Bebout and Garcia-Pichel, 1994). In the SL and EP mats, *M. chthonoplastes* trichomes appeared well

developed and healthy. In the FB mat, however, *M. chthonoplastes* trichomes showed frequent fragmentation (Fig.1A dashed arrow) and weak autoflourescence of its cells. The environmental conditions in this mat were evidently not optimal for the growth of this organism.

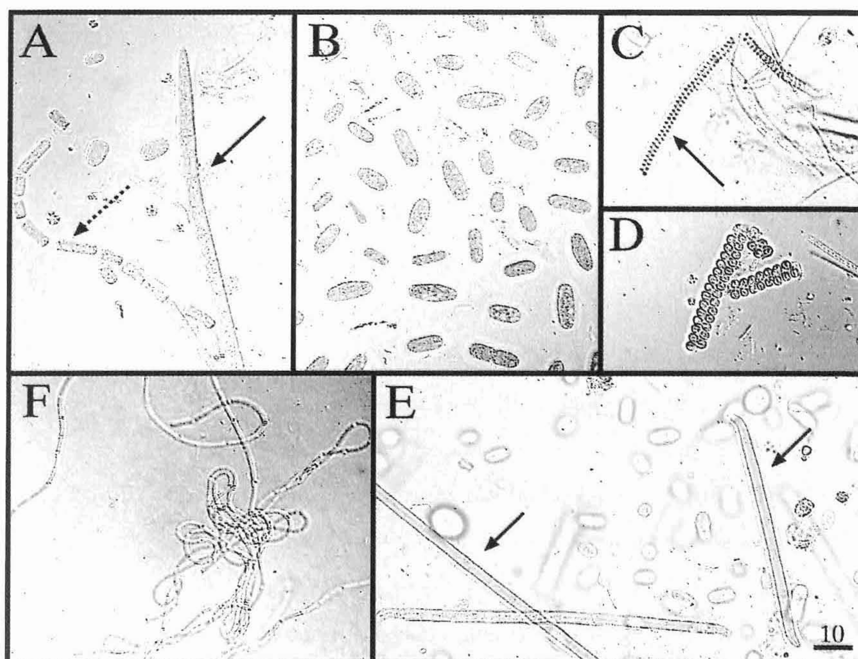

Fig 1. Filamentous and unicellular Cyanobacteria from fresh and transplanted solar lake samples (scale bar 10μm).

A. *Microcoleus chthonoplastes* (arrow); dashed arrow indicates trichome fragmentation in FB mat.
B. Unialgal culture of unicellular cyanobacteria, which probably belongs to the recently described "Halothece" cluster, growing in ASN III medium at 7% salinity and 28 °C.
C. *Halospirulina* sp. (ex. *Spirulina labyrinthiformis*).
D. *Halospirulina tapeticola* (ex. *Spirulina subsalsa*).
E. *Oscillatoria*-like morphotype with curved apicall cells (arrows).
F. Unialgal culture (strain TFEP-A) of a filamentous cyanobacterium with extremely thin filaments (1μm) grown in ASN III medium at 7% salinity and 28°C.

The second dominant morphotype, unicellular cyanobacteria (3 μm diameter or larger) showed variable cell shapes, ranging from coccoid to bacilloid (Fig.1B) with cell division in one plane. The physiology and the phylogeny of similar organisms has been studied before (Garcia- Pichel et al., 1998; Margheri et al., 1999). These studies showed that extremely halotolerant unicellular cyanobacteria of similar morphology formed a diverse but monophyletic cluster based on the analysis of 16S rRNA genes. This Halothece cluster is characterized by phylogeny, morphology and physiology and is separate from taxa with similar morphology occurring outside hypersaline ranges.

Minor microscopically conspicuous components in the mat samples studied included filamentous morphotypes with tightly coiled trichomes corresponding to the botanical species *Spirulina labyrinthiformis* (trichome diameter ca.1.5-2 μm) (Fig.1C) and *Spirulina subsalsa* (trichome diameter ca 2.5-4 μm) (Fig.1D). Similar morphotypes from hypersaline waters have been shown to cluster together, justifying the establishment of a new genus *Halospirulina*, based on morphology, high salt tolerance and 16S rRNA signatures (Nübel et al., 2000).

Variations in cyanobacterial dominance were observed in all samples removed from the original site in Solar Lake, transferred to experimental ponds, and kept there for extended periods of time. The most conspicuous change was the presence of extremely thin (ca 0.75-1 μm diameter) filaments (Fig.1F) with elongated cells. This *Phormidium*-like morphotype dominated the EP and FB mat samples in terms of biomass, but it was not observed in the original Solar Lake mat sample (SL mat).

Another prominent change in dominance occurred in sample M. Here the biomass was dominated by an *Oscillatoria*-like morphotype with typically curved end-cells (Fig.1E). It was also observed in FB mat, but was absent in our SL and EP mats samples. While neither the *Phormidium*-like thin filaments nor the *Oscillatoria*-like morphotype were observed in the sample collected directly from Solar Lake (SL mat), morphologically similar organisms have been reported earlier from Solar Lake mat systems (Campbell and Golubic, 1985).

Among the mat samples studied, the experimental pond FB mat showed the greatest cyanobacterial diversity based on morphotypic examination, since it harbored all morphotypes identified in the mat samples studied here. A number of diatoms including *Nitzschia thermalis* and *Navicula* species inhabited all these mat samples, but were not taken into consideration here.

Community differences revealed by DGGE analysis

The DGGE analysis showed noticeable differences in the banding patterns among the four studied mat samples (Fig.2 and Fig.3, right panel), indicating very significant community differences following transfers and maintenance either in the laboratory or in open experimental pond in Eilat for extended periods of time. Three samples (SL, EP

and M) generated a banding pattern with three separate bands each, whereas the FB-mat showed many visible bands and, thus, a higher apparent richness of cyanobacteria. Most of the bands halted migration at a denaturant gradient 45-55%, except for two bands (SL1 and M1), which showed melting at a denaturant concentration of about 30-35%.

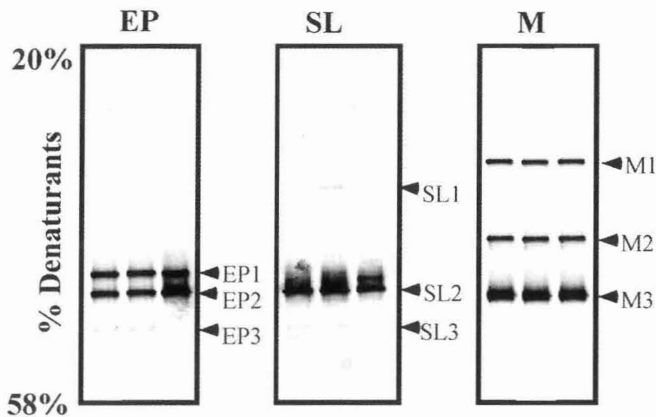

Fig 2. DGGE banding patterns of PCR-amplified 16S rRNA fragments obtained from triplicate, independent samples of mats EP, SL, and M. see *experimental procedures* for sample codes. Labelled bands were excised, reamplified and sequenced

All visible bands were recovered from the DGGE gel, re-amplified, sequenced and aligned to the 16S rRNA cyanobacterial sequences available in the ARB database. An additional band above SL2 repeatedly failed to give products after re-amplification. This band did not appear again upon reamplification of the original DNA extracts, indicating that it was possibly a one-time artifact. The placement of these sequences in a phylogenetic tree of the cyanobacteria and plastids is shown in Fig.4. The sequences derived from bands halting around 30-35% denaturant were related to plastid 16S rRNA sequences. Phylogenetic analysis of the sequences EP2, SL2 and FB3 showed that all mat samples except for M mat contained 16S rRNA band sequences closely affiliated with the previously described *Microcoleus chthonoplastes* cluster (Garcia-Pichel et al., 1996). The correlation between *M. chthonoplastes* morphology and phylotypes thus shows a good degree of correspondence. It has been previously shown that *M. chthonoplastes* isolated from geographically distant environments had a high degree of phylogenetic coherence (Garcia-Pichel et al., 1996). The intensity of this band in FB mat, however, was much lower than in SL and EP mats, a fact consistent with the microscopic observation showing "ailing" *Microcoleus chthonoplastes* remains with fragmented trichomes. Although known to be the dominant mat-forming species in

many moderately hypersaline environments, *M. chthonoplastes* did not count among the most abundant cyanobacteria in this semi-artificial community. DGGE analysis of reverse transcribed 16S rRNA (Fig 3, left panel) which should reflect the growth or activity status of the different phylotypes, indicated that *M. chthoplastes* played even a more restricted role than one could surmise from its relative DNA abundance in the overall cyanobacterial activity of this mat.

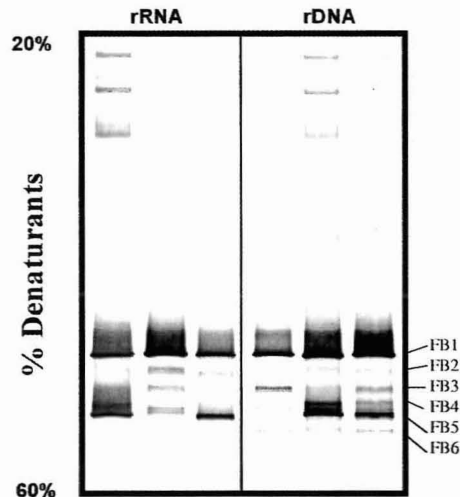

Fig 3. DGGE profiles of 16S rDNA snd reverse-transcribed 16S rRNA fragments obtained from triplicate samples of FB mat. The labelled fragments were subsequently analysed. Labelled bands were exised, reamplified and sequenced.

Other sequences retrieved such as EP3, SL3, FB4 and FB6 fell within the recently described phylogenetic Halothece cluster (Garcia-Pichel et al., 1998). This finding was also consistent with microscopic observations. Two different types of 16S rRNA signatures identified unicellular cyanobacteria (bands FB4 and FB6) in the FB mat, suggesting the presence of local microdiversity within the Halothece Cluster.

The freshly collected Solar Lake mat sample SL showed a community structure characterized by the bands produced by *Microcoleus chthonoplastes* and *Halothece* sp. In contrast, sample M, which grew for 3 years in the laboratory, showed a completely different and unique 16S rRNA band pattern indicating a community structure with three major populations. Phylogenetic analysis identified two different cyanobacterial, and one plastid 16S rRNA bands. The sequence from Band M3 was closely related to the marine filamentous strain *Oscillatoria* sp. MPI 95 OS 01 isolated from the mats of Guerrero Negro, Baja California, Mexico (F. Garcia-Pichel and U. Nübel, unpublished).

This observation is consistent with microscopic observations of the same mat sample. The second cyanobacteria band, M2, fell well within the cluster of heterocystous cyanobacteria, even though we found no heterocystous morphotypes in microscopic observation.

Possible causes for community shifts upon transplant

While our results do not offer direct information regarding the possible environmental parameters that promoted the shifts in community structure, some remarks on this issue may be granted. In the case of mat M, low light conditions likely played a significant role in promoting the dominance of the *Oscillatoria*-like morphotype. Such morphotypes have been shown to dominate in deep, shady mats in Guerrero Negro and to be adapted to very low light intensities, with saturation of photosynthesis below 100 µmol photons m^{-2} s^{-2}. (Garcia-Pichel et al. 1993) The presence of molecular signals for heterocystous (nitrogen-fixing) cyanobacteria, suggests significant changes in the availability of N in such mat. For the rest of the samples, differences in the hydrological regime, or in the chemical composition of the brine, may have played a role as environmental agents forcing the shifts. A study of the physiological capacities of isolated strains from the novel populations may shed some light on this issue.

Novel populations in the experimental pond mats

The most characteristic and unique feature of the experimental pond mat communities (EP and FB) was the development of novel populations (EP1, FB1 and FB2 bands), with sequences that were not particularly close to those of described strains. These populations were not observed in either the Solar Lake sample SL or in the mat sample maintained in the laboratory (M). In the FB mat, the intensity of the bands (FB1 and FB2) both from the 16S rDNA and the reverse transcribed 16S rRNA indicated that this FB1 population was the most abundant (DNA) and active (RNA) cyanobacterium in this community, whereas FB2 population showed less abundance but rather high activity (Fig 3). The microscopic studies suggested that a possible candidate for this unknown population could be the thin filaments that made up much of the biomass in the FB and EP samples, but were absent in the rest. We succeeded in obtaining an isolate of such

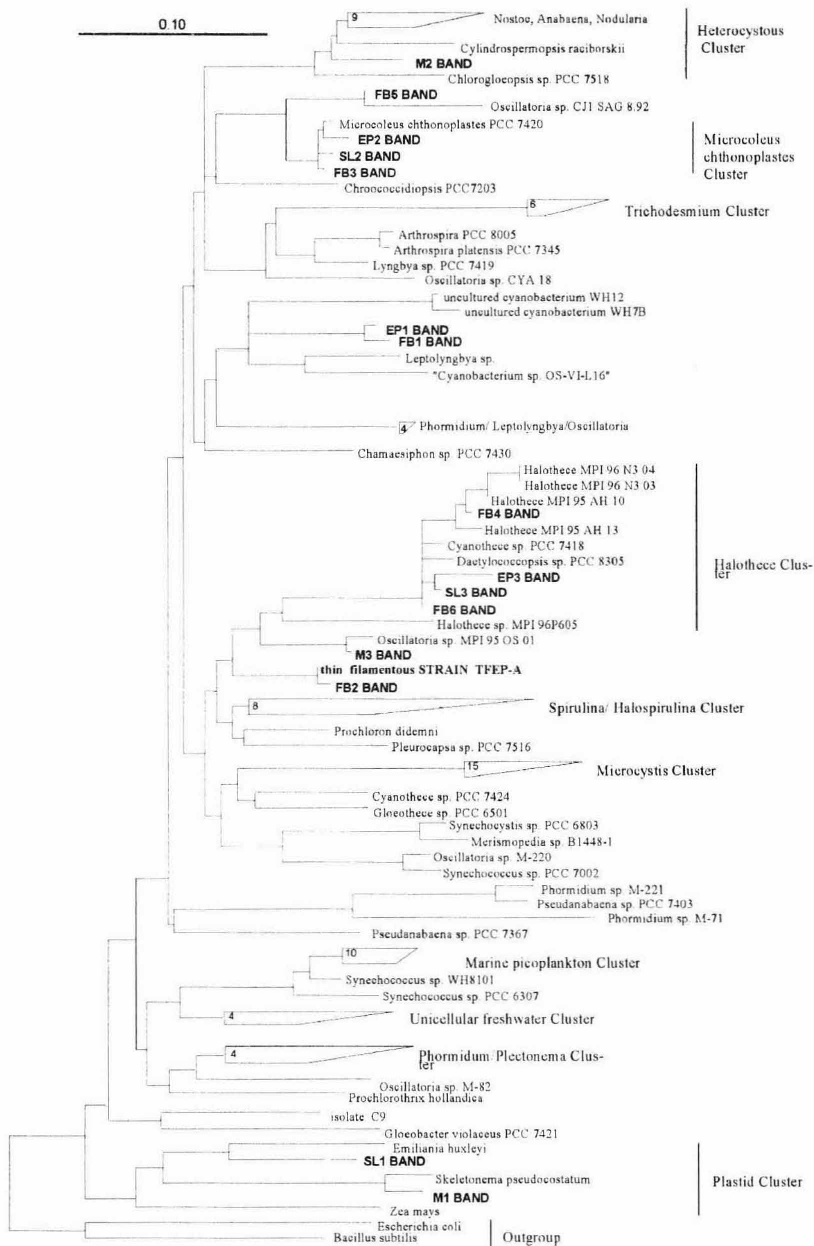

Fig 4. Phylogenetic reconstruction of the cyanobacteria and plastids (maximum likelihood) based on publicly availbale, almost complete 16S rRNA genes from the cyanobacterial line using those of *E. coli* and *B. subtilis* as outgroups. The numbers next to the collapsed clusters indicate the number of sequences contained in each cluster. The phylogenetic placement of 400 bp long 16S rRNA sequences from our excised DGGE bands was carried out using parsimony criteria without changing the topology of the pre-established tree using the tool in the ARB software. Sequence TFEP-A was obtained from an isolated strain. the bar indicates 10% sequence divergence. Strain denomination of particular strains correspond to those given in the databases and we do not imply that they are necessarily taxonomically correct.

morphotypes (strain TFEP-A). Its 16S rDNA sequence corresponded well to that of Band FB2 (fig 4). This population was morphologically indistinguishable from those observed in field samples of the FB mat.
Thus, it is likely that a novel group of filamentous cyanobacteria with extremely thin trichomes 0.75-1.5 µm in diameter, with rounded end cells and with cross walls without constrictions, but with a gas vesicle at each side of the cross wall (Fig.1F) needs to be defined. We are presently expanding such cultivation studies to better define the diversity and physiological traits associated with these organisms.

The second novel population was represented by EP1 and FB1 bands in the DGGE analyses. The phylogenetic analyses of the respective sequences showed that they were close to each other but not closely related to any well described cyanobacterium (Fig 4). According to the database, the closest relatives include a thin filamentous form (*Leptolyngbya*) and some unidentified environmental sequences. According to the molecular analyses, this population co-dominated the EP mat community along with *M. chthonoplastes*, while it was predominant in the FB mat. Renewed microscopic efforts in both mat samples could not distinguish a particular morphotype that could account for such mismatch between microscopy and molecular analyses. A microscopic quantification of major groups of cyanobacteria in the FB community yielded 46 % of the biovolume to filamentous cyanobacteria with wide trichomes, 43% to cyanobacteria with extremely thin trichomes, and 11% to Halothece. Judging from these data alone, the chances of this population being thin or thick filamentous cyanobacteria is almost equal. A survey from enrichment cultures obtained from the mat (not shown) did not yield any closely matching sequences. *In situ* hybridization analyses (Schönhuber et al., 1999) are in progress in order to elucidate the identity of this mysterious cyanobacterium.

Concluding remarks

This study supports the results of Nübel et al (1999, 2000) indicating that the polyphasic approach can yield congruent results in cyanobacterial community analyses. However, the correspondence between molecular and morphotypic analyses is not complete. The results provided strong evidence that the Solar Lake cyanobacterial community underwent significant changes following long-term transfer from its natural environment to experimental ponds or to laboratory settings, particularly with regard to the loss of dominance by *M. chthonoplastes*. The changes comprised both shifts in dominance as well as replacements in composition of the dominant cyanobacteria. The extent of these changes was commensurate with the extent of difference in conditions between the original site and the sites of transfer. The most dramatic shifts occurred in transfers to laboratory conditions over a long period of time. A prevalence of two novel populations (filamentous cyanobacteria with very narrow trichomes and an unknown, but dominant, cyanobacterial group) were to be the most significant change following the transfer of natural mat community to the experimental ponds of Eilat.

The comparison between sites shows an apparent increase in Shannon's richness following transfer of microbial mats to experimental ponds (FB mat), paralleling a decline in viability of the previously dominant species *Microcoleus chthonoplastes*. In our judgment, this increase can be explained as follows: the original mat used in the transfer experiment is only one of several zonally arranged microbial mat communities, which characterize the Solar Lake (Campbell and Golubic, 1985). Accordingly the total richness of Solar Lake cyanobacteria is significantly higher than that of the particular microbial mat community that was used for the transplant experiments. The relatively small size and regular holomictic turnover of Solar Lake water column (Krumbein et al., 1977) provided ample opportunity for dissemination of propagules of various species, while growth and dominance was granted only to those best adjusted to local conditions in each particular mat zone. Transplant of the mat and associated environmental change resulted in re-setting of conditions for competitive growth of species previously suppressed and out-competed, and only present at undetectable levels. The possibility cannot be discounted, however, that the novel populations were indeed not present in the

original inoculum, and were selected for from environmental propagules.

The differences observed between the community structure and composition of the original Solar Lake mat and the transplants into experimental ponds or laboratory settings demonstrate that the experimental systems develop their own unique community structure and composition and, thus, cannot be considered representative of the system operating in Solar Lake. Accordingly, the results obtained from experimental systems after long-term incubation are not necessarily directly applicable to natural environments of origin.

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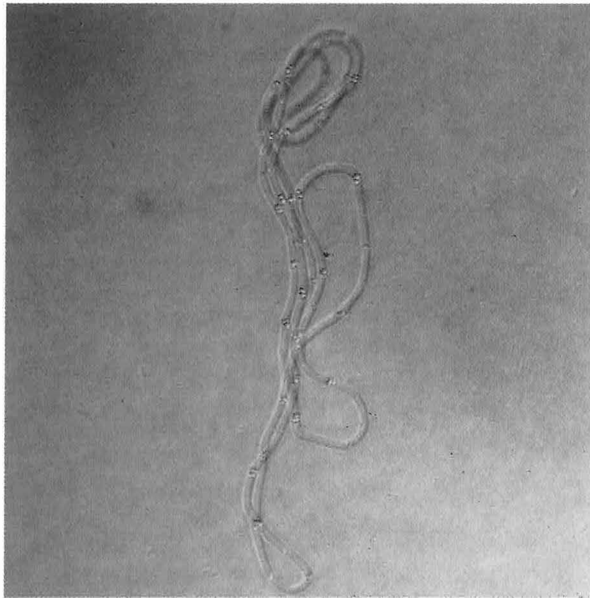
Chapter 3

Polyphasic characterization of benthic, moderately halophilic, moderately thermophilic cyanobacteria with very thin trichomes and the proposal of *Halomicronema excentricum* gen. nov., sp. nov.



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Front page:
Microscopic slide of the strain TFEP4 used for the description of the new genus *Halomicronema*. Note the size of the filaments and the distribution of gas vesicles.

Polyphasic characterization of benthic, moderately halophilic, moderately thermophilic cyanobacteria with very thin trichomes and the proposal of *Halomicronema excentricum* gen. nov., sp. nov.

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Abstract

A new genus of moderately halophilic, moderately halotolerant and moderately thermophilic cyanobacteria with very thin trichomes is described. The four strains included in this genus were isolated from benthic microbial mats in a man-made hypersaline pond. Trichomes were around 1 µm thick, with small constrictions at the cross-walls, and diffluent colorless sheaths. Thylakoids were parallel to the cell wall, but thylakoids and nucleoid were often excentrically arranged within the cytoplasm with respect to the main trichome axis. Strains grew between 3.2 and 12-15% (w/w) salinity with optima between 3.2 and 12%. They showed lower temperature limits around 20 °C and upper limits between 45 and 50 °C, with optima between 28 and 45-50 °C. Carotenoid and mycosporine amino-acid complements were identical among strains. Phylogenetic analyses based on 16S rRNA gene sequence analyses showed that all strains were closely related (99% or higher similarity) and distantly related to other cyanobacteria (91% or lower similarity). We propose the new genus and species *Halomicronema excentricum* for these strains. The type strain is TFEP1.

Introduction

Non-heterocystous thin filamentous cyanobacteria having trichome diameter of less than 2 µm, are known to occur in diverse habitats including thermal, subaerial, freshwater, marine and hypersaline environments (see for example, Whitton and Potts 2000). The taxonomic classification of such oxygenic phototrophs is next to that of the small unicellular forms (i.e. genus *Synechococcus*), one of the most controversial among the cyanobacteria. This is because the morphological features that make up the base of all cyanobacterial taxonomic systems in use do not suffice to differentiate morphologically simple cyanobacteria (Wilmotte et al. 1992; Garcia-Pichel et al. 1998). Very thin filamentous cyanobacteria are typically assigned to the genus *Phormidium* in the traditional botanical Geitlerian system (Geitler 1932) and to the genus *Leptolyngbya* in the botanical revision of Anagnostodis and Komarek (1988) and in the Bergey's Manual (Castenholz and Waterbury 1989). The latter genus is considered to be synonymous to the *ad hoc* LPP-B (*Lyngbya/Plectonema/Phormidium*-group B) in the bacteriological system of Rippka et al (1979).

The use of 16S rRNA sequence similarities for taxonomic placement is well established (Woese 1987). This molecular information has a strong potential in the characterization and taxonomy of cyanobacterial strains when combined with other traditional approaches (Wilmotte 1994; Garcia-Pichel et al, 1998; Nübel et al, 2000). 16S rRNA sequence comparisons have revealed that cyanobacteria with *Phormidium*-like morphologies do not form a monophyletic group, but occur within several clusters that are not closely related (see the phylogenetic trees presented by Giovanonni et al. 1988; Wilmotte 1994 and Garcia-Pichel, 2000). This indicates that there exist several different groups sharing *Phormidum*-like morphology, and that a phylogenetically sound taxonomy of these organisms will need redescription on the basis of characters other than simple morphological traits. Indeed, Wilmotte et al. (1992) demonstrated the 16S rRNA-based phylogenetic relatedness of several cultured marine oscillatorian cyanobacteria with narrow trichomes that have common ecological origin.

Using a combination of cultivation-independent molecular methods and microscopy, we have recently identified a potentially novel population of thin filamentous *Phormidium*-like cyanobacteria in a hypersaline microbial mat (Abed and Garcia-Pichel 2001). In microbial mats, even the recognition of very thin filamentous cyanobacteria as such can be problematic, since they cannot be easily distinguished from

morphologically very similar filamentous anoxygenic phototrophs. In this contribution, we present a polyphasic analysis of four novel strains isolated from these populations. We characterized the isolates based on their morphology, ultrastructure, 16S rRNA-based phylogeny, studies on temperature and salinity physiology, and chemotaxonomic markers (carotenoids and mycosporine-like amino acids). We propose a new genus to classify these novel isolates and populations.

Materials and Methods

Origin of the isolated strains

Samples for isolation were obtained from microbial mats maintained in artificial ponds in Eilat, which had originally been transplanted from Solar Lake (Sinai, Egypt) (Abed and Garcia-Pichel, 2001). We have shown that these mats contain prominent populations of thin filamentous cyanobacterium of uncertain phylogeny. These populations were not observed in the original Solar Lake transplants. DGGE analysis of the reverse transcribed 16S rRNA showed that these populations have rather high activity in the field (Abed and Garcia-Pichel, 2001).

Cultivation, isolation and microscopy

Medium (1/2 PES) was prepared by adding one-half strength nutrients, vitamins and trace elements according to Starr and Zeikus (1987) to a base of natural seawater. Hypersaline media of 70, 100, 120, and 150‰ final total salinity were obtained by adding appropriate amounts of NaCl to the formulation. All media were autoclaved. Solid media were prepared with 1% (w/v) agarose. Small pieces of mat were directly inoculated onto solid and liquid media at different salinities. These enrichments were incubated at different temperatures and checked microscopically for the growth of cyanobacteria. Four monospecific cultures with extremely thin filaments were isolated and further purified by successive transfers at the optimal conditions of growth (see below). Microscopic examination of the cultures revealed that they were free of other cyanobacteria, diatoms and to a large extent, heterotrophic bacteria. These four strains were designated as TFEP1, TFEP2, TFEP3 and TFEP4. Strain TFEP1 was obtained in axenic culture after repeated self-isolation on agar (Castenholz, 1988).

Transmission electron microscopy

Samples of growing cultures were prepared for electron microscopy according to Hernández-Mariné (1996). Briefly, specimens fixed with 2.5% glutaraldehyde in cacodylate buffer were post-fixed with 1% osmium tetroxide in the same buffer. They were dehydrated in a graded acetone series, embedded in Spurr's resin, and sectioned at 70 nm with an Ultracut E (Reichert-Jung, Nussloch, Germany); the sections were stained with 2% uranyl acetate and lead citrate. Sections were examined in a Hitachi-800 MT electron microscope operating at 100 kV.

Growth rate versus salinity and temperature

Strains were inoculated in deep Petri dishes filled with liquid medium of various salinities. BG11 medium was used to check the ability to grow under freshwater conditions (Rippka and Herdman 1992). They were incubated at 45 °C and illuminated constantly with fluorescent tubes at an intensity of 50 μmol (photons) $m^{-2}s^{-1}$. The increase in phycobilin and chlorophyl *a* fluorescence with time was measured as a proxy for biomass using a fluorimeter specially designed for cultures that grow attached to the vessel walls (Karsten et al. 1996). Growth was monitored in triplicate, independent cultures during 1-2 weeks, during which at least 3-4 doublings occurred. The instantaneous growth rates, μ, were estimated by linear regression of the natural logarithm-transformed fluorescence values. The coefficient of correlation in these regressions was typically around 0.95 (and never below 0.9). Means and standard deviations of three independent measurements are presented. The growth rates of the four strains were also measured at different temperatures (15, 22, 28, 35, 40, 45 and 50 °C). Strains were grown liquid medium at a salinity of 60‰ and incubated for 1-2 weeks at a light intensity of 50 μmol (photons) $m^{-2}s^{-1}$. Growth rates were determined as above. At the end of the experiments, all the cultures were kept for extra 2 months in order to check for slow growth rates at the given conditions.

Extraction and analysis of carotenoids and mycosporine-like amino acids (MAAs)

Cultures for carotenoid analysis were grown in liquid medium at 60‰ salinity and incubated at 45 °C. Cells were collected by centrifugation and the pellets obtained were homogenized with ice-cold 100% acetone to extract the carotenoids. The extracts were concentrated by evaporation under a stream of nitrogen and re-dissolved in 100 μl 100%

acetone. Strong light was avoided throughout the extraction procedure. Carotenoids were then determined by HPLC coupled to on-line ultraviolet/visible spectroscopy and identified by co-chromatography and spectroscopic matching with authentic primary or secondary standards (Karsten and Garcia-Pichel 1996). Cultures for the determination of mycosporine-like amino acids (MAAs) were pre-exposed to UV-B radiation according to Garcia-Pichel and Castenholz (1993) since the synthesis of these compounds has been shown to be UV inducible in some cyanobacteria. The cells were recovered by centrifugation and MAAs were extracted by submersion in 25% (v/v) aqueous methanol for 3 h at 45 °C. The extracts were concentrated in a vacuum evaporator. The residual matter was dissolved in 25% (v/v) aqueous methanol. MAAs were determined by HPLC and on-line spectroscopy as described by Karsten and Garcia-Pichel (1996).

Direct PCR amplification of 16S rDNA gene fragments

Nucleic acids were extracted from cell pellets of the four isolated strains by multiple sequential freeze-thaw cycles. PCR was carried out for the amplification of 16S rRNA genes directly on these extracts. Partial 16S rRNA sequences were obtained using cyanobacteria-specific oligonucleotide primers CYA359F (with a 40 nucleotide GC clamp at the 5′ end for DGGE purposes, see below) and CYA781R (Nübel et al. 1997). Approximately 10 ng of DNA was used as template for the PCR. Thermocycling was performed using Mastercycler gradient cycler (Eppendorf, Hamburg, Germany), the denaturation temperature was 95 °C for 5 min followed by 80°C for 1 min at which 1 µl of Super Taq DNA polymerase (HT Biotechnology, Ltd. Cambridge UK) was added. Thirty-five cycles were performed at 94 °C for 1 min, 60°C for 1 min (annealing temperature) and 72 °C for 1 min. In order to check that one and only one amplification product was obtained from each culture, we analyzed aliquots thereof by denaturing gradient gel electrophoresis as described before (Muyzer et al. 1998). The amplificates were then commercially sequenced.

Cloning of 16S rRNA genes

Cloning was done to obtain the full sequence of strain TFEP1, as it was not axenic at that time. The culture was first subjected to freeze and thaw cycles 3-5 times and the extract was amplified directly by PCR using the GM3 and GM4 primers (Hicks et al. 1992; Kanne et al. 1993). The PCR products were purified using the QIAquick PCR

purification kit (Diagen, Düsseldorf, Germany) and were cloned by applying pGEM-T plasmid vector system and Competent JM 109 cells (Promega, Heidelberg, Germany). The clones obtained were screened for the presence of cyanobacterial inserts, as described before (Garcia-Pichel et al. 1998). Several plasmids from different clones having cyanobacterial inserts were prepared using the Wizard Plus Minipreps Kit (Promega) and mixed together. The full sequence of the 16S rRNA from the plasmid inserts were re-amplified by PCR using the same primers (GM3 and GM4) and subsequently sequenced commercially.

Sequence analysis and phylogenetic affiliation

Complete cyanobacterial 16S rRNA gene sequences available from GenBank were imported and aligned to the sequences in the ARB database (Ludwig et al. 1998). A phylogenetic tree was constructed based on long 16S rRNA sequences (more than 1300 bp) by applying different methods integrated in the ARB software such as maximum likelihood, maximum parsimony and neighbor joining. The 16S rRNA sequences of *Escherichia coli* and *Bacillus subtilis* were included in the calculations as outgroup sequences. The partial sequences obtained from our strains with the cyanobacteria-specific primers, as well as the almost complete sequence obtained by cloning were aligned to the sequences in the ARB database using the alignment tool of the ARB software package. These sequences were then inserted into the pre-established tree using the parsimony ARB tool and maintaining the overall tree topology without changes. Additionally, a BLAST analysis (http://www.ncbi.nlm.nih .gov/BLAST) was carried out against the complete GenBank database before submission (23 April 2001)

Results

The four strains were isolated in liquid medium of 70‰ salinity and a temperature of 28°C; they grew poorly on solid agar media compared to liquid media. Growth was also possible in other seawater-based media such as f/2 (Guillard and Ryther 1962) and in chemically defined media such as ASN III (Rippka 1988).

Morphology and microscopic observations

The four isolates had very similar morphologies (Fig. 1), although minor differences in cell dimensions were present. All strains were filamentous, with trichome width around

1μm. These are among the narrowest trichomes described for filamentous cyanobacteria. Cells were always longer than wide (2-8 μm long). Trichomes did not appear constricted at the crosswalls under optical microscopy. Using Nomarski interference optics the presence of small inclusions less dense than the cytoplasm (usually interpreted as gas vesicles) at each side of the cross-walls was typical in all strains. End cells were rounded, without any wall thickening and also with gas vesicles, which sometimes results in a slight bulbousness of the end cell. The sheath was colorless, very thin and diffluent and only observed with phase contrast microscopy. Slow gliding motility was present and cultures displayed long-term populational phototaxis towards a light source by gliding on solid substrates. These isolates were morphologically indistinguishable from the thin filamentous cyanobacterial populations that we had observed in the microbial mats of origin (Abed and Garcia-Pichel 2001) and their morphology fits well the description of *Phormidium hypersalinum* (Campbell and Golubic 1985).

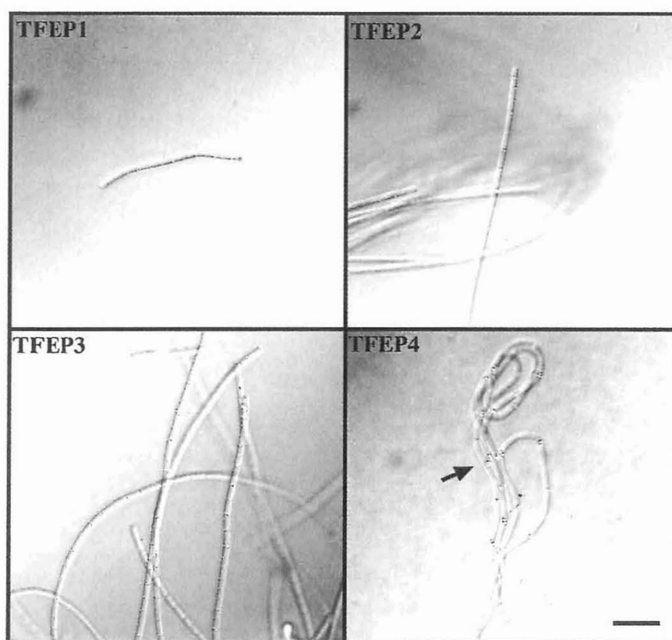

Figure 1. Photomicrographs of the thin filamentous, non heterocystous cyanobacterial strains investigated grown in ½PES medium at 7% salinity and 28°C. Strain denominations are indicated in each panel. All strains are shown at the same magnification. (Bar indicates 10μM).

Ultrastructure

All four strains had very similar ultrastructural features, differing only in quantitative aspects of such features. Interestingly, electron microscopy revealed characteristics that are usually considered morphological, such as the presence of trichome constrictions at the cross walls, which, due to their small size, could not be detected with optical microscopy (Figs 2, 4). The presence of gas vesicles next to the cross-walls (Fig 4) was confirmed. Thylakoids were arranged concentrically and parallel to the cell wall, but unlike in other cyanobacteria, their distribution was often asymmetrical with respect to the trichome long axis (Fig 3, Fig 4), with several thylakoids on one side but only one on the opposite side. As a result of this, the nucleoid was also asymmetrical with respect to the trichome axis. This feature resulted in cells that had strong autofluorescence on one side and weak on the opposite side when observed under confocal epifluorescence microscopy (not shown). In elongated (older) cells, large extensions of the intrathylakoidal space could be observed close to the septum (Fig 4). Pores were distributed irregularly on the cell wall and septum, and no junctional pores or central perforations were seen. Polyhedral bodies (carboxysomes), cyanophycin granules and lipid inclusions were also observed. No polyhydroxyalkanoate granules were seen.

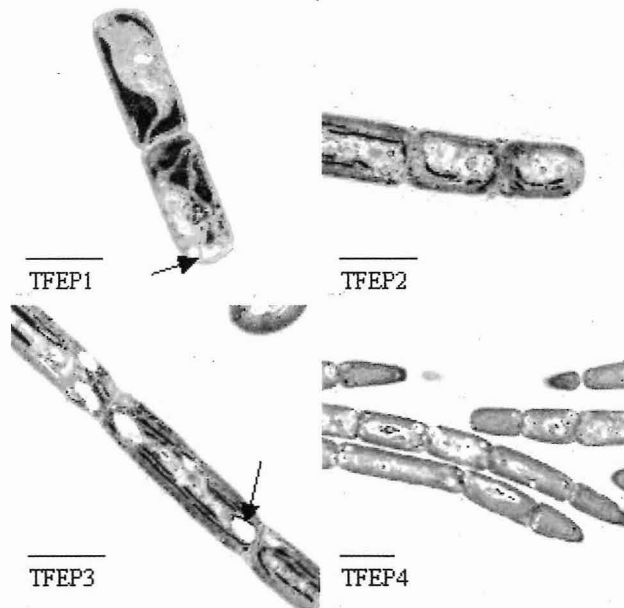

Figure 2. Ultrastructural aspects of the four strains studied. For strain TFEP1, a two celled hormogonium is depicted, which contains apically placed gas vesicles (arrow). Notice the small diameter of the trichomes (black lines are 1μm scales), the rounded ends of apical cells, and the small constrictions at the cross walls, about 100 nm deep, not visible with optical microscopy. Visible in strain TFEP3 are dialations of the intrathylakoidal space next to the cross walls (arrow), typical of older, elongated cells.

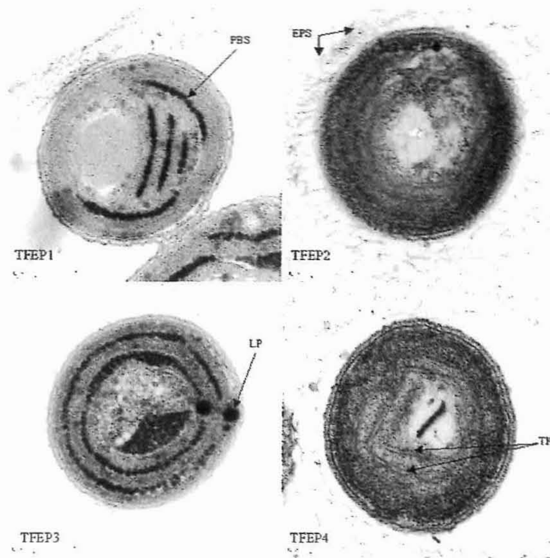

Figure 3. Cross-sectional views of the four strains. Strain denominations are indicated in each panel. Note the disposition of the thylakoids (TK), concentrical and parallel to the cell wall. In many cells, the thylakoids are asymmetrically placed with respect to the central trichome axis, as exemplified here in strain TFEP1. EPS: Extracellular polymeric substance. PBS: phycobilisomes. LP: lipid inclusions. Black bars indicate 0.1 μm

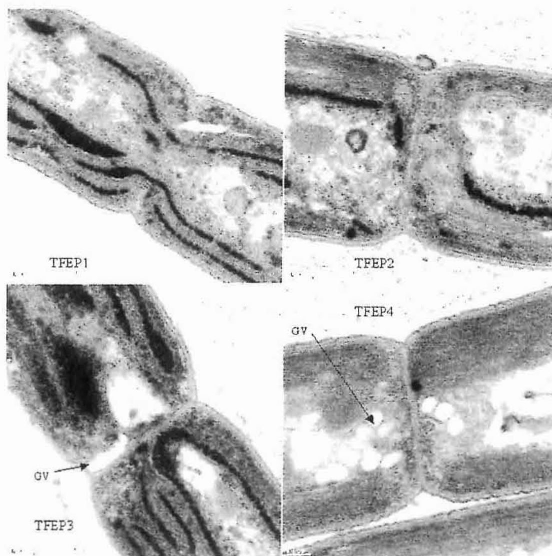

Figure 4. Transversal sections of the four strains. Strain denominations are indicated in each panel. Note the incipient, symmetrical cell division in the section of strain TFEP1. The outer membrane is not involved in the formation of the septum or cross-wall. Typical are the groups of a few gas vesicles (GV) placed close to the septum. Black bars indicate 0.1μm

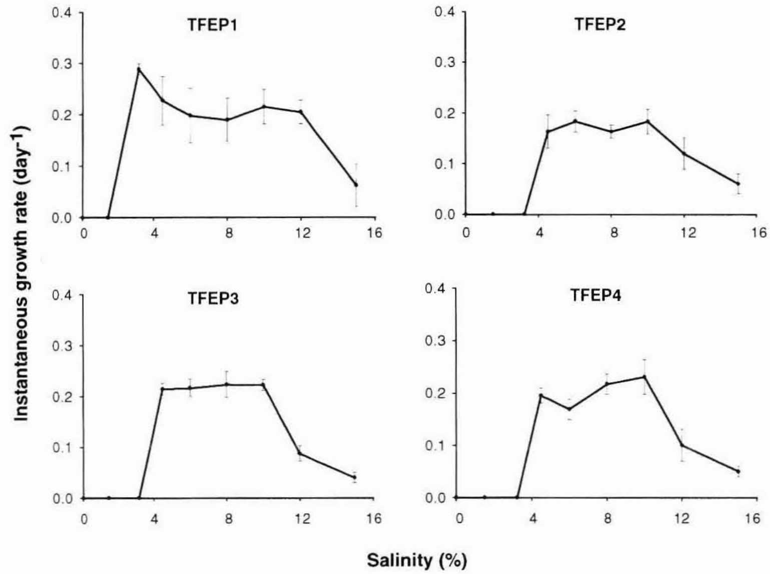

Figure 5. Growth rates of the four studied strains grown at different salinities. All measurements were performed at 45°C. Error bars indiacte 2 SD of triplicate measurements. Strain denominations are indicated in each panel.

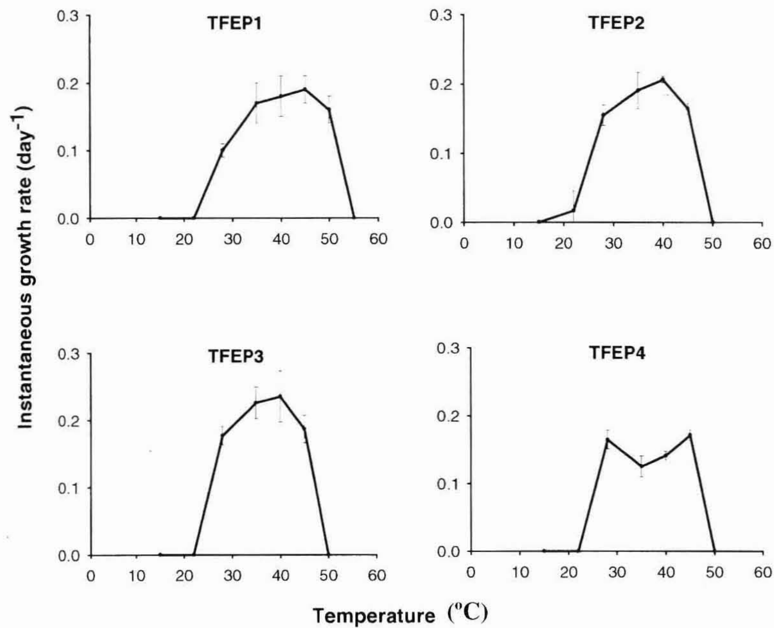

Figure 6. Growth rates of the four studied strains grown at different temperatures. All measurements were performed at 6% salinity. Error bars indiacte 2 SD of triplicate measurements. Strain denominations are indicated in each panel.

Growth rates and salinity

None of the four strains grew in fresh-water medium (Fig 5). Thus, elevated ionic concentrations were a requirement for growth, a characteristic typical of truly marine cyanobacteria. We could not measure significant growth of strains TFEP2, TFEP3 and TFEP4 at 15 and 32‰ salinity during the duration of our measurements. However, under long-term incubation (ca. 2 months), growth was positive for all strains at 32‰ but not at 15‰ salinity. These same three strains exhibited optimal growth between 45-100‰ salts, decreasing remarkably at 120‰ salinity and above. Strain TFEP1 had a somewhat broader salinity range for growth, with high growth rates observed at 32 and up to 120‰. This strain showed a decreased rate of growth at 150‰ salinity and above. Therefore, the four investigated isolates can be described as strictly marine and moderately halotolerant, with optima between 45-120‰ salinity (moderately halophilic). In comparison to other halotolerant cyanobacteria such as those in the Halothece cluster (Garcia-Pichel et al. 1998) or *Halospirulina* strains (Nübel et al. 2000), they were relatively stenohaline.

Growth rates and temperature

The growth rates of the four strains at different incubation temperatures are shown in Fig 6. The estimated lower temperature limit for growth in all strains was around 20 °C. The temperature range of growth was quite homogenous among three isolates (TFEP2, TFEP 3 and TFEP 4) but again slightly different for the fourth (TFEP1). Strains TFEP2, TFEP 3 and TFEP 4 exhibited optimal growth within the temperature range 28 – 45 °C but growth was not possible at 50 °C. Strain TFEP1 had a somewhat deviant behavior since it could grow optimally from 28 up to 50°C. At 55 °C this strain lost pigmentation and subsequently died.

16S rRNA phylogeny

Partial 16S rRNA gene sequences of the four strains and virtually complete sequence of strain TFEP1 were deposited in the GenBank database under the accession numbers AF320089-AF320093. The phylogenetic relationships of the four strains and other cyanobacteria are shown in a tree calculated applying the maximum likelihood method (Fig 7). All these sequences consistently clustered together and were distant to other 16S rRNA cyanobacterial sequences available in the database. Similarity matrix calculation showed that the 16S rRNA partial sequences of the four strains were more than 99%

similar. The divergence of these sequences from the closest cyanobacteria in the tree (*Phormidium* sp. N182, *Phormidium* sp. D5, and *Plectonema* sp. F3) was found to be 10% or more (Fig 7). The 16S rRNA gene sequences of the four strains shared more than 98% sequence similarity to the field DGGE band FB2 (Abed and Garcia-Pichel 2001). This indicates that these strains are representative of the field populations and not a cultivation bias.

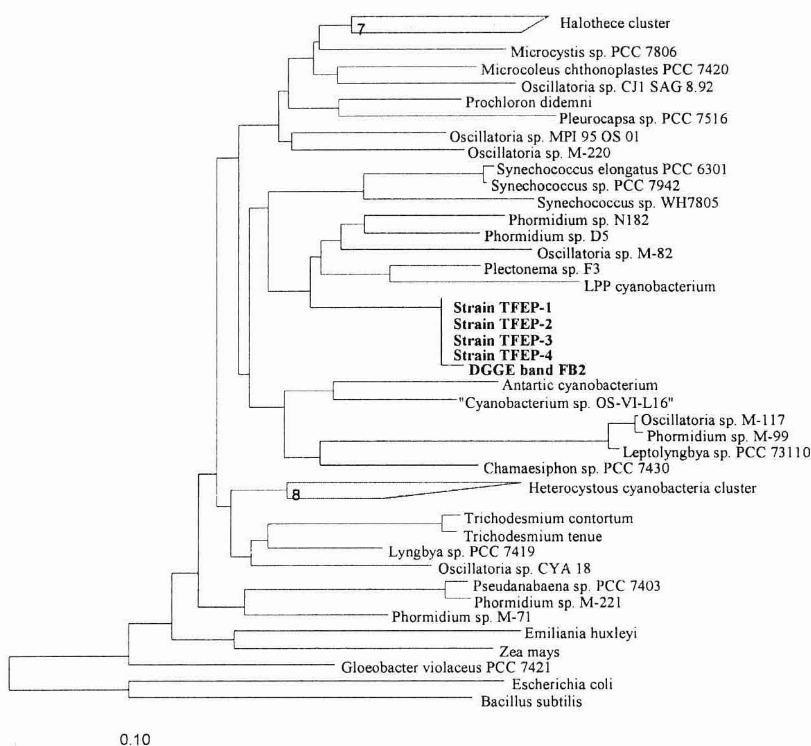

Figure 7. Phylogenetic reconstruction of the cyanobacteria based on 16S rRNA sequences showing the affiliation of the 16S rRNA gene sequences obtained from the four investigated strains (TFEP1, TFEP2, TFEP3, and TFEP4) either by direct amplification of gene fragments or by cloning of the complete genome. The tree was constructed using almost all available 16S rRNA cyanobacterial complete sequences but was simplified here for clarity. The affiliation was done using the parsimony ARB tool (Ludwig et al. 1998) without changing the overall tree topology. The 16S RNA gene sequences from *Escherichia coli* and *Bacillus subtilis* were used as the outgroup sequences. The bar indicates 10% sequence divergence. The domination of particular strains corresponds to those in the database and we do not imply that they are taxonomically correct.

Carotenoids and MAAs analysis

Three carotenoids were found to account for most of the carotenoid complement with several other minor ones present. All four strains contained the same major carotenoids in the exponential phase of growth, although in different ratios. Zeaxanthin, having an

absorption maximum at approximately 450 nm and retention time of ca. 6 min, constituted about 44% of the total carotenoid pool. ß-carotene, having absorption maximum at 480 nm and retention time of ca. 18 min, constituted 18% of the pool. The third major carotenoid had a retention time at 7.58 and constituted about 9.5% of the total carotenoids. We were not able to identify this carotenoid in comparison to available standards but on the basis of published spectra it was probably ß-cryptoxanthin. Echinenone and canthaxanthin, which occur in many cyanobacterial strains, were not found in any of ours. Mycosporine-like amino acids (MAAs), known to function as ultraviolet sunscreens (Garcia-Pichel and Castenholz 1993), were not detected in any of the four studied strains even after exposure to UV radiation for more than a week. Such compounds were previously reported to occur in various groups of cyanobacteria isolated from hypersaline environments (Karsten and Garcia-Pichel 1996; Garcia-Pichel et al. 1998).

Discussion

Taxonomy of thin filamentous cyanobacteria: problems and challenges

The simple morphology of thin filamentous cyanobacteria, and the absence of consensus in delimiting alternative traits of taxonomic relevance, has resulted in diverging, unstable concepts for their taxonomic treatment in the botanical as well as in the bacteriological approach. The Geitlerian system (Geitler 1932) recognized the genus *Phormidium* to include those non-heterocystous filamentous cyanobacteria with very thin trichomes. But Anagnostidis and Komarek (1988), acknowledging the simplicity of this approach, established several additional new genera (*Leptolyngbya*, *Pseudophormidium*) to accommodate some former *Phormidium* species. In the bacteriological approach of Rippka et al. (1979), a temporary, non-generic group (LPP-group) encompassing strains from the botanical genera *Lyngbya*, *Plectonema* and *Phormidium sensu* Geitler (1932) was proposed. In their treatment of the Oscillatoriales (subsection III), Castenholz and Waterbury (1989) redefined previously established boundaries of the LPP group, recognizing the genus *Lyngbya*, and transferring some strains to the genus *Oscillatoria*, but the genus *Phormidium* was not explicitly treated or recognized. This abridged account conveys the difficulties that have plagued the classification of non-heterocystous filamentous cyanobacteria, in general, and of the thin filamentous forms in particular. When one looks at the results obtained from phylogenetic reconstructions based on 16S rRNA sequences, the data do not provide support for any of the taxonomic groups

established (generic or otherwise). Thus, for example, Giovanonni et al. (1988) demonstrated that diverse strains from the LPP group did not cluster phylogenetically. Also in their treatment, it was obvious that thin filamentous strains (*Oscillatoria (Phormidium) limnetica*, "*Microcoleus* 10mfx", *Phormidium ectocarpi* PCC7375, *Plectonema* PCC73110) are also polyphyletic. Similar conclusions can be obtained by analyses of more recent and complete sequence datasets (Wilmotte 1994; Garcia-Pichel 2000; our Fig 3). Thus, it becomes evident that a successful, phylogenetically coherent classification of the filamentous forms will require the use of criteria other than morphology for the establishment of genera.

A polyphasic taxonomic approach integrating data and information from ecology, morphology, phylogeny and physiology, has provided a strong potential in the classification and definition of phylogenetically coherent groups of microorganisms (Wilmotte 1994; Vandamme et al. 1996). Such comprehensive approach has successfully been applied in the study of extremely halotolerant unicellular cyanobacteria (Garcia-Pichel et al. 1998; Margheri et al. 1999) which led to the formation of the phylogenetically and physiologically coherent *ad hoc* "Halothece" cluster. Indeed, new cyanobacterial genera have been described based on this approach, such as *Halospirulina* encompassing the extremely halotolerant filamentous cyanobacteria with tightly coiled trichomes (Nübel et al. 2000). These and other studies underscore the importance of ecological origin and physiological capacities, such as halotolerance, in delimiting low rank taxonomic units.

Extremely thin filamentous cyanobacteria from hypersaline environments

That the 16S rRNA molecular signatures of the four strains shared more than 99% similarity among themselves and with its corresponding field populations (Abed and Garcia-Pichel 2001) confirms that our strains are closely related and representative of such a population and not a result of enrichment bias. The molecular analysis correlated with the fact that morphologically and ultrastructurally all four strains were very similar. Carotenoid and MAA analyses, which have proven useful as chemotaxonomic markers for closely related strains (Wilmotte et al. 1992; Garcia-Pichel et al 1996; Garcia-Pichel et al. 1998; Nübel et al. 2000) confirmed the close relatedness of the four studied strains, since they share a common pattern of carotenoid complement and an absence of mycosporine-like amino acids (MAAs). Taken together all these similarities in morphology, phylogeny, halotolerance and biomarker composition, indicate that these

isolates form a phylogenetically and phenotypically coherent group of cyanobacteria and they belong in a single genus.

On the other hand, this cluster exhibited more than 9% genetic divergence to other cyanobacteria including *Phormidium*-like strains. The closest relatives in the tree were the marine strains *Phormidium* sp. D5 and *Phormidium ectocarpi* sp. N182 which had sequence similarities of about 87 and 90% respectively, to the sequences in this cluster. A BLAST analysis (May, 2001) against all the sequences (including partial sequences) in GenBank yielded as closest match 3 sequences with similarities of 91% corresponding to filamentous cyanobacteria. This indicates that our strains exhibit sufficient genetic divergence from all other *Phormidium*-like strains from which full 16S rRNA gene sequences are available to warrant a new generic-level taxonomic separation. In a recent study by Nübel et al. (2000), a new genus *Halospirulina* was proposed to describe halotolerant cyanobacteria with *Spirulina*-morphology, which had 7.7% or more 16S rRNA gene divergence to all other cyanobacteria. The growth limits for salinity in our strains are consistent with their habitat of origin, which may have provided the opportunities for a separate line of evolution. Their apparent thermotolerance is a characteristic that has been also found in many halotolerant cyanobacteria such as the unicellular forms in the Halothece cluster (Garcia-Pichel et al. 1998) and the tightly coiled *Halospirulina* isolates (Nübel et al. 2000). This might be an adaptation to life in such hypersaline waters, which due to the high specific heat of brines easily reach elevated temperatures when sunlit. However the sensitivity of our strains to cold temperatures (less than 20 °C) is certainly an unusual trait.

We therefore propose *Halomicronema* gen. nov, to classify these strains. This genus could be further extended to include more isolates from other hypersaline environments, which are already known to harbor such thin filamentous non-heterocystous cyanobacteria populations (D´Amelio et al. 1989; Ward et al. 1989).

Description of *Halomicronema* gen. nov.

Halomicronema gen. nov. (Ha.lo.mi.cro.ne' .ma. Gr. N. *hals, halos*, salt;Gr. *mikros, small*, Gr. N. n. *nema, filament; N. L. n.Halomicronema* salt-tolerant small filament)

Moderately halophilic and halotolerant, benthic, marine, filamentous, non-heterocystous cyanobacteria of very thin trichomes. Growth is optimal at salinities between 45 and

120‰ (w/v) total salts and ceases above 170‰ and below 30‰. Trichomes are cylindrical, around or slightly less than 1 μm wide. Thylakoids are arranged concentrically and parallel to the plasma membrane, single or stacked. Pores are distributed irregularly on cell wall and septa. No junctional pores observed. Found in sunlit hypersaline environments.

Description of *Halomicronema excentricum sp nov.*
Halomicronema excentricum sp. nov. (ex.cen'tri.cum L. preff. *ex*, away from; L. n. *centrum*, center; *excentricum* , away from the center, excentrical)

Moderately halotolerant, benthic, marine, filamentous, non-heterocystous cyanobacteria of extremely thin trichomes. Growth is optimal at salinity between 45 and 120‰ total salts and ceases above 170‰ and below 30‰. Moderately thermophilic, grow at 45-50 °C and are sensitive to temperatures below 20 °C. Trichomes are cylindrical, around or below 1 μm wide. Cells are quadratic to elongated, 2-8 μm long. Apical cells are rounded. Trichomes are slightly constricted at cross-walls (visible under electron microscopy only). Sheaths are thin or diffluent and colorless. Thylakoids are arranged parallel to the plasma membrane, single or stacked but distribution is typically asymmetrical with respect to the trichome axis, giving an excentrical cross-sectional appearance to cellular ultrastructure. Groups of a few gas vesicles are typically present next to cross-walls. Pores are distributed irregularly on cell wall and septa. No junctional pores present. Trichomes and cultures are blue-green in color and phycoerythrin is absent. Gliding motility is present, but motility is slow. Cell populations are phototactic. Found in sunlit hypersaline benthic environments. Type strain is TFEP1^{T}, isolated from a microbial mat in man-made solar ponds in Eilat, deposited in the Culture Collection of Microorganisms from extreme Environments, Eugene, OR, USA.

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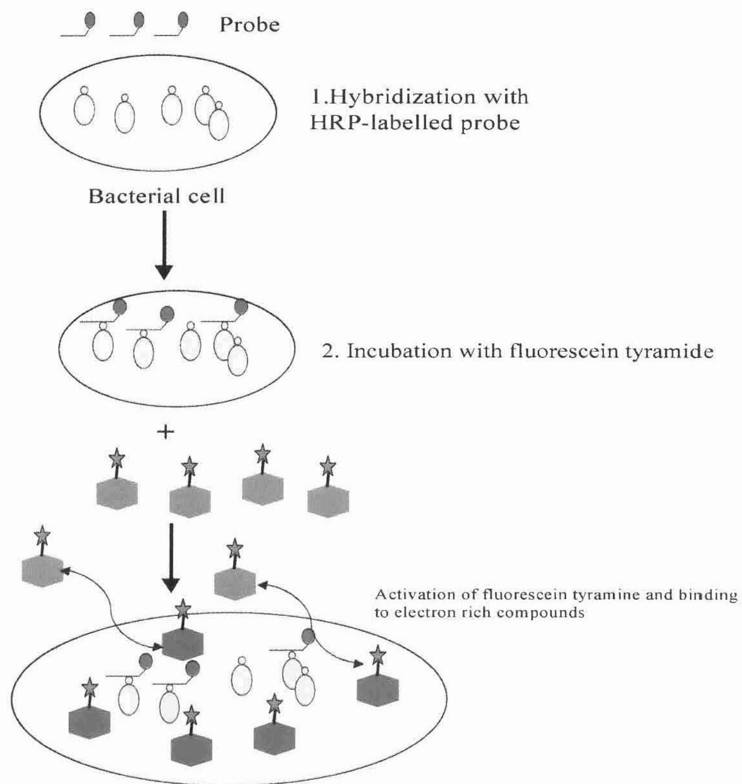
Chapter 4

Discovery of Picobenthic Cyanobacteria by 16S rRNA Targeted *In Situ* Hybridization



This chapter has been submitted to Applied and Environmental Microbiology

Front page:
Detection of cyanobacterial populations by *in situ* hybridization with horseradish-peroxidase labeled oligonucleotide probes

Discovery of Picobenthic Cyanobacteria by 16S rRNA-Targeted *In Situ* Hybridization

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Abstract

We report on the morphological identification of a population of benthic cyanobacteria from microbial mats known previously only from molecular analyses of field samples based on the retrieval of environmental 16S rRNA sequences. We used *in situ* hybridization with horseradish peroxidase-labeled oligonucleotide probes designed to target the 16S rRNA of our unidentified population. Two probes were designed and were checked for target binding ability and specificity using membrane hybridization against electroblotted bands from a denaturant gradient gel electrophoresis (DGGE) fingerprint of 16S rDNA gene fragments from the original cyanobacterial community. Under *in situ* hybridization, these probes bound specifically to extremely small, unicellular, colony forming cyanobacteria, 0.75 to 1µm in diameter, which were embedded in abundant mucilagenous investments. While picoplanktonic cyanobacteria are abundant in ocean and freshwaters, picobenthic (< 1 µm) unicellular cyanobacteria have not been previously described. The small size and low levels of photopigment autofluorescence from these cells probably rendered them cryptic to routine microscopic observations. It is not known how widespread picobenthic cyanobacteria may be in other environments.

Introduction

Culture-independent approaches have led to the retrieval of a large number of novel bacterial 16S rRNA gene sequences directly from environmental samples (4, 12,15, 23). Identification of the corresponding populations of these gene sequences in the field by direct microscopy is typically difficult due to their simple morphology. Cultivation has also proven difficult for many environmentally important microorganisms (9, 28, 29). However, in case of cyanobacteria, relating 16S rRNA signatures to corresponding organisms in the field can be less problematic because in many cases they have distinct morphology. Recent polyphasic characterization of certain groups of cyanobacteria resulted in good agreement between cyanobacterial 16S rRNA sequence and morphology (13, 14, 19). In microbial mats in general and hypersaline ones in particular, cyanobacteria have been well characterized and identified based on their phylogeny and morphology (1, 21). Indeed, it has also been shown that phylogenetic sequences obtained directly from the field were well represented by cultivated cyanobacteria (18). There are, however, particular cases in which the morphological identification of a cyanobacterial 16S rRNA obtained directly from the field could not be achieved (10).

While using culture-independent molecular methods to describe the cyanobacterial community changes in a Solar Lake hypersaline microbial mat (Sinai, Egypt) that had been transplanted and maintained in an experimental pond (1), we observed the development of two unidentified novel cyanobacterial populations represented by two unique 16S rRNA sequences. Phylogenetic analysis did not point to any close affiliation to the 16S rRNA of any known cultivated cyanobacteria in public databases. The first cyanobacterium was successfully isolated in culture and corresponded to an extremely thin filamentous form (1 μm diameter), which probably conforms a new genus (R. M. M. Abed, F. Garcia-Pichel, and M. Hernández-Mariné, submitted for publication). The second population exhibited the most dominant molecular signature but we could neither relate it to a particular morphology by direct microscopy, nor bring it into culture. This cyanobacterium remained cryptic.

In this study, we report the morphological identity of this mysterious cyanobacterium, by using *in situ* hybridization with horseradish peroxidase (HRP) labeled oligonucleotide probes designed specifically for its 16S rRNA (24).

Materials and Methods

Nucleic acids were extracted from different cores of a hypersaline microbial mat growing in an experimental pond in Eilat as described before (1). PCR was carried out for the amplification of 16S rRNA genes directly on these extracts. Partial 16S rRNA sequences were obtained using cyanobacteria-specific oligonucleotide primers CYA359F (with a 40 nucleotide GC clamp at the 5´end) and 781R (20). DGGE (Denaturant Gradient Gel Electrophoresis) was carried out in order to separate cyanobacterial 16S rRNA fragments of different community members. These DGGE gels were used for electroblotting and membrane hybridizations (see below). The DGGE bands were sequenced commercially and their phylogeny was studied using the ARB software (16) available at http://www.mikro.biologie.tu-muenchen.de. A complete description of the cyanobacterial community of the experimental pond mats using microscopy, cultivation and phylogenetic reconstruction, is given elsewhere (1).

Probe Design

Probe design was done with the appropriate tool in the ARB program package on the basis of environmental sequences obtained previously. From a selection of potential oligonucleotide sequences two were chosen (see Fig. 1) to optimize *in situ* accessibility of the corresponding target region (11) and after verifying the theoretical specificity with 'Probe match', another tool of ARB. Probes were labeled with horseradish peroxidase (HRP) as described before (26).

Electroblotting of DGGE gels

Transfer of the DNA bands from DGGE gels to nylon membranes was carried out according to Muyzer et al. (17). Briefly, a nylon membrane (Hybond N+, Amersham) and filter papers were cut to the dimensions of the DGGE gel. After equilibration in 0.5X TBE buffer, the nylon membrane was placed on top of the DGGE gel. Together, they were embedded between two piles of pre-soaked filter papers. The electro-transfer was performed at 400 mA for 1 hour. The membrane was then incubated in denaturing solution (0.4 M NaOH, 0.6 M NaCl) and subsequently washed twice with 2.5X SSC buffer (3 M NaCl, 0.3 M sodium-citrate, pH 7.0). It was exposed to 302-nm UV

radiation for 45 sec to cross-link the DNA fragments to the membrane, which were then used for hybridization.

Blot hybridization

Hybridization of the blotted DGGE gels was done as described for dot blots (24). In short, the membrane filters were prehybridized for at least 1 h with 10 ml solution containing 10 × SSC, 4% blocking reagent (Roche Diagnostics, Germany), 0.2% N-lauroylsarcosine, and 0.04% SDS at 35°C. This temperature was used throughout the hybridization and washing steps. Hybridization was done by replacing this solution by 2 ml hybridization buffer (20 mM Tris/HCl [pH 8.0], 900 mM NaCl, 2% blocking reagent [Roche Diagnostics], x% formamide, 0.01% SDS) containing 100 ng HRP-labeled probe and incubating for 30 min. The formamide concentration was increased stepwise from 20 to 70% for successive hybridizations in order to determine an appropriate stringency for each of the two probes. Subsequently, the filters were washed twice for 15 min with 20 ml of washing buffer (20 mM Tris/HCl [pH 8.0], 0.01% SDS) containing NaCl at molarities equivalent to the stringency of the formamide concentrations used during hybridization and finally rinsed in 1×PBS. Specifically bound nucleic acid probes were visualized via HRP-induced chemiluminescence (ECL, Amersham, Braunschweig, Germany). Emitted light was recorded by exposure of membranes to X-ray films (Hyperfilm, Amersham, Braunschweig, Germany). Before reusing the membranes, they were incubated with a solution containing 0.5% SDS and 0.1 × SSC for 20 min at 80°C, afterwards rinsed in 2 × SSC, and finally either prehybridized again or air dried and stored at –20°C for further experiments.

Sample preparation

Core samples from the microbial mat were frozen by immersion in liquid N2 in the field and stored at –80°C prior to usage. 10 µl-aliquots of mat suspensions were then spotted onto wells of Teflon coated microscopic slides and air-dried. Alternatively, cores were individually incubated in embedding medium (Tissue Tec; Miles Inc., Elkhart, IN, USA) at 4°C overnight on a rotating device prior to cryosectioning. Sections of 10 µm thickness were prepared using a cryomicrotome (HM 505E, Microm, Walldorf, Germany) at a temperature of –16°C and placed onto the wells of Teflon-coated slides. After air drying, the embedding medium was removed by spotting sterile distilled H_2O onto the sections, incubating them for a few minutes, draining off the water, and air

drying them. Slides containing either aliquots or sections were submitted to an increasing ethanol series (50, 80, and 96%) and air-dried again.

In situ hybridization

The hybridization buffer used was as above, with the stringency set as determined in the membrane hybridizations (65% formamide for probe DGGE 413 and 50% for probe DGGE 573). Different stringencies from 20-70% were also tried. 50 ng of HRP-labeled probe in 10 μl hybridization buffer was used for each of the individual wells and the hybridization was performed in a humid chamber at 35°C for 2 h. The slides were then briefly rinsed with preheated washing buffer (as above but in addition containing 5 mM EDTA), and incubated then for 20 min at 35°C in the same buffer. A fluorescent detection of the bound HRP-labeled probes was done using the TSA method described earlier (24), except that incubation times were extended to 1 h. A Zeiss Axiophot2 (Carl Zeiss, Jena, Germany) equipped for epifluorescence with a high-pressure mercury bulb (100 W) and Zeiss filter sets 09 and 15 was used. Epifluorescence micrographs and the corresponding phase contrast images were taken with Axiophot2 imaging system (Zeiss, Germany). For confocal microscopy a Zeiss LSM 510 (Carl Zeiss, Jena, Germany) was used.

Cultivation attempts

Different approaches were tried to isolate this cyanobacterium and bring it into culture. Pieces of the mat were inoculated directly into different media (1/2 PES, ASNIII, and MN) (22, 25) at different salinities (0.5-15%) and those cultures were incubated at different temperatures (15-45 °C). Intact cores of the microbial mats were homogenized in seawater-based buffer and aliquots of that suspension were filtered through 5 μm Millipore filters in order to check for the growth of very small cyanobacteria. The filtrates were used to inoculate different media and incubated at different conditions. After 1-3 months of inoculation the cultures were screened for the growth of cyanobacteria other than the ones already observed by direct microscopy.

Results

Phylogenetic reconstruction based on 16S rRNA gene sequences

The phylogenetic affiliation of the 16S rRNA gene sequence of this unknown cyanobacterium was previously shown (1). The phylogenetic study was repeated using a newly updated database in order to check for recently described potential closer relatives. Our sequence showed less than 90% similarity to all sequences available in the database except for an uncultivated cyanobacterium from a hot spring microbial mat ("Cyanobacterium sp. OS-VI-L16"; 91.5% similarity) (27). The closest relatives included a group of thin filamentous cyanobacteria (*Phormidium* and *Leptolyngbya*) and unidentified environmental clones WH7B and WH12 retrieved from microbial mats of Great Sippewissett Salt Marsh, USA (8). The next closest sequences to our unknown belonged to a group of *Synechococcus* species among which was the sequence of *Synechococcus lividus* dominant in hot spring mats. The similarity between our sequence and those sequences was not more than 88%. An exhaustive survey of our microbial mats for sequence variability in our unknown cyanobacterium yielded sequences that invariably shared more than 98% similarity to each other.

Probe design and specificity

Two oligonucleotide probes were designed to be complementary to the sequences obtained from DGGE bands, probe DGGE 413 and DGGE 573. Using partial sequences of the 16S rRNA for the probe design limited our choice of probes because of the few potential target regions available. However, the uniqueness of our partial 16S rRNA sequence, which had more than 10% divergence to all available sequences in the database, enabled appropriate probe design. The positions of the corresponding target regions were chosen for good *in situ* accessibility according to (11). The brightness classes for the respective probes in E.coli were II (probe DGGE 413; 80-61% relative fluorescence compared to Eco1482 probe) and III (probe 573; 60-41%) respectively. This means that the signals obtained by these two probes should be theoretically bright enough for detection. The probes were then checked for their specificity using the ´probe match` tool of ARB, the matching results are shown in Fig. 1. Probe DGGE 413 was highly specific even if two mismatches were allowed and only the sequences from our unidentified DGGE band could serve as a target. Probe DGGE 573 was less specific and showed matching with other database sequences shown in Fig. 1. Nevertheless, this

probe showed at least three mismatches, which is enough for descrimination, with sequences obtained from other cyanobacteria known to inhabit the same mat sample. This indicates that this probe was very specific within the diversity contained in the mat sample.

Probe **DGGE 413**	3' CTGCCGGATTACCAACAT 5'	
Target (E. coli position 413-431)	5' GACGGCCUAAUGGUUGUA 3'	
FB1 band*	....	Sequences obtained either directly (DGGE bands) or from isolates from the same kind of mat
EP1 band*	....	
Microcoleus chthonoplastes	..A...UCUUG......	
Thin filamentous cyanobacteria	..A.....CUC.......	
Unicellular cyanobacteria (Halothece)	..A..UCUUG.C...C	
Oscillatoria sp.	..A....UUG..C...	

Probe **DGGE 573**	3' TTCGCAGGCATCCACCAA 5'	
Target (E. coli position 573-591)	5' AAGCGUCCGUAGGUGGUU 3'	
FB1 band*	....	Sequences obtained either directly (DGGE bands) or from isolates from the same kind of mat
EP1 band*	....	
Microcoleus chthonolplastes	.........C......GG	
Thin filamentous cyanobacteria	.........C...C..C.	
Unicellular cyanobacteria (Halothece)	.........C...G.GG	
Oscillatoria sp.	.........C......CC	
Cyanothece aeruginosa	....	Sequences retrieved from the ARB database
Uncultured cyanobacterium WH12	....	
Uncultured cyanobacterium WH7B	....	
Phormidium sp.	....	
Oscillatoria sp.	....	
Cyanothece PCC 7424	....	
Synechocystis sp. PCC 6803	....	
Leptolynghya foveolarum	....	
"*Chamaesiphon* PCC 7430"	....	
Plectonema sp. F3	....	

Figure 1. Probe match results against sequences available in the database and cyanobacterial sequences obtained either from DGGE bands or isolates from mat samples. FB1 and EP1 band sequences correspond to our unknown cyanobacterial population. Probe positions correspond to the *E.coli* 16S rRNA numbering of Borsius et al. (6). Dots in the alignments indicate bases that are identical to those in the target sequences.

Membranes containing electroblotted DGGE pattern of bands were hybridized with the two probes in order to confirm probe specificity and optimize the hybridization conditions (Fig. 2). Probe DGGE 413 did not exhibit a specific hybridization pattern on membranes at stringencies corresponding to formamide concentrations between 20% to 50% and bound to more than two bands (Fig. 2A). The total number of bands bound to the probe apparently decreased at concentrations higher than 50%. At 65 and 70%, the probe bound only to the target band suggesting that at these concentrations the stringency was sufficiently high to prevent probe binding to a not fully complementary target site. Also probe DGGE 573 showed non-specific hybridizations at low stringency (formamide concentrations 25, 35, 45%) and bound to other 16S rDNA fragments on the membrane (Fig. 2B). At 50% formamide concentration, the probe bound not only to the band of

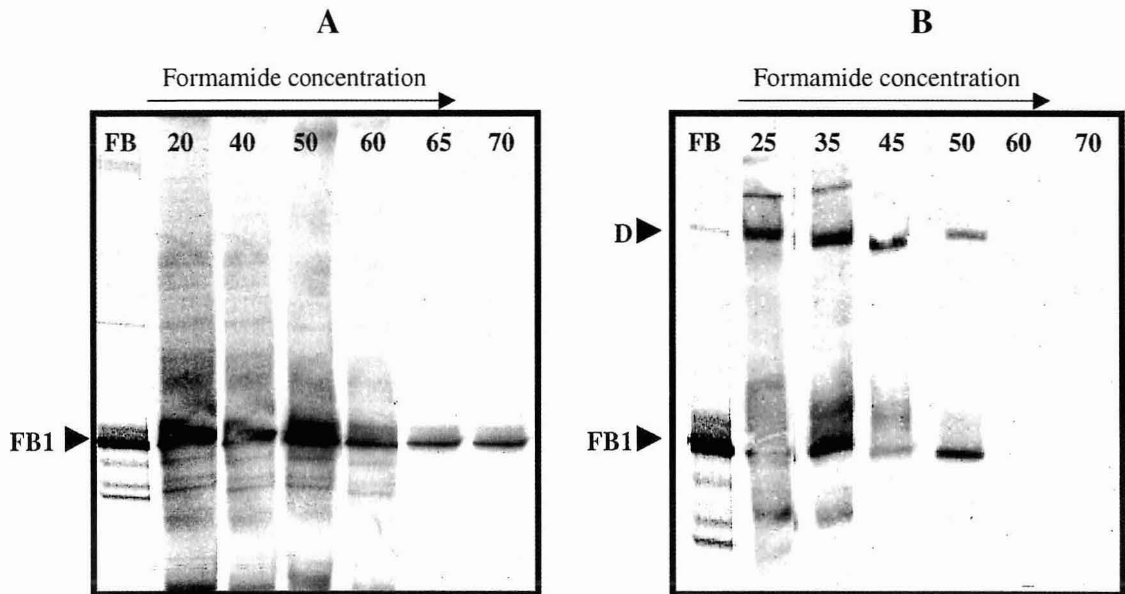

Figure 2. Membrane hybridization of the probes DGGE 413 (panel A) and DGGE 573 (panel B) onto electroblotted DGGE gel pattern at different formamide concentrations. FB lane shows all the bands composing the cyanobacterial community in those mats before hybridization. FB1 band is the target band representing the unknown cyanobacterial population.

interest (FB1) but also to another additional band D whereas at higher stringencies both signals were lost (60 and 70%). Narrowing the stringency conditions between 50-60% did not result in excluding this additional band. Re-amplification and sequencing of this band (Accession number AF393620) showed that it belongs to 16S rRNA of a diatom plastid and it has only one mismatch with this probe. From the above results, the dissociation temperature of probe DGGE 413 was higher than that of probe DGGE 573 which might be due to GC richness of the 3′-half of probe DGGE 413.

In situ hybridization

Both HRP labeled probes were checked for whole-cell hybridizations at different stringencies. The best hybridization signal for probe DGGE 413 was at 65% formamide concentration whereas it was at 50% for probe DGGE 573. This was consistent with the optimum stringencies of the two probes determined by membrane hybridizations. Unspecific hybridization of the two probes to other morphotypes in the mat could be detected below 50% formamide concentration. At the proper stringencies, they hybridized clearly only with extremely small unicellular cyanobacteria (Fig 3). The diameter of these cells was between 0.75-1 μm but never more than 1μm. The spatial arrangement of these cells showed that they formed a cluster embedded within mucilagenous investments, in a *Gloeocapsa*-like arrangement (Fig 3C).

This probably indicates that the cells divide by binary fission within their sheaths but remain attached to each other to form a colony. These cyanobacteria were found to autofluoresce red upon green light excitation (optimum excitation for cyanobacteria). This confirmed that the probe hybridized to photosynthetic cyanobacteria but not bacteria. In comparison to other cyanobacteria in the mat, however, their levels of autofluorescence were very weak. Hybridization with a *Synechococcus lividus* specific probe (W. Schönhuber, unpublished) yielded no signal, demonstrating that our signals with DGGE 413 and DGGE 573 probes were not false positives.

Cultivation attempts

Trials to bring this cyanobacterium into culture were unsuccessful. All enrichments inoculated directly with microbial mat pieces did not show any growth of small sized unicellular cyanobacteria. Only thin filamentous and big unicellular cyanobacteria (3-6 μm diameter) were enriched for in those cultures. Some of the cultures inoculated with 5 μm-filtrate of mat suspensions showed some growth of small coccoid cyanobacteria especially on MN medium at 7% salinity but not in case of ½ PES or ASNIII. Small unicellular cyanobacteria grew there in association with other eukaryotic algal cells. Trials to purify these cultures were made by carrying out several transfers and treating some of them with cycloheximide, which is an antibiotic causing specific blocking in the protein machinery of eukaryotic cells. The cultures could not survive long and trials to revive them failed.

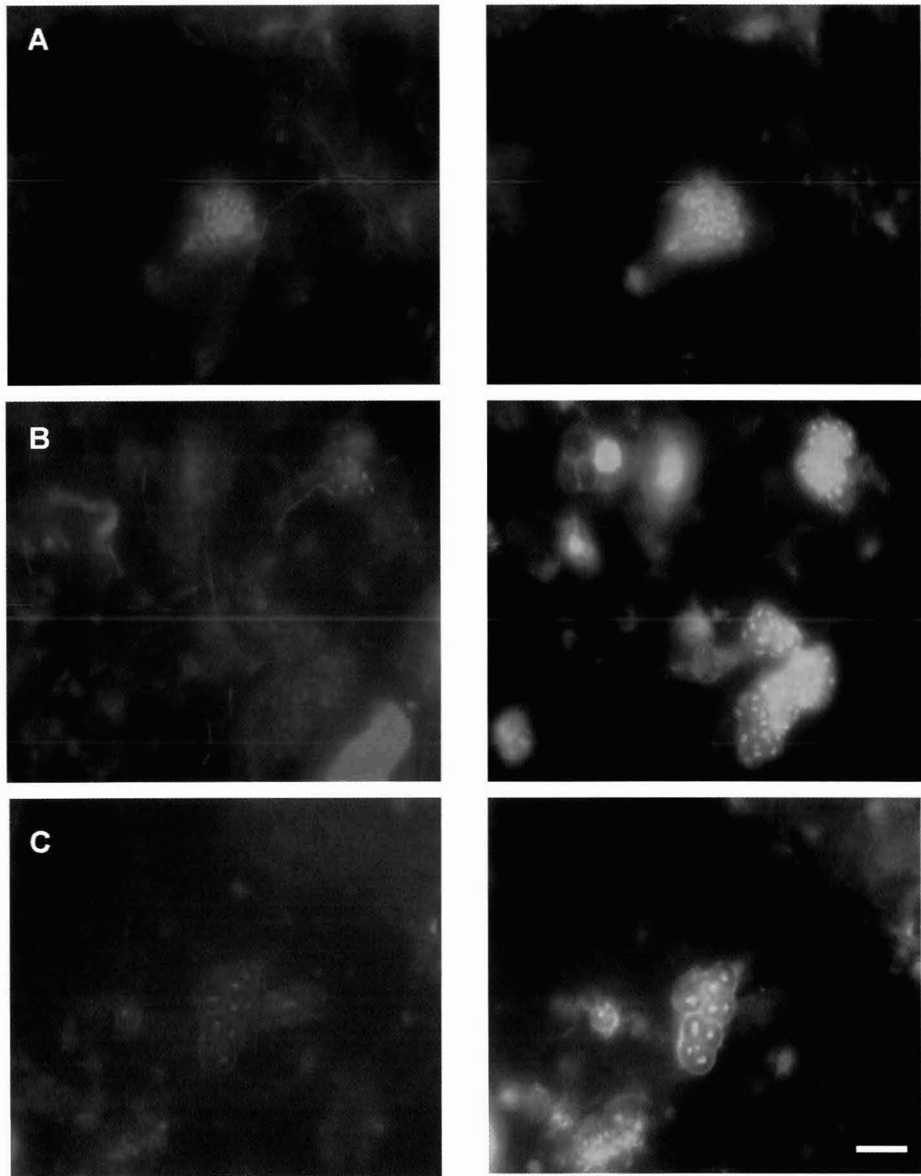

Figure 3. Photomicrographs of thin sections of a microbial mat after *in situ* hybridization with Probe DGGE 413 (A) and Probe DGGE 573 (B and C). Autofluorescence (left) and probe signal (right) detected with fluorescein-tyramide are shown for identical microscopic fields. The bar represents 10 μm.

Discussion

The PCR amplificates of our unknown cyanobacterium typically accounted for more than 35% of the total cyanobacterial PCR amplificate displayed on DGGE gels from the microbial mats. This suggests that it was a dominant cyanobacterium in those mats, and yet, we could not identify it by direct microscopy. Hybridization with probe DGGE 413 targeting our sequence, revealed the cryptic, unidentified nature of the cyanobacteria in question as colony-forming, extremely small unicells. Hybridization with a second probe (DGGE 573), which had broader specificity for cyanobacteria corroborated these results. In this case, all other sequences matching probe DGGE 573 belong to either filamentous (*Phormidium*, *Oscillatoria*, *Leptolyngbya* and *Plectonema*) or larger unicellular cyanobacteria (*Cyanothece aeruginosa*, *Cyanothece* sp. PCC 7424 and *Synechocystis* sp. PCC 6803). Even the additional band (D) that hybridized to this probe on the membrane was found to share more than 96% sequence similarity to the plastids of filamentous diatoms belonging to the genus *Skeletonema*. The sequence of this band had only one mismatch with the probe and hence was difficult to discriminate on the membrane. Based on the hybridization results of both probes, we conclude that the main DGGE band found in our mats corresponds to a hitherto uncultured, very small unicellular cyanobacterium. The morphology of this cyanobacterium was apparently unique and different from all other cyanobacteria inhabiting the same mat sample, which is congruent with the uniqueness of the sequence. This also offers an explanation to the fact that we have overlooked their presence in previous studies. Due to their small size, atypical for cyanobacteria but within the range of heterotrophic bacteria, they are difficult to differentiate by microscopic observation. The mucilagenous sheath surrounding these cells made their focus under the microscope somewhat difficult. In addition to that, their low levels of autofluorescence made them hard to identify as oxygenic phototrophs even under epifluorescence microscopy, particularly in the glare of highly fluorescent larger cyanobacteria (such as *Microcoleus chthonoplastes*, *Oscillatoria* spp. and Halothece). Such a cyanobacterium was not reported in any of the Solar Lake microbial mats (Sinai, Egypt) surveys from which this mat sample originated and was transplanted (6). Moreover, unicellular cyanobacteria that small have not been reported from any benthic environments. Free living picoplanktonic cyanobacteria, belonging to the genera *Prochlorococcus* and *Synechococcus*, inhabiting modern oceans

(23) are within this size range. We can thus describe our population as picobenthic. Interestingly, these small-sized planktonic cyanobacteria also remained unknown during many decades of plankton research, only to be regarded presently as major contributors to the global oceanic primary production (7, 30).

Our results show that *in situ* hybridization with HRP-labeled rRNA probes is a well-suited technique for specific identification of individual cyanobacterial populations particularly, when dealing with very simple morphologies. With the help of this technique we are now able to link bacterial sequences retrieved directly from the environment to their corresponding populations with known morphology and ecological distribution without cultivating them (3, 24). This is a substantial step in molecular ecology in order to confirm that these sequences are obtained from natural microbial communities and not a bias (2). These data also suggest that traditional techniques such as microscopy and enrichment cultivation may fail to recover these very small microorganisms from the environment leading to underestimation of the microbial diversity. Indeed, Ferris et al. (1996) (10) demonstrated that traditional techniques conceal the true extent of diversity in hot spring populations of *Synechococcus*. It is interesting to speculate if such picobenthic cyanobacteria have been overlooked in other environments as well. Our results suggest that it may be worth paying special attention to this possibility. *In situ* hybridization has now the potential to shed light on the distribution of picobenthic populations in other environments.

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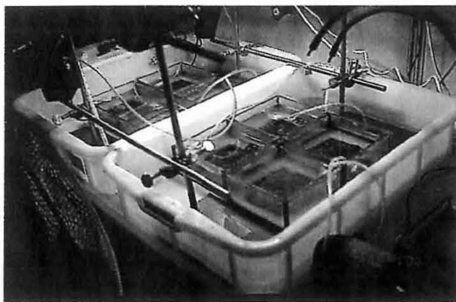
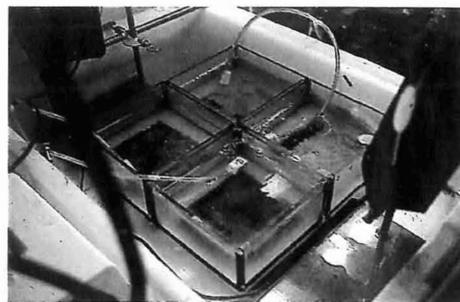
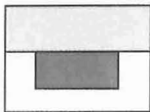
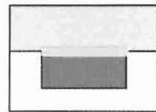
Chapter 5

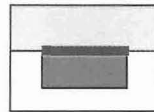
Biodegradation of oil components by a hypersaline microbial mat and by cyanobacterial strains

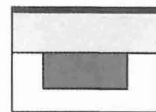












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Manuscript in preparation

Front page:
Biodegradation experiment with intact microbial mat pieces from a hypersaline environment. The mats were incubated with model petroleum compounds immobilised on clay particles (top). Different controls were maintained (bottom).

Biodegradation of oil components by a hypersaline microbial mat and by halotolerant cyanobacterial strains

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Abstract

We investigated the potential of a hypersaline microbial mat and of typical-mat forming cyanobacterial cultures to degrade five main components of petroleum, phenanthrene, pristane, octadecane, perhydrofluran and dibenzothiophene. This model compounds were applied to montmorillonite clay with hydrophobic surface to form organo-clay complexes (OCC) in order to enhance their bioavailability. A mixture of 6 mg/cm^2 OCC, containing ~33.3 µg of each compound/mg OCC, was applied to the intact mat pieces. Degradation was followed under controlled laboratory conditions by gas chromatography. Mats were followed for 4 months and cultures for 2 months. Microbial community changes were followed by DGGE analyses of PCR-amplified 16S rDNA genes. In mats, the aromatic compounds phenanthrene and dibenzothiophene were preferentially degraded compared to the alkanes pristane and *n*-octadecane. Population analysis, however, did not reveal any major community changes concomitant with the decrease of the petroleum model compounds. From 8 cyanobacterial strains tested, only *Oscillatoria sp.* was able to degrade the alkanes partially. We conclude that these mats represent a robust ecosystem with limited ability to degrade petroleum compounds and that degradation of these pollutants by normal cyanobacterial components of hypersaline mats is not common.

Introduction

Microbial mats are laminated benthic microbial communities, which develop under a wide range of extreme environmental conditions (Van Gemerden 1993, Stal 1995). They have been observed to inhabit polluted sites and develop remarkably fast after oil spill incidents, i.e., after the Gulf War in 1991, indicating a possible role of such communities in biodegradation of pollutants (Hoffmann 1996, Höpner et al. 1996). The identification of hydrocarbon-oxidizing bacteria in natural environments has received considerable attention because of the possibility of utilizing their biodegradation potential in the treatment of oil spills. Microbial mats are believed to be interesting systems for that purpose as they are inhabited by physiologically diverse micro-organisms including cyanobacteria and diatoms as oxygenic phototrophs, colorless sulfur and purple sulfur bacteria, aerobic heterotrophs and anaerobic sulfate-reducing bacteria (Stal et al. 1985, Van Gemerden 1993). Several reports have demonstrated the ability of micro-organisms belonging to such physiological groups to biodegrade petroleum compounds. Several cyanobacterial strains were shown to degrade both aromatic and aliphatic petroleum compounds (Cerniglia et al. 1979, Cerniglia et al. 1980a, Al-Hasan et al. 1998, Radwan et al. 2000). Two non-axenic cultures of *Microcoleus chthonoplastes* and *Phormidium corium*, isolated from oil-rich sediments of the Arabian Gulf degraded n-alkanes (Al-Hasan et al. 1998). Studies on *Oscillatoria* sp. and *Agmenellum quadruplicatum* showed their ability to oxidize naphthalene to 1-naphthol (Cerniglia et al. 1979, Cerniglia et al. 1980a). Other studies showed the ability of *Oscillatoria* sp. strain JCM to oxidize biphenyl to 4-hydroxybiphenyl (Cerniglia et al. 1980) and *Agmenellum quadruplicatum* to metabolize phenanthrene to trans-9,10-dihydroxy-9,10-dihydrophenanthrene and 1-methoxy-phenenthrene (Narro 1985). Aerobic organotrophic bacteria associated to cyanobacteria such as *Acinetobacter calcoaceticus* and *Micrococcus* sp. were also isolated from the oiled Gulf mats and found to degrade oil compounds (Radwan et al. 1999). In addition, anaerobic degradation of petroleum compounds has also been demonstrated (Rabus and Widdel 1995). Recent studies have reported efficient degradation of petroleum compounds such as alkanes, benzenes, alkylbenzenes and biphenyls by sulfate-reducing bacteria (Rabus et al. 1993, Rabus and Widdel 1995). Other less common micro-organisms in mats such as denitrifying and iron-reducing bacteria were shown to be able to degrade different hydrocarbons anaerobically (Rabus and Widdel 1996, Rabus et al. 1999). In addition, both oxic and anoxic processes, which

may contribute to oil degradation, occur within the mats as they shift from supersaturation of oxygen during daytime due to high photosynthetic activity, to anoxia during the night (Revsbech et al. 1983).

Therefore, in this study we tested the ability of an intact microbial mat originated from an experimental pond in Eilat and several bacterial and cyanobacterial strains to degrade five model compounds: phenanthrene, pristane, perhydrofluorene, *n*-octadecane and dibenzothiophene. Organo-clay complexes, i.e., hydrophobic clay coated with the model compounds were used in order to establish direct contact between these model compounds and benthic cyanobacterial mats. The biodegradation of the five model compounds by the intact mats and cyanobacterial isolates was followed by gas chromatography over a period of 4 and 2 months, respectively. Changes in community structure of the mats were monitored by molecular techniques.

Materials and Methods

Origin and maintenance of mat samples

Eight cyanobacterial mat samples (15 cm x 15 cm) were collected from a hypersaline experimental pond, Eilat (Abed and Garcia-Pichel 2001) and transferred to the laboratory (Bremen, Germany) within 24 hours. In the laboratory, the mats were incubated in a glass aquarium filled with aerated artificial seawater (ASW, Wiegandt, Germany). The salinity of the artificial seawater was adjusted to 7.5% (wt/vol) and the pH to 8.2. The aquaria were incubated at 27° C with a light regime of 12 h light/12 h dark and 600 μmol photons $m^{-2}s^{-1}$.

Preparation of organo-clay complexes

Phenanthrene, pristane, perhydrofluorene, *n*-octadecane and dibenzothiophene were used as model compounds for petroleum constituents representing straight chain alkanes, branched alkanes, aromatic hydrocarbons, and organo-sulfur compounds, respectively. Hydrophobic clay was used as a carrier substance for the petroleum model compounds in order to overcome the low accessibility of the compounds when directly added. 2% aqueous suspension of montmorillonite KSF (Aldrich) was prepared and 0.8 mmol benzyl-trimethylammmonium (BTMA) chloride per gram of clay was added slowly as a 10 mM solution (modified after El-Nahhal et al. 2000). The mixture was stirred for 24 h, washed three times to remove excess BTMA chloride and then freeze-dried. To adsorb the model compounds, the hydrophobic clay (BTMA- montmorillonite) was suspended

in n-hexane. The mixture of the model compounds (in total 20 mg per 100 mg of hydrophobic clay dissolved in n-hexane) was slowly added under continuous stirring. The slurry was dried in a vacuum rotary evaporator, which yielded homogenous powder of hydrophobic clay loaded with 16.67 wt-% of petroleum model compounds (further designated as organo-clay complex, OCC). The adsorbed amount of model compounds was determined by gas chromatography after extraction with dichloromethane (DCM).

Contamination experiment with intact mats

Mat pieces (10 x 10 cm) were transferred to small glass aquaria (16 x 16 x 8 cm) and fixed in agar (1.5%, ASW). Each aquarium was filled with 1 l ASW and placed into a basin containing water for temperature control. The mat samples were incubated in the aquaria for five days before the addition of the model compounds for acclimatisation. The following treatments were performed in duplicates.

1) Microbial mats incubated in the presence of OCC (hereafter referred to as CO). For each of the two mats 0.48 g hydrophobic clay coated with 0.12 g petroleum model compound mixture (i.e. 6 mg/cm² OCC) was 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 organo-clay complex cover to the mats surface only. The organo-clay complex settled immediately to the mat surface and the frame was removed.

2) Microbial mats contaminated with the petroleum model compounds via the water phase (hereafter referred to as O). A 0.12 g mixture of the petroleum model compounds was dissolved in 1 ml dichloromethane and transferred to a glass slide. After evaporation of dichloromethane, one slide was placed in each of the two aquaria.

3) As controls for the effect of the hydrophobic clay, two other mats samples were covered with 4,8 mg/cm² of the hydrophobic clay without model compounds (hereafter referred to as C). To account for physical removal of the petroleum model compounds from the OCC, one aquarium contained ASW and agar with OCC on the surface was maintained.

4) Untreated mat samples (hereafter referred to as B).

The experiment was run for 4 months and sampling was done at weeks 0, 1, 5, 9, 13, and 18. Water and mat samples were collected to follow the degradation of the added substance. Small cores (ca. 7 mm in diameter) were also collected to monitor microbial community changes by molecular techniques. The mat cores of CO experiment were sectioned into 1-2mm layers to check for vertical distribution of the compounds.

Culture experiment with bacterial isolates

Several bacterial isolates (Table 1) were investigated for their potential to degrade the same model compounds. They include four unialgal cyanobacterial strains isolated from the experimental pond mat (TFEP1, EPUG, EPUS and OSC), two axenic strains of aerobic heterotrophs isolated on glycolate from the same mats (AH2 and AH7) and three axenic strains of cyanobacteria from the original Solar Lake mats (Sinai, Egypt) (MT, AH, and DCS). The cyanobacterial cultures were obtained from the experimental pond microbial mats by enrichment on ASNIII medium (Garcia-Pichel et al. 1998). The degradation experiments were carried out in 250 ml sterile Erlenmeyer flasks. Each Erlenmeyer flask received 99 ml of autoclaved ASNIII medium, 1 ml of culture suspension and 100 mg of OCC. Controls without culture suspension were also maintained. All flasks were incubated at 28°C with constant shaking at 100 rpm and a light regime of 12 h light and 12 h dark. The light intensity was 80 μmol (photons) $m^{-2}s^{-1}$. A similar set of flasks was maintained in the dark. The experiment was run for 60 days and samples for chemical analysis (2 ml each) were taken every 10 days.

Table 1. Cyanobacterial strains screened for their biodegradation capability of petroleum compounds

Strain	Description	Source
Axenic strains		
MT	*Microcoleus chthonoplastes* PCC 7420	Solar Lake
DCS	*Dactylococcopsis salina*	Solar Lake
AH	*Aphanothece halophyletica*	Solar Lake
Unialgal strains		
EPUS	Unicellular cyanobacteria (Halothece)	Eilat mat
EPUG	Unicellular Halothece	Eilat mat
OSC	*Oscillatoria* spp.	Eilat mat
TFEP1	Thin filamentous cyanobacteria (*Halomicronema*)	Eilat mat
Aerobic heterotrophs		
AH2	Aerobic heterotrophs isolated on glycolate	Eilat mat
AH7	Aerobic heterotrophs isolated on glycolate	Eilat mat

Chemical analysis

Samples (cores from the experiment with intact mats and 2 ml suspension from the culture experiment) were extracted ultrasonically with a 1:0.5:0.4 (v/v/v) mixture of methanol (MeOH), dichloromethane (DCM), and water [modified after Bligh and 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 MeOH:DCM:H_2O at a 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 analysed 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 m x 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 min. 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.

Molecular analysis

Mat cores obtained from all treatments were subjected to nucleic acid extraction, polymerase chain reaction (PCR) and denaturing gradient gel electrophoresis (DGGE) as described before (Abed and Garcia-Pichel 2001). The PCR was carried out for the amplification of 16S rRNA using two sets of oligonucleotide primers. CYA359F (with 40 nucleotide GC clamp at the 5′ end) and CYA781R were used as specific primers for cyanobacteria (Nübel et al. 1997) and GM5F with GC-clamp in combination with the universal 907R for all bacteria. A hot start program was performed for the cyanobacterial-specific primers as described by Nübel et al. (Nübel et al. 1997), whereas in case of bacterial universal primers (GM5 and 907R), a hot-start touch-down program was used to minimize nonspecific amplification (Santegoeds et al. 1998). The DGGE was performed at 60°C and a constant voltage of 200 V for 3.5 h as described before (Muyzer at al. 1998).

Results

Mat surface appearance differed significantly immediately after initiation of the experiment. 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 added showed the sandy color of

the clay material. At the end of the experiment, all mats showed the same green-beige surface color.

Degradation of the model compounds by intact mats

In the first experiment with intact mats, perhydrofluorene could not be detected in reproducible amounts because it was too volatile. However, the other four model compounds were initially present at nearly equal concentrations of ~33.3 (±0.1) mg/g OCC each (Fig. 1). The concentrations of all other four petroleum model compounds decreased continously. The aromatic substances (phenanthrene and dibenzothiophene) decreased faster than the aliphatic compounds (pristane and *n*-octadecane). The decrease proceeded linearly for all four compounds but in no case a complete degradation could be detected.

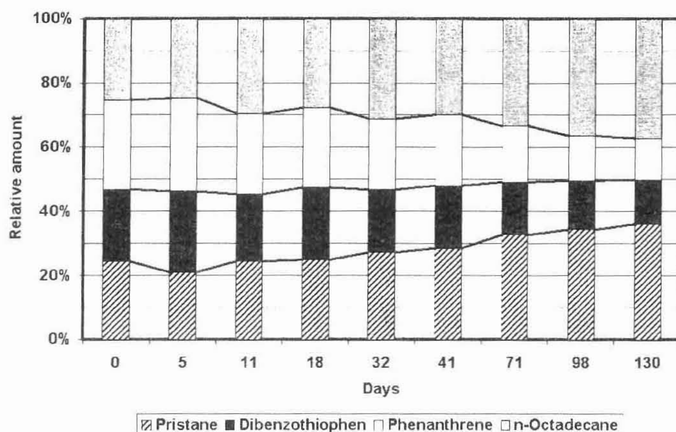

Fig. 1. Relative amounts of petroleum model compounds (normalized to 100 %: average value of the experiment CO). The diagram shows the amount of aromatic compounds dibenzothiophene (black) and phenenthrene (white) decreased in course of the experimental relative to the aliphatic compounds (pristane: hatched; *n*-octadecane: grey). For comparison the initial composition of the OCC is shown (left column).

At the end of the degradation experiment, pieces of the polluted mats (CO) and the control mats (B) were sectioned into layers of 1 or 2 mm thickness in order to analyze the vertical distribution of the petroleum model compounds. In the treated mats (CO), the major portion of the petroleum model compounds was present in the top layer (Fig. 2). This layer was characterized by filamentous cyanobacteria which grew through the applied OCC, and 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 prevail against the aliphatic compounds below that layer. In the treatment (O), none of the compounds could be detected by GC analysis. The substances sublimated to solid form 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 concentrations of the petroleum model compounds did not change.

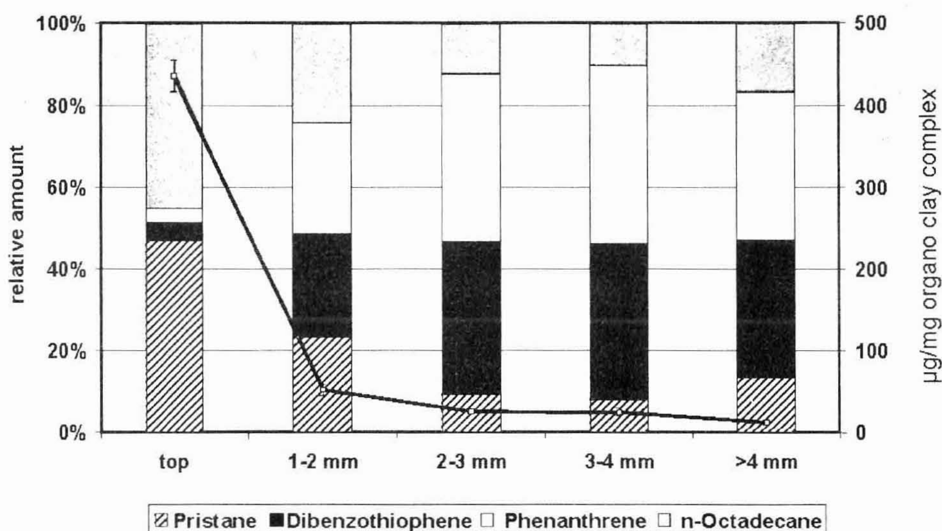

Fig. 2. Concentration of petroleum model compounds, pristane: (hatched), dibenzothiophene (black), phenanthrene (white), *n*-octadecane: (grey), present in different layers of the microbial mat at the end of the experiment (right vertical axis) and their relative composition (normalized to 100%, left vertical axis).

Molecular analysis

The microbial community changes after incubation with the five model compounds are shown in Fig. 3. In case of cyanobacteria, the community composition at the beginning of the experiment, as assessed by DGGE patterns, was identical in all mat pieces. A complete description of the cyanobacteria inhabiting these mats is given elsewhere (Abed and Garcia-Pichel 2001). The community was dominated by two cyanobacterial populations; *Microcoleus chthonoplastes* and the recently described "picobenthic" unicellular cyanobacteria (Abed et al., 2001), as revealed by subsequent sequencing and phylogenetic analysis of the two bands displayed on the gel. At the end of the experiment, the same community members persisted with the development of one additional population represented by band D. Phylogenetic analysis of this band revealed that its 16S rRNA sequence belonged to a diatom plastid. In the treatment (O), where the

model compounds are applied directly to the water without a carrier, the development of an extra population represented by band O1 was observed. This change in the community could not be attributed to degradation activities as the chemical analysis showed that the model compounds did not dissolve in the water phase and thus, did not reach the mats. Microscopic quantification of dominant morphotypes at the beginning and at the end of the experiment showed no changes in the abundance of these morphotypes (data not shown).

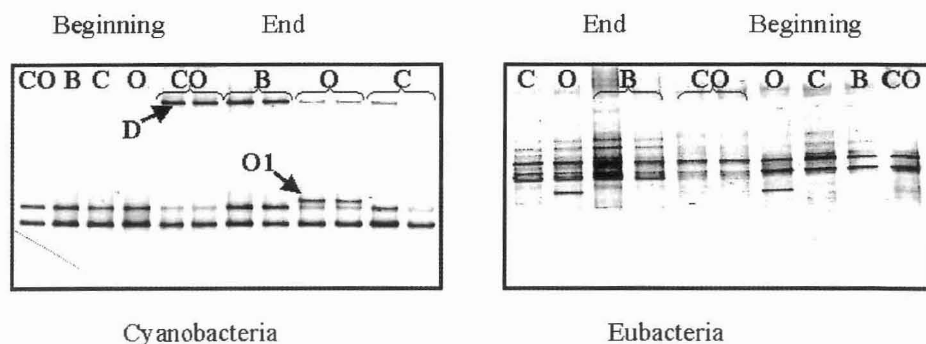

Fig. 3. DGGE profiles of PCR-amplified 16S rRNA fragments, using cyanobacterial (panel A) and universal bacteria primers (panel B), obtained from microbial mats before and after the different treatments. B: blanck, O: organics applied directly to the mats via water, C: hydrophobic clay only (no organics) and CO: organo-clay complex.

The banding pattern obtained with the universal bacterial primers was more complex compared to that with cyanobacterial primers. This is due to the fact that this primer set does not only amplify 16S rRNA of bacteria but also those of cyanobacteria. The banding pattern at the beginning of the experiment suggested a slight difference in the community composition between the mat subjected directly to the petroleum model compounds (O) and the other mat pieces (C, B, and CO). At the end of the experiment, as in 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 higher than at the beginning of the experiment. The mats exposed to the OCC (experiment CO) exhibited a lower number of bands in comparison to other mats.

Degradation of the model compounds by cyanobacterial cultures

No pronounced decrease in the concentration of the model compounds at the end of the experiment was detected in all tested isolates except for the *Oscillatoria* culture (Fig. 4 and 5). This was observed in all cases both in light and dark. The extraction of the suspension in the controls yielded more than 80% of the theoretical concentration of the

model compounds. In some cases, the concentration of the model compounds was lowered as less OCC could be sampled. In case of *Oscillatoria* culture the concentration of *n*-octadecane and pristane decreased after 60 days to 25% of the initial concentration both in the light and in the dark (Fig. 5).

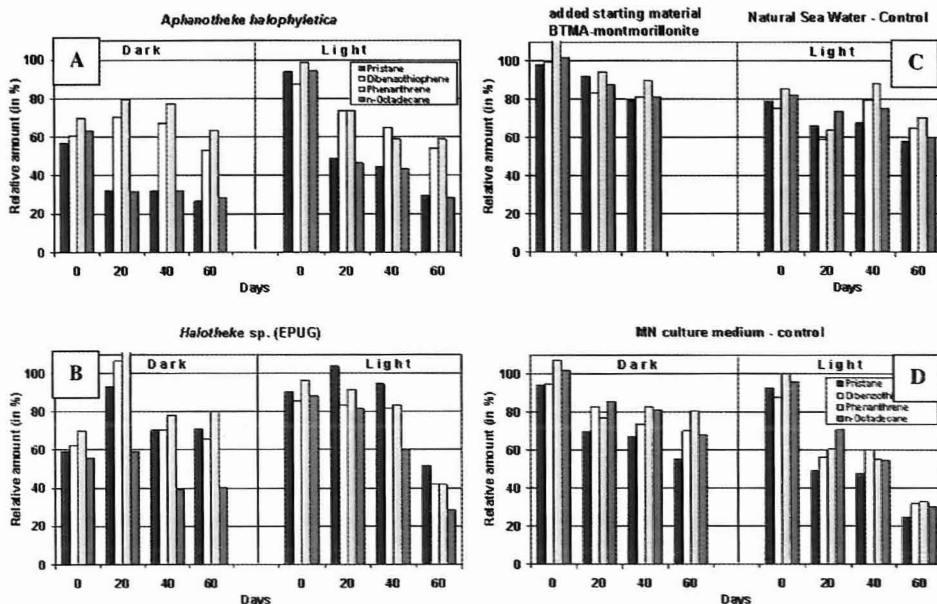

Fig. 4. Quantities of model compounds in slurry experiment with cyanobacterial strains vs. time: A: *Aphanothece halophyletica*, B: unicellular Halothece C and D: controls. The results are shown for light (right panels) and dark treatments (left panels).

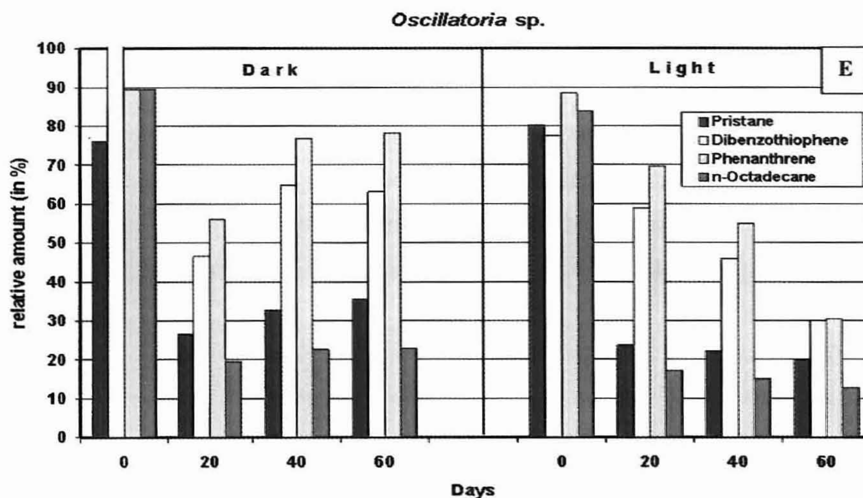

Fig. 5. Quantities of model compounds in culture experiment with *Oscillatoria*. Culture (E) vs. time. The results are shown for light (right panels) and dark treatments (left panels).

Discussion

The decrease in relative concentration of the petroleum model compounds in course of the experiment with intact mats indicated a slow biodegradation of these compounds. Photooxidation, which might account for the selective disappearance of aromatic compounds, is excluded since the agar control experiment without mat material did not show the compositional changes that were observed in the experiment with the organo-clay complex. Furthermore, losses due to water solubility are unlikely since regular water analysis of the experiments with petroleum model compounds or OCC did not show relevant concentrations of the compounds dissolved in the water phase. The fresh water 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). Our solubility tests using OCC and water with 7% salinity yielded values <0.5 mg/l for the four model compounds. Therefore, significant non-biological losses from the OCC, as well as transport of model compounds to the mats in case of the direct addition to the water phase are excluded.

In our experiment the aromatic compounds were degraded faster than aliphatic ones. Similarly, it was reported after the Gulf War in 1991 that aromatic compounds were degraded in the Gulf seawater at much higher rates than aliphatic compounds (Fayad and Overton 1995). Nevertheless, the turnover of these model compounds by this microbial mat system was considered to be slow. This could be attributed to the fact that these mats were pristine and have not been pre-exposed to contamination with petroleum compounds thus oil-degrading bacteria are unlikely to inhabit such mats. In contrast, degradation of the same model compounds in a slurry experiment by microbial mats already exposed to a high level of pollution from Wadi Gaza was achieved within five days (Abed et al. 2001, submitted). For comparison, assuming a linear pattern of degradation, the calculated half-life time of phenanthrene and dibenzothiophene in case of Gaza mats was less than 3.5 days for both while in case of Eilat mats, they had 74.3 and 72.4 days, respectively (Grötzschel et al. 2001, submitted). This indicates that pre-exposure to pollution gives the oil-degrading bacteria the opportunity to grow in such systems and, therefore, they are considered to be better models for biodegradation studies.

Molecular data showed no relevant community response to incubation with the model compounds. Previously, it was presumed that the microbial communities inhabiting these

mats are very robust to various treatments. Incubation of these mats in the presence of different carbon sources such as glucose, glycolate, and acetate induced dramatic changes in the physiology of the mats but without affecting the community structure (Grötzschel et al. 2001, submitted). The community analysis in our experiment supported this assumption, as both cyanobacterial and bacterial communities did not show any changes attributed to the degradation of the aromatic compounds. This suggested that the addition of petroleum compounds to these mats did not selectively favor the growth of certain micro-organisms over others. Comparison of the community structure of both cyanobacteria and bacteria before and after the experiment suggested that the only community changes observed were attributed to experimental settings. Such changes were observed earlier when mats were maintained under laboratory conditions (Abed and Garcia-Pichel, 2001). In the presence of OCC, a decrease in the intensity (cyanobacteria, panel A) or in the number (bacteria, panel B) of some bands was observed. This indicates that the petroleum compounds have a toxic effect on the growth of certain micro-organisms. Many studies demonstrated that crude oil includes constituents that are inhibitory to cyanobacteria even at low concentrations (Radwan and Al-Hasan 2000 and references therein). For example, it was demonstrated that crude oil exerts reversible as well as irreversible inhibition of microbial photosynthesis (Vandermeulem and Ahern 1976, Winters et al. 1976, Batterton et al. 1978). Pollution with aromatic compounds was shown to inhibit photosynthesis and growth, reduce enzyme activity and microbial biomass and induce changes in cyanobacterial species composition (Megharaj et al., 2000).

Degradation of petroleum compounds does not seem to be a common trait among cyanobacterial isolates, both unialgal and axenic. Cyanobacteria are phototrophic micro-organisms, capable of fixing atmospheric CO_2 and, therefore, they do not necessarily need an external carbon source. In most biodegradation studies with cyanobacteria, it was not made clear that the tested cyanobacterial cultures were axenic and many studies were carried out on non-axenic cultures (Radwan and Al-Hasan 2000). Therefore, it is likely that heterotrophic bacteria associated with the cyanobacteria, and not the cyanobacteria, were responsible for the degradation activities Indeed, in one particular study, 30 cyanobacterial axenic isolates representing 17 different genera were tested for the biodegradation of polychlorobiphenyls (PCBs). None of these isolates was capable of degrading these compounds (R. Rippka, person. commun., unpublished). In another study, two non axenic cyanobacterial isolates, *Microcoleus chthonoplastes* and

Phormidium corium, were shown to degrade n-alkanes (Al-Hasan et al. 1998). However, it was concluded that the cyanobacteria and not the organotrophic bacteria degraded the compounds as they accumulated fatty acids of equivalent chain lengths to those of the alkanes within their cells (Al-Hasan et al. 1998). In our *Oscillatoria* unialgal culture, the aliphatic compounds were partially degraded. It was previously demonstrated that *Oscillatoria* species such as strain JCM was able to metabolize napthalene and biphenyl (Cerniglia et al. 1980a, Cerniglia et al. 1980). Nevertheless, in our experiment the degradation of the compounds could be either performed by the *Oscillatoria* or by the attached organotrophs. Axenic cultures of *Oscillatoria* and the respective aerobic heterotrophs need to be checked independently for their biodegradation potential of the model compounds.

Conclusion

It is concluded that the pristine hypersaline microbial mats from the experimental pond do not have a strong biodegradation potential. They are able to degrade petroleum compounds incompletely and at a slow rate. The use of OCC as a carrier system for the model compounds is, however, a useful technique for further biodegradation studies with mats. The investigated mats have a very stable microbial community, which did not change after four months of exposure to petroleum compounds as determined by DGGE. We also conclude that biodegradation of petroleum compounds is not a common capacity among cyanobacteria even though a few strains may be able to perform it.

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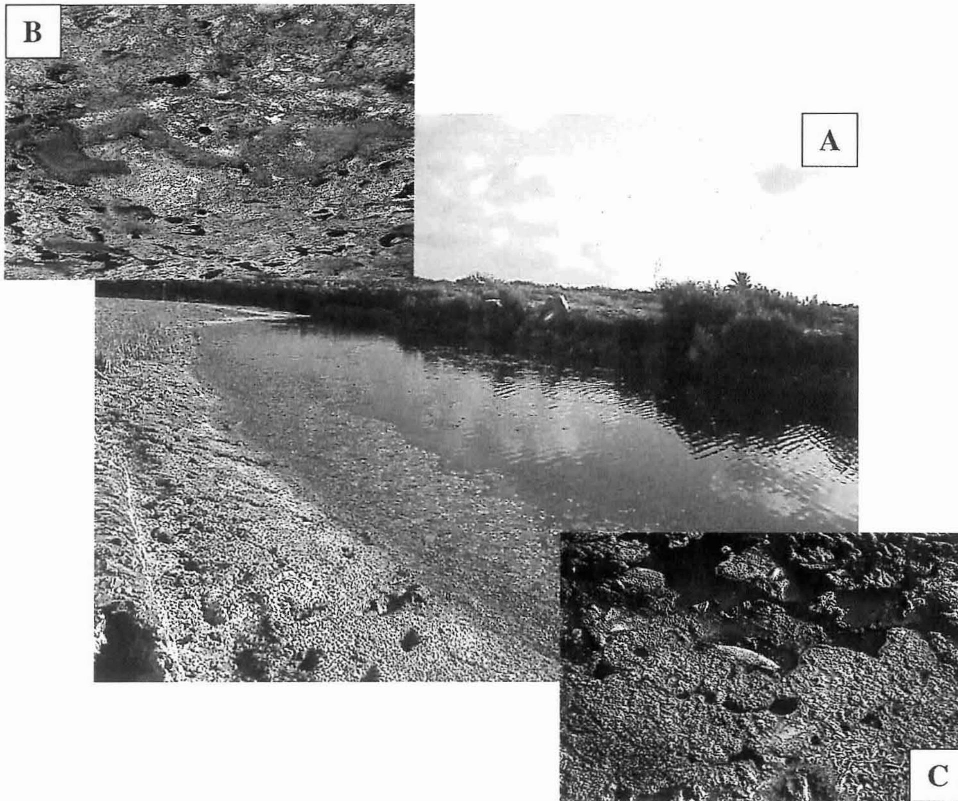
Chapter 6

Microbial Diversity of a Heavily Polluted Microbial Mat and Its Community Changes Following Degradation of Petroleum Compounds



This chapter has been submitted to Applied and Environmental Microbiology

Front page:
The heavily polluted site of Wadi Gaza (Gaza, Palestine) located at the Mediterranean coast (A). The site shows a remarkable development of microbial mats dominated by cyanobacteria in the presence of petroleum pollution (B and C)

Microbial Diversity of a Heavily Polluted Microbial Mat and Its Community Changes Following Degradation of Petroleum Compounds

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Abstract

We studied the microbial diversity of benthic cyanobacterial mats inhabiting a heavily polluted site of a coastal stream (Wadi Gaza) and monitored microbial community response induced by exposure and degradation of four model petroleum compounds in the laboratory. *Phormidium* and *Oscillatoria*-like cyanobacterial morphotypes were dominant in the field. Bacteria belonging to different groups mainly, *Cytophaga/Flavobacterium/Bacteriodes*, γ and β subclasses of the proteobacteria, and green non-sulphur bacteria were also detected. In slurry experiments, these communities efficiently degraded phenanthrene and dibenzothiophene completely in 7 days both in the light and in the dark. *n*-Octadecane and pristane were degraded to 25 % and 34 %, respectively, within 7 days but without further degradation till 40 days. Both cyanobacterial and bacterial communities showed noticeable changes concomitant to the degradation of the compounds. Populations enriched by exposure to petroleum compounds included a cyanobacterium affiliated phylogenetically to thin filamentous forms. Bacteria enriched both in the light and in the dark treatments, but not in any of the controls belonged to the newly described phylum *Holophaga/Geothrix /Acidobacterium*. In addition, another bacterial population, found to be a member of green-non sulfur bacteria, was only detected in the light treatment. All or some of these populations may play a significant role in metabolizing the petroleum compounds. We conclude that the microbial mats from Wadi Gaza are rich in micro-organisms with strong biodegradative potential.

Introduction

There is a general interest in studying the diversity of indigenous microorganisms capable of degrading pollutants such as crude oil, polycyclic aromatic hydrocarbons (PAHs) and polychlorinated biphenyls (PCBs) in different environments (32, 33, 43, 48). 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 response to pollutants (17, 43), to isolate potential degraders (9) and to identify functional genes involved in particular degradation processes (37, 40, 45, 57). Evidence has been presented that microbial communities dominated by phototrophic cyanobacteria can be actively involved in the degradation of petroleum and its derivatives. Observations after oil spills in the Arabian Gulf showed intensive colonization of polluted sites by cyanobacteria-dominated microbial mats that correlated with the disappearance of hydrocarbons (29, 30). Other studies have demonstrated the capacity of cyanobacterial isolates to degrade hydrocarbons (2, 10-12). While many studies were performed to describe bacterial diversity and community changes in various pollutant-degrading communities using recent culture-independent molecular techniques, little research focused on microbial communities dominated by photosynthetic organisms in relation to oil degradation. We studied microbial diversity of a benthic cyanobacteria-dominated community inhabiting a heavily polluted site in Wadi Gaza (Gaza Strip/Palestine), a river that receives a variety of pollutants such as diesel oil and other petroleum products, sewage, pesticides, solid waste as well as agricultural and industrial discharges. We also investigated the ability of native communities to degrade selected model compounds (*n*-octadecane, pristane, phenanthrene and dibenzothiophen) in the laboratory, and we monitored changes in the microbial community linked to the exposure to these compounds. The selected compounds represent the most important groups of petroleum constituents (straight-chain alkanes, branched alkanes, aromatic hydrocarbons and organo-sulfur compounds). These pollutants were found to occur in Wadi Gaza sediments in variable quantities (53). In general, alkanes and polycyclic aromatic hydrocarbons are ubiquitous environmental pollutants. Particular attention has been paid to polycyclic aromatic hydrocarbons (PAHs) as they exhibit toxic, mutagenic and carcinogenic properties (14, 16, 38). Combustion of sulfur-containing organic constituents, such as dibenzothiophene, emits noxious sulfur dioxide, which contributes to acid rain and air pollution (19, 36).

Petroleum compounds are considered to be recalcitrant to microbial degradation and persist in ecosystems because of their hydrophobic nature (low water solubility) and low volatility, posing a significant threat to the environment. To overcome the low accessibility of these compounds in our experiment, we used organo-clay complexes: the model compounds were adsorbed to clay particles with hydrophobic surfaces, in order to enhance the contact of the pollutants with the microbial community and to increase their bioavailability.

Materials and Methods

Environmental setting of the microbial mats

The mat samples used in this study were obtained from Wadi Gaza, Gaza Strip, Palestine. This wadi originates from Hebron city in West Bank and runs in East-West direction across the Gaza Strip. It is the only surface water in Gaza Strip, and is heavily used for disposal of sewage, solid waste, as well as agricultural and industrial wastewater. In the west, Wadi Gaza reaches the Mediterranean Sea. The connection to the sea is intermittent depending on rainfall. Here, most of the cyanobacterial mats develop in the presence of a high level of pollution mainly by diesel oil and other petroleum products (53). Salinity, temperature and water level of the Wadi change seasonally, leading to marked changes in the appearance of the mats. In the western part, salinity ranges between 1 % (wt/vol total salts) in winter and 3.5 % in summer and the average daily temperature varies between 15°C in winter and 35°C in summer. At the time of sampling (May 2000), the mats were submerged, the measured water temperature was 25°C, and the salinity was 2 %.

Preparation of organo-clay complexes

n-Octadecane, pristane, phenanthrene, and dibenzothiophene were selected as model compounds for petroleum constituents. Hydrophobic clay was used as a carrier substance for the petroleum model compounds in our experiments. 2 % aqueous suspension of montmorillonite KSF (Aldrich) was prepared and 0.8 mmol benzyl-trimethylammmonium (BTMA) chloride per gram of clay was added slowly as a 10 mmol solution [modified after (20)]. The mixture was stirred for 24 h, washed three times to remove excess BTMA chloride and then freeze-dried. To adsorb the model compounds, the hydrophobic clay (BTMA-montmorillonite) was suspended in n-hexane.

The mixture of the model compounds (in total 20 mg per 100 mg of hydrophobic clay dissolved in *n*-hexane) was slowly added under continuous stirring. The slurry was dried in a vacuum rotary evaporator, which yielded homogeneous powder of hydrophobic clay loaded with 16.67 wt-% of petroleum model compounds (further designated as organo-clay complex). The adsorbed amount of model compounds was re-extracted with dichloromethane (DCM) and analyzed by gas chromatography.

Experimental design

Experiments were carried out in sterile 250 ml Erlenmeyer flasks. The medium used was prepared by mixing equal volumes of natural seawater with distilled water. The salinity was adjusted to 2 % to mimic the salinity of the site at the time of sampling. Nitrogen and phosphate sources were added to the medium as 1 mM ammonium chloride and 8 μM sodium dihydrophosphate, respectively. Each Erlenmeyer flask received 100 ml of autoclaved medium, 1 g of Wadi Gaza mat material (hereafter referred to as WG) and 100 mg of organo-clay complex, except for controls. The following four controls were maintained, two for chemical analysis and two for community structure comparison: 1) medium and organo-clay complex without mat material (in the light and in the dark), 2) medium and organo-clay complex with autoclaved mat material, 3) medium and mat material without organo-clay complex (hereafter referred to as G), and 4) medium, and mat material + hydrophobic clay (without model compounds) (hereafter referred to as GC). Controls 1 and 2 were maintained to check for photo-oxidation of the model compounds and to account for adsorption of the organo-clay complex particles to the mat material, respectively. The other two controls (3 and 4) were maintained to follow changes in the community in the absence of organic model compounds. All flasks were incubated at 28°C with constant shaking at 100 rpm and a light regime of 12 h light and 12 h dark (the light treatment is hereafter referred to as L). The light intensity was 80 μmol (photons) $m^{-2}s^{-1}$, PAR. One flask containing medium, organo-clay complex and mat inoculum was incubated in complete darkness (hereafter referred to as D). The experiment was run for 40 days, samples for chemical analysis (2 ml each) were taken every four days during the first two weeks and every 10 days till the end of the experiment. At the end of the experiment, the contents of the flasks were collected by centrifugation.

Microscopy and cultivation

Small pieces of microbial mats from Wadi Gaza were torn apart and mounted in water on microscopic glass slides. The slides were observed using transmitted light, phase-contrast and fluorescence microscopy. Different cyanobacterial morphotypes were identified and photographed. Cultivation of different cyanobacteria was carried out in BG11 defined medium (52) enriched with the four model compounds (1 mg organo-clay complex/1 ml medium). The salinity was adjusted to 2 % and incubation was performed at 28°C.

Chemical analyses

Samples of 2 ml volume were taken from the Erlenmeyer flasks with a disposable pipette. The flasks were shaken vigorously to suspend the solid material in order to achieve sufficient homogeneity. The samples were extracted ultrasonically with a 1:0.5:0.4 (v/v/v) mixture of methanol (MeOH), dichloromethane (DCM), and water [modified after (4)]. 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 analysed using a Hewlett Packard 6890 gas chromatograph equipped with a Gerstel KAS3 temperature-programmable injector, a flame ionisation detector and a fused silica column (J&W DB-5HT, 30 m x 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 min. 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 analysis

The field material (WG) as well as the biomass collected from both controls (G and GC) and the two treatments in the light (L) and in the dark (D), were subjected to nucleic acid extraction, polymerase chain reaction (PCR) and denaturing gradient gel electrophoresis (DGGE) as previously described before (1). PCR for the amplification of 16S rRNA was carried out using two sets of oligonucleotide primers. CYA359F (with 40 nucleotide GC clamp at the 5´ end) and CYA781R were used as specific primers for cyanobacteria (49) and GM5F with GC-clamp in combination with the universal 907R for all bacteria (54). A hot start program was performed for the cyanobacteria-specific primers as described by Nübel et al. (49), whereas in case of bacterial universal primers (GM5 and 907R), a hot-start touch-down program was used to minimize nonspecific amplification (54). DGGE was run at 60°C and at a constant voltage of 200V for 3.5 hours (1). The DGGE bands were excised manually, the DNA let to diffuse out in buffer overnight, and PCR re-amplified. Products were then commercially sequenced. The sequences were deposited in GenBank under accession numbers **AF266657 to AF266672**.

Phylogenetic affiliation

Cyanobacterial phylogenetic trees were constructed based on long 16S rRNA sequences (more than 1300 bp) by applying different methods integrated in the ARB software (42) such as maximum likelihood, maximum parsimony and neighbor joining (a maximum likelihood tree is presented in Fig. 2). The 16S rRNA sequences of *Escherichia coli* and *Bacillus subtilis* were included in the calculations as outgroup sequences. Cyanobacterial partial sequences (ca. 400 bp) obtained in this study were aligned to the sequences in the ARB database using the alignment tool of the ARB software package (42). These sequences were then inserted into the pre-established tree using the parsimony ARB tool and maintaining the overall tree topology without changes. Bacterial partial sequences were also inserted within a pre-established stable tree, containing all bacterial sequences in the ARB database without allowing changes of the overall tree topology. The final tree was minimized for simplicity in presentation.

Results

Cyanobacterial community composition of the native microbial mats from Wadi Gaza

Microscopic observation of the native mat sample revealed the presence of at least three morphotypes of filamentous cyanobacteria with different trichome widths. Among these was a cyanobacterium with thin filamentous, *Phormidium*-like morphology (trichome width 1 µm). A second *Phormidium*-like morphotype with wider trichomes (2-4 µm) and clearly visible crosswalls was also observed. The cells of this cyanobacterium were longer than wide (3-6 µm long) with prominent constrictions at the crosswalls. The third morphotype was an *Oscillatoria*-like cyanobacterium with a trichome width of 6-8 µm and discoid cells. In addition to these cyanobacterial morphotypes we also observed some pennate diatoms and unicellular green algae at a very low abundance.

DGGE analysis of the community using cyanobacteria-specific primers showed three distinguishable bands (Wa, Wb and Wc; Fig. 1). The banding pattern was very similar among triplicate extracts from the same mat sample. The sequence represented by Band Wa was affiliated closely to a group of 16S rRNA gene sequences from thin filamentous cyanobacteria belonging to the genera *Phormidium* and *Pseudanabaena* (Fig. 2). Upon enrichment cultivation, a thin filamentous cyanobacterium (1 µm width) was isolated (strain TFWG), and its sequence was found to affiliate closely to this DGGE band with 99% sequence similarity. This indicates that this cultivated cyanobacterium is most probably a representative of the field population represented by DGGE band Wa. The sequence of DGGE band Wb fell next to a cluster of sequences of filamentous cyanobacteria, which includes strains assigned to the genera *Phormidium*, *Leptolyngbya*, *Plectonema,* and *Oscillatoria*. However, the sequence from band Wb was at least 9 % dissimilar from any of the sequences in this cluster. The sequence of DGGE band Wc mapped phylogenetically close to that of *Oscillatoria* sp. M-82 (31) with about 94% sequence similarity.

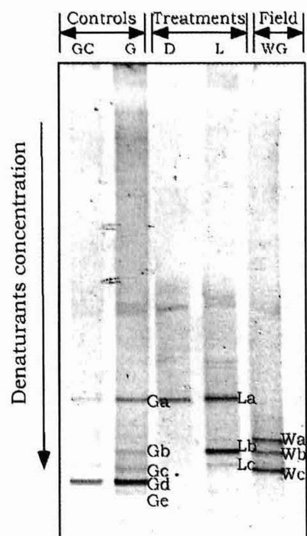

Fig. 1. DGGE banding pattern of PCR-amplified 16S rRNA fragments, using cyanobacteria-specific primers, obtained from the field microbial mats of Wadi Gaza (WG) and mats collected at the end of the experiment from the microbiological controls (G and GC) and both treatments in the light (L) and in the dark (D). Labeled bands were excised, re-amplified and sequenced.

Bacterial community structure of the native microbial mats from Wadi Gaza

The banding pattern generated using the universal eubacterial primers (GM5 and 907RC) was more complex than that generated with the cyanobacterial primers (Fig. 3). This was expected, as these primers not only amplify 16S rRNAs of dominant bacteria but also those of cyanobacteria. Eight clear bands were obtained, three of them being cyanobacterial, as assessed by subsequent sequencing and phylogenetic analysis. This analysis yielded results virtually identical to those obtained with the cyanobacteria-specific primers and is not discussed further. For the remaining bands, a phylogenetic reconstruction is shown in Fig. 4. Band W1 sequence mapped to the *Cytophaga/ Flavobacterium/Bacteriodes* group and was affiliated to the marine bacterium *Polaribacter glomeratus* (ex. *Flectobacillus*) with 93.7% sequence similarity (25). Band W3 sequence fell within the γ subclass of the proteobacteria next to a group of environmental bacterial sequences obtained from activated sludges (95.7% similarity) (6). On the other hand, band W4 sequence fell within the β-subclass of the proteobacteria and was closely affiliated to *Hydrogenophaga palleroni* (97.6% sequence similarity) and *Hydrogenophaga* sp. DSM 5680 (95.7% similarity). These two strains have the ability to aerobically degrade the sulfonated azo compounds 4-carboxy-4'-sulfoazobenzene (5) and 4-aminobenzenesulfonic acid (21), respectively.

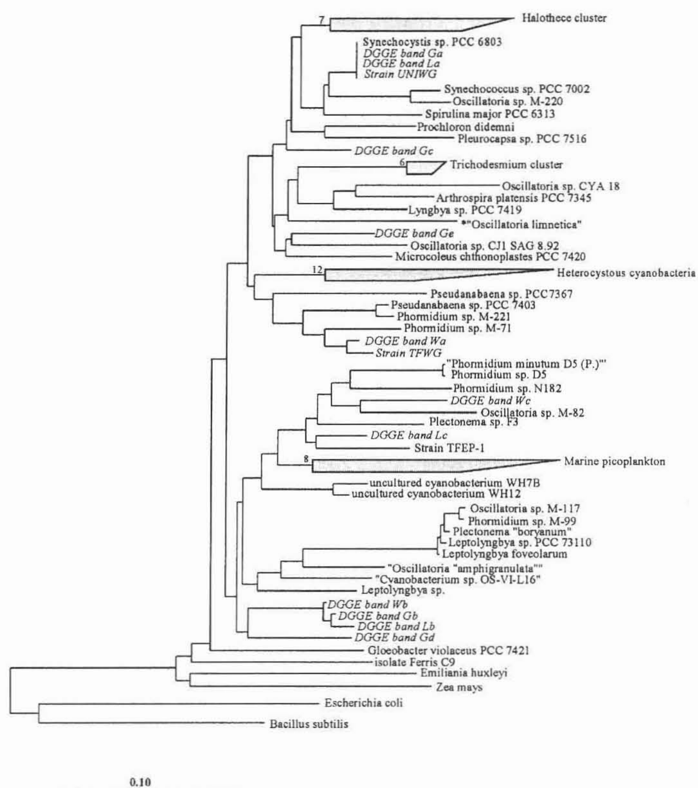

Fig. 2. Phylogenetic reconstruction of the cyanobacteria and plastids (maximum likelihood) based on publicly available, almost complete 16S rRNA genes from the cyanobacterial line using those of *E. coli* and *B. subtilis* as outgroup. The numbers next to the collapsed clusters indicate the number of sequences contained in each cluster. The phylogenetic placement of 400 bp long 16S rRNA sequences from our excised DGGE bands was carried out using parsimony criteria without changing the topology of the pre-established tree. The bar indicates 10 % sequence divergence. Strain denominations of particular strains correspond to those given in the database, and we do not imply that they are necessarily taxonomically correct.

Sequences from bands W2 and W5 had at most 82 % similarity to any other sequences available in the database. Band W2 fell next to sequences from *Rhodothermus* sp. while band W5 fell next to a group of sequences belonging to green non-sulphur bacteria (chloroflexaceae) suggesting that this is probably a member of this group. Some of the environmental sequences, closely related to our sequences, have been implicated in pollutant-degrading microbial consortia (6, 18).

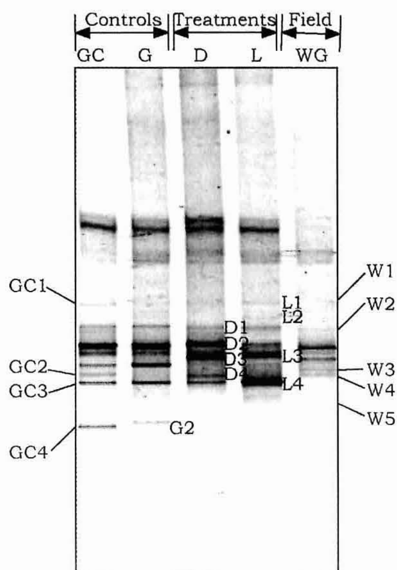

Fig. 3. DGGE profiles of PCR-amplified 16S rRNA fragments, using universal bacterial primers, obtained from the field microbial mats of Wadi Gaza (WG) and mats collected at the end of the experiment from the microbiological controls (G and GC) and both treatments in the light (L) and in the dark (D). The labeled fragments were excised, re-amplified and sequenced.

Degradation of four model compounds

Degradation of pristane, dibenzothiophene, phenanthrene, and *n*-octadecane was studied by their disappearance in the course of the experiments (Fig. 5). The amount of each model compound added to the experimental flasks was 3.33 mg (corresponding to 13.1, 13.9, 18.7 and 18.1 μmol of *n*-octadecane, pristane, dibenzothiophene and phenanthrene, respectively). The extraction of the suspension at day 0 yielded 76 to 84 % of the theoretical amount of the four model compounds. This level of recovery is commonly obtained as a result of experimental and analytical conditions. In the control samples with and without dead biomass (Fig. 5c and d), the amounts and unaltered relative composition of the model compounds indicate that no degradation had occurred. Slightly lower yields were obtained in the control

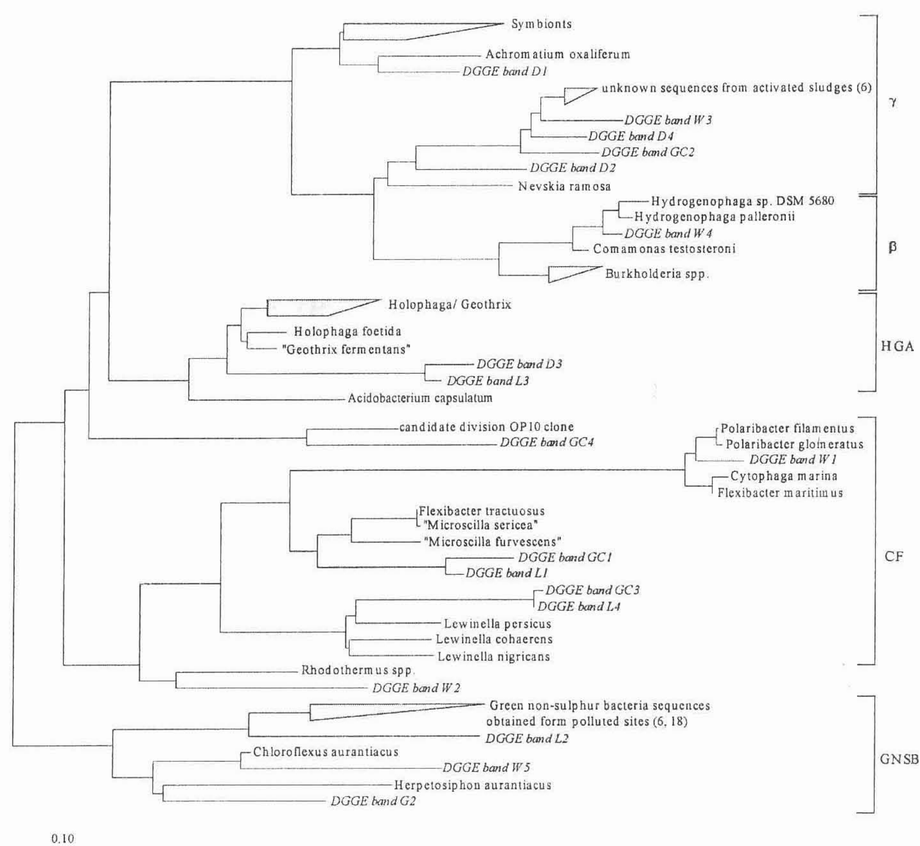

Fig. 4. Unrooted phylogenetic tree showing the affiliation of bacterial 16S rRNA partial sequences to selected sequences from different bacterial clusters; γ and β subclasses of the proteobacteria, *Cytophaga/Flavobacterium/Bacteriodes* (CFB), the newly described phylum *Holophaga/Geothrix/Acidobacterium* (HGA) and green non sulfur bacteria (GNSB). The tree was simplified for clarity; all sequences between clusters have been omitted. The scale bar indicates 10 % estimated sequence divergence.

with dead biomass (Fig. 5c). A lower recovery and a slight decrease of *n*-octadecane relative to the other model compounds was also found in the light control without biomass at day 11; this pattern remained unaltered until 40 days. After three days treatment in the light, phenanthrene and dibenzothiophene were degraded to 3 and 5 % of the initially added amount, respectively. In the dark, the amounts of these two aromatic compounds decreased to 11 and 20 % (Fig. 5a and b). In the samples of day 7 and later, these compounds were absent both in the light and the dark. After three days, the aliphatic compounds, pristane and *n*-octadecane, were also degraded. In case of *n*-octadecane, the remaining amount after three days was ca. 25 % both in the light and in

the dark. Similarly, pristane was degraded to 34 % in the light and 38 % in the dark (Fig. 5b). During the rest of the experiment until day 40, the amounts of pristane and *n*-octadecane remained constant at 30 and 25 %, respectively.

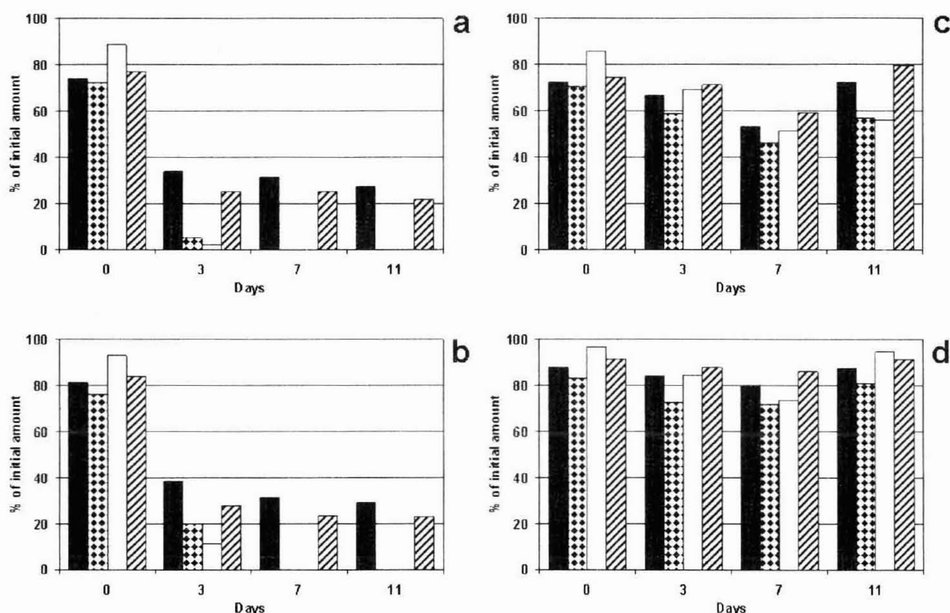

Fig. 5. Quantities of model compounds in slurry experiment with Wadi Gaza microbial mats vs. time: a) 12 h light/12 h dark; b) 24 h dark; c) control with autoclaved biomass, 12 h light/12 h dark; d) control without biomass, 24 h dark. The data are presented in percent relative to the amount of compounds initially introduced into the system (i.e. 3.33 mg per model compound adsorbed on 100 mg organo-clay complexes). Black: pristane, dotted: dibenzothiophene, white: phenanthrene, cross-hatched: *n*-octadecane.

Community changes of cyanobacteria following exposure to pollutants

DGGE analysis showed noticeable changes in cyanobacterial community both in the controls and the treatments (Fig. 1). The banding pattern suggested the replacement of dominant species of cyanobacteria by others. This was observed earlier when mats were taken under laboratory conditions (1). Some field bands such as Wa and Wc disappeared from the treated as well as the control mats; Wb persisted in one treatment (L) and one control (G). A new population represented by band La developed in all treatments and controls. The sequence of this band was phylogenetically affiliated to *Synechocystis* sp. PCC 6803 with 100 % sequence similarity. This population was successfully isolated on a medium enriched with model compounds, and found to be, as expected, a unicellular cyanobacterium (cells are 2-3 µm in diameter) with the morphology of *Synechocystis* species. In the presence of model compounds, the mat incubated in the light showed a

development of three bands, one of them affiliated to the *Syenchocystis* sp. PCC 6803. The second band had the same DGGE position and a closely related sequence to the Wb band from the native mat. The third band (DGGE band Lc) was only observed in this treatment and not in any of the controls or the mats. This indicates that it was a direct development in response to the addition of the model compounds. The sequence of this band was close to that of strain TFEP1, a representative of the newly proposed genus *Halomicronema*, which has extremely thin filaments (94.5% sequence similarity) (R.M.M. Abed, F. Garcia-Pichel and M. Hernández-Mariné, submitted for publication). In the dark, all field cyanobacteria disappeared and amplification of DNA using cyanobacteria-specific primers yielded a product with very low concentration. Upon DGGE of a concentrated product from several PCR reactions, only one cyanobacterial band was displayed on the gel, which was represented by a sequence affiliated to *Synechocystis* sp. PCC 6803. The controls showed banding pattern different from those of the treatments. The mat incubated in the absence of the model compounds exhibited a relatively higher diversity represented by the development of five distinguishable bands. Two of these bands (Ga and Gb) were similar to those detected in the mat incubated in the presence of the model compounds in the light. The other three bands (Gc, Gd, and Ge) did not show more than 93% sequence similarity to any of the cyanobacterial sequences available in the database. In the presence of clay without model compounds only two bands were detected, which were similar to those detected in the G control. This implies that the only change directly attributable to the addition of the model compounds was the enrichment of the population represented by the Lc band in the light treatment.

Community changes of bacterial community following degradation of model compounds

Almost all DGGE bands corresponding to the field bacteria were replaced by others during the treatments, demonstrating dramatic shifts in their community structure. Some of the new bands were common in all experiments including the controls, whereas others were specific for certain treatments (Fig. 3). The total number of bands was higher in the controls than in the treatments indicating that the model compounds inhibited the growth of some bacteria. The new common bands fell phylogenetically within different groups

such as the γ subclass of *Proteobacteria* (band D1), the β subclass of *Proteobacteria* (band D2), and the *Cytophaga/Flavobacter* cluster (band GC1, L1, GC3 and L4). The sequence of these bands did not exhibit more than 85 % to any of the sequences available in the database. In the presence of the model compounds, four bands appeared which were not detected in any of the controls L3, D3, L2 and D4. L3 and D3 bands were detected in the light and in the dark treatments, respectively. Phylogenetically, they fell within a group of sequences belonging to the newly described *Holophaga/Geothrix/ Acidobacterium* phylum, which was defined mainly on the basis of environmental sequences (41). This phylum includes only three cultivated isolates, one of them with aerobic (*Acidobacterium capsulatum*) (34) and the other two with anaerobic (*Holophaga foetida* and *Geothrix fermentans*) mode of life (15, 39). Band L2 was detected only in the light and was related to sequences from green non-sulfur bacteria. On the other hand, band D4 was only detected in the dark and was close to the sequence of the native band W3, which shared around 95.7% sequence similarity to environmental bacterial sequences from phosphate-removing and non-phosphate-removing activated sludges (6). Bands G2 and band GC4 were detected only in G and GC controls, respectively. Their phylogeny is shown in Fig. 4.

Discussion

The indigenous microorganisms inhabiting the highly polluted site of Wadi Gaza seem to have a strong potential to effectively degrade petroleum compounds. This supports previous studies, which indicated that chronic exposure to high levels of petroleum results in microbial communities adapted to hydrocarbons with correspondingly higher concentrations of hydrocarbon-degrading bacteria (7, 8).

The use of organo-clay complex as a carrier

Most petroleum constituents are poorly water-soluble limiting their biodegradation in nature. For example, the water solubility of phenanthrene is less than 1.3 mg/L (50) and that of dibenzothiophene is 1.47 mg/L (28). Therefore, in biodegradation studies, different strategies have been used to make them accessible to microorganisms. This includes either enhancing the solubility of the compounds using natural or synthetic surfactants and emulsifiers (3, 26), or adsorbing the compounds to solid substrates like clay or resins (22, 35). We selected organo-clay complexes since the inoculum was taken

from a surface biofilm of fine-grained Wadi Gaza sediment, and it is likely that the bacteria are adapted to growth on solid surfaces. Furthermore, by the addition of organo-clay complex we simulated the natural situation in which hydrophobic contaminants are mainly present in adsorbed form. The use of organo-clay complex in our experiment was successful as we could recover more than 75% of the loaded model compounds. This level of recovery was influenced by the experimental procedures such as sampling and chemical analysis. Higher yields were obtained (more than 90%) when the compounds were extracted directly from suspensions of organo-clay complexes (J. Köster, unpublished data). In the dark control without biomass, the constant recovery of more than 75 % of the model compounds till the end of the experiment indicates that the organo complex was stable throughout the experiment and there was no biodegradation (Fig. C). Comparison of light control (not shown) and dark control (Fig. 5d) without biomass also demonstrated that the model compounds were not photo-oxidized. The control experiment with autoclaved biomass (Fig. 5c) showed that adsorption was not significant, even though the recovery of the compounds was reduced in some cases (Fig 5d). This is most likely due to coagulation of clay particles and biomass leading to lower organo-clay complex concentration in the sampled suspension. Consequently, we attribute the disappearance of the model compounds in the presence of living biomass to biodegradation by micro-organisms but not to adsorption or photo-oxidation. The aromatic compounds were completely degraded indicating complete accessibility of these compounds to the degrading bacteria. In contrast, the amount of aliphatic hydrocarbons decreased after 3 days and then remained constant up to 40 days. We assume that these compounds were only partially accessible to bacteria. It is likely that they were trapped by strong binding in the clay mineral interlayers, which inhibits desorption or direct biodegradation (35, 58). This degradation-resistant portion could be extracted with dichloromethane by ultrasonication since this procedure causes a disintegration of aggregates and swelling of the organo-clay complexes. In our experiment we observed that both cyanobacterial and bacterial communities in the absence of clay were different from samples containing either hydrophobic clay or organo-clay complex. The hydrophobic clay seems to inhibit the growth of some cyanobacteria (Fig. 1) but enhances the growth of other bacteria (Fig. 4). Therefore, in the presence of organo-clay complex, the community changes induced by the hydrophobic clay could be discriminated by the control containing only this hydrophobic

clay. Such difference in microbial communities in the presence of a sorptive phase compared to those in the absence of a sorbent was previously reported (27, 22).

Role of cyanobacteria in biodegradation

Recent reports demonstrated that photosynthetic microorganisms, particularly cyanobacteria, may play a direct or indirect role in the metabolism and degradation of hydrocarbons. Cyanobacteria such as *Anabaena cylindrica*, *Phormidium faveolarum*, *Oscillatoria* sp., strain JCM, and *Agmenellum quadruplicatum* can degrade different aromatic compounds (10, 11, 51). Narro (1985) demonstrated the ability of *Agmenellum quadruplicatum* to oxidize phenanthrene to trans-9,10-dihydroxy-9,10-dihydrophenanthrene and 1-methoxy phenanthrene involving a monooxygenase system (47). Degradation of *n*-alkanes by two non-axenic strains, *Microcoleus chthonoplastes* and *Phormidium corium* isolated from oil-contaminated sediments was also demonstrated (2). These *n*-alkanes were oxidized to fatty acids, which were subsequently incorporated into cell lipids. In mat systems, cyanobacteria are present in association with oil-degrading bacteria and prevent them from being washed out by immobilizing them in their mucilage. In addition, cyanobacteria also supply these bacteria with the oxygen produced by photosynthesis and the fixed nitrogen needed for their activity in the degradation processes. This indirect role of cyanobacteria can be important to the overall successful biodegradation process.

In our experiment, shifts in cyanobacterial community composition were very dramatic with elimination of two field species. These species might have been sensitive to cultivation conditions in the laboratory. However, new cyanobacteria became conspicuous in the experiment, although they were not detectable in the field sample. These species were probably present in the field at very low abundance. The maximum number of species was detected in the control in which the mat sample was cultivated in the absence of model compounds, lending credibility to the notion that cyanobacteria exhibit maximum diversity in the absence of petroleum compounds. Many studies have demonstrated that crude oil includes constituents that are inhibitory to cyanobacteria even at low concentrations [see reference (51) and references therein]. Aromatic compounds were shown to have more drastic effects than alkanes. Pollution with these compounds inhibits photosynthesis and growth, reduces enzyme activity and microbial biomass and induces changes in cyanobacterial species composition (44). In the presence of model compounds in the light experiment, the cyanobacterial community favored the

growth of three cyanobacterial populations. The population represented by band Lc, which appeared only in this experiment, might play a role in detoxification of any of the model compounds. In order to demonstrate this, experiments with axenic cultures of the respective population are needed. Another population affiliated to *Synechocystis* sp. PCC 6803 developed in this treatment, but also in the controls. A search of the complete genome of *Synechocystis* sp. PCC 6803 (http://www.ncbi.nlm.nih.gov) revealed that this cyanobacterium possesses dioxygenases genes, which catalyze the incorporation of both atoms of an oxygen molecule into an aromatic ring producing dihydrodiols (23). This suggests that this cyanobacterium might also play a role in the aerobic degradation of aromatic compounds like dibenzothiophene and phenanthrene in our experiment. In any case, these three populations seem to tolerate the supplied concentrations of the model compounds indicating that sensitive species in the field were replaced by more resistant ones. As cyanobacteria are sensitive to pollution, any alteration in their community composition could be useful as a bio-indicator of pollution (44).

Bacteria in Wadi Gaza

DGGE analysis suggested a less diverse bacterial community in Wadi Gaza compared to those communities developed during the experiment. This is apparently owing to the environmental stress caused by the high level of pollutants, which allows for a restricted number of species tolerating such conditions. However, the site seems to be very rich in novel micro-organisms since in many cases the phylogenetic analysis of sequences obtained from the bacterial bands did not yield close matches to any known organisms. Many sequences fell within the *Cytophaga/Flavobacterium* and the proteobacteria (γ and β) groups. These groups were previously reported to be present in marine ecosystems (24), waste waters (13) and oil-polluted sites (43, 59). The retrieval of some sequences (bands W3, D4, GC2 and D2) related to sequences from activated sludge is consistent with the sewage pollution in the site which must favor the growth of such species. The field species represented by band W4 fell within a group of organisms having degradation abilities suggesting a possible role of this organism in biodegradation of pollutants in the field. In the experiments, where the model compounds were degraded, phylogenetic characterization of the major bands indicated that they do not belong to genera containing known hydrocarbon-degrading bacteria such as *Sphingomonas*, *Pseudomona*, *Burkholderia* and *Mycobacterium*. Instead, these bands

were found to belong to the newly described *Holophaga/Geothrix/Acidobacterium* phylum (band D3 and band L3), γ subclass of the proteobacteria (band D4) and green non-sulphur bacteria (L2). The appearance of these bands only in the presence of the model compounds and not in the controls implies that these species might contribute to the degradation of the compounds. The two sequences L3 and D3 were obtained from the treatments in the light and the dark, respectively, and found to have similar sequences and phylogeny. These sequences fell within the *Holophaga/Geothrix/Acidobacterium* phylum (41). One of the representative isolates of this group is the Fe(III)-reducing bacterium *Geothrix fermentans*, which was isolated from a contaminated aquifer that has been adapted to rapid anaerobic oxidation of toluene coupled to Fe(III) reduction (15). Another member of the group is the obligatory anaerobic bacterium *Holophaga foetida*, which has the ability to degrade methoxylated aromatic compounds (39). This suggests that these compounds might have also been metabolized anaerobically. Anaerobic degradation of petroleum compounds has been previously demonstrated (60). Our experiments were performed in an open, oxic system, but the possibility of anaerobic niches and the presence of anaerobic micro-organism is possible. It is likely that anaerobic processes are more important in the natural environment of Wadi Gaza where the fine-grained sediment is oxygen-depleted below a few millimeters. The presence of such organisms and their metabolic activity in mainly oxic zones of microbial mats has been demonstrated (46, 55, 56). The appearance of band L2, phylogenetically shown to be a member of green non-sulphur bacteria, only in the presence of organo-clay complex in the light opens the unexplored possibility of implicating anoxygenic phototrophs in bioremediation studies particularly those of known photoheterotrophic mode of growth such as *Chloroflexus*.

Conclusions

Our chemical results support the observations after oil spills in the Arabian Gulf that microbial communities dominated by cyanobacteria can be involved in the degradation of petroleum pollution. Microbial communities inhabiting Wadi Gaza degraded effectively both aliphatic (pristane and *n*-octadecane) and aromatic compounds (phenanthrene and dibenzothiophene). This site seems to harbor phylogeneticlly diverse and novel microbial populations, able to tolerate heavy pollution. Organo-clay complexes have been successfully applied as a carrier system for the model compounds

and allowed the complete degradation of aromatic compounds and most of the aliphatic compounds. The degradation of these compounds was accompanied by changes in both cyanobacterial and bacterial communities. The development of certain populations was attributed directly to the incubation with the model compounds and thus believed to play a role in the metabolism of those compounds. Among these the apparent involvement of the members of the *Holophaga/Geothrix/Acidobacterium* and green non-sulfur bacteria are novel findings. This shows that polluted sites contain a significant hidden diversity of yet unknown and uncultured micro-organisms that contribute to biodegradation. It is, however, needed to isolate such key microorganisms in order to fully explore their individual potential to degrade different petroleum compounds.

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List of Publications

Contributions to the Manuscripts Presented in this Thesis

Abed, R. M. M., and F. Garcia-Pichel. 2001. Long-term compositional changes after transplant in a microbial mat cyanobacterial community revealed using a polyphasic approach. Environ. Microbiol. **3(1):**53-62.

Concept by R. A., practical work and writing by R. A., editorial help by F. G. P.

Abed, R. M. M., F. Garcia-Pichel, and M. Hernández-Mariné. 2001. Polyphasic characterization of benthic, moderately halophilic, moderately thermophilic cyanobacteria with very thin trichomes and the proposal of *Halomicronema excentricum* gen. nov., sp. nov. submitted to Archives of Microbiology.

Concept by F. G. P. and R. A., experiments and practical work by R. A., electron microscopy by M. H. M., writing by R. A and editorial help by F. G. P. and M. H. M.

Abed, R. M. M., W. Schönhuber, R. Amann and F. Garcia-Pichel. 2001. Discovery of Picobenthic Cyanobacteria by 16S rRNA-Targeted *In Situ* Hybridization. Submitted to Applied and Environmental Microbiology.

Concept by R. A., practical work by R. A., W. S. introduced me to the technique, writing by R. A. and editorial help by F. G. P., R. A. and W. S.

Abed, R. M. M., J. Köster, S. Grötzschel, D. de Beer and F. Garcia-Pichel. 2001. Biodegradation of oil components by a hypersaline microbial mat and by cyanobacterial strains. In prep.

Concept by D. d. B. (with intact mats) and R. A. (experiment with cyanobacterial strains), experiments and practical work by R. A. and S. G., chemical analysis by J. K., writing by R. A., J. K. and S. G. and editorial help by F. G. P.
(This is part of a detailed study, which includes microsensor analysis by S. G., molecular work by R. A. and chemical work by J. K. The detailed chapter will be presented in S. G. thesis)

Abed, R. M. M., N. M. D. Safi, J. Köster, D. de Beer, J. Rullkötter and F. Garcia-Pichel. 2001. Microbial Diversity of a Heavily Polluted Microbial Mat and Its Community Changes Following Degradation of Petroleum Compounds. Submitted to Applied and Environmental Microbiology.

Concept by R. A., Experiment by R. A., chemical analysis by J. K. and N. M. D. S., writing by R. A. and J. K. (chemical part), and editorial by F. G. P., D. d. B and J. R.