Physiological reactions of hypersaline cyanobacterial mats upon degradation of organic pollutants

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Physiologische Reaktionen hypersaliner Cyanobakterienmatten im Zuge des Abbaus organischer Schadstoffe

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Titelseite: Gipskruste aus einer Salzgewinnungsanlage am Roten Meer in Israel als Beispiel für die charakteristische Zonierung mikrobieller Matten. Die farbliche Abstufung wird durch die unterschiedliche Pigmentierung der phototrophen Mikroorganismen hervorgerufen.

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Chapter 1

General Introduction

MARINE POLLUTION

Marine environments represent important ecological and economical resources all over the world. For centuries huge amounts of anthropogenic pollutants were released to the atmosphere, pedosphere and hydrosphere, which often ended up in the oceans (EEA 2001). Harmful effects of many of those substances are defused by dilution, sorption and the activity of microorganisms which in the course of evolution have developed mechanisms to tolerate and to mineralize pollutants (Hemleben 1997). Bioremediation of contaminants in nature depends on the environmental conditions at the polluted site. Some substances, however, are resistant to microbial breakdown, e.g. toxic components like heavy metal compounds or persistent organic pollutants (POP). Their recalcitrance to degradation allows them to accumulate to toxic levels in the environment. The only way to limit the threat from non-degradable pollutants is to control and finally stop their release. The importance of preventing the proliferation of e.g. POP´s was taken into account just recently in the "Stockholm Convention on Persistent Organic Pollutants" treaty which was signed by 91 nations in May 2001 (IPEN 2001). Other groups of pollutants also threaten marine environments despite their potential biodegradability, simply because of the relatively high amounts released. Petroleum hydrocarbons or halogenated aromatics play a role in that context. The sources of marine hydrocarbon pollution are mainly runoff from land and municipal/industrial wastes (13.74×10^8 l/a), routine ship maintenance like bilge cleaning (5.19×10^8 l/a), air pollution from cars and industry (3.48×10^8 l/a), natural seeps (2.35×10^8 l/a), tanker accidents (1.4×10^8 l/a) and offshore oil production (0.57×10^8 l/a) (US Coast Guard 1990). Degradation of oil by microorganisms is one of the most important long-term natural processes for removal of oil from the marine environment (NRC 1985).

The degradative capabilities of microorganisms have been exploited for bioremediation. There are two main approaches for *in situ* bioremediation of polluted sites: bioaugmentation and biostimulation. The former involves the addition of bacteria with the ability to degrade pollutants in order to supplement the existing microbial population. This approach has only been successful in controlled

bioreactors. In the open environment, the number of autochthonous degrading bacteria have usually been found to increase after contamination, e.g. in sediments (Prince 1993, Venosa et al. 1997), open waters (Pierce et al. 1975, Atlas 1993) and sea ice (Delile et al. 1997). Furthermore, the rates of biodegradation by allochthonous microorganisms did not exceed those achieved by nutrient enrichment alone, no matter if commercially available mixtures of microorganisms (Lee and Levy 1987) or enriched cultures of indigenous pollutant-degrading bacteria (Fayad et al. 1992, Venosa et al. 1996) were used. Allochthonous microorganisms are generally unable to compete with the natural microflora (Venosa et al. 1996, Lee and Merlin 1999). Biostimulation involves the addition of nutrients, growth-enhancing co-substrates and improvements in habitat quality, including oxygen supply, to stimulate the growth of indigenous pollutant degraders (Lee 2000). Bioaugmentation is often more successful in *ex situ* cleaning approaches, but the maintenance of reactors or plants is expensive, due in particular to the costs of aeration. Oxygen plays a major role in the biodegradation of organic pollutants. For example, in an experimental reedbed with the macrophyte *Typha*, the C-9 to C-26 alkane fraction of a diesel oil was preferentially removed in areas of greater oxygen supply (Omari et al. 2001). In natural systems like microbial mats where oxygenic photosynthesis occurs, the production of oxygen in large amounts during the day may obviate the need for additional aeration.

MICROBIAL MATS

Although the term microbial mat has been used also for benthic microbial communities that do not form tough coherent structures, most researchers agree on the definition of cohesive, accretionary biofilms growing on solid surfaces with typical macroscopically observable vertical laminations which arise from the different pigments of the distinct physiological groups of microorganisms in the mats (Stal and Caumette 1994). Microbial mats can be found all over the world in a wide spectrum of environments. This includes hypersaline lakes and lagoons, freshwater lakes and streams, alkaline lakes, hot springs, intertidal coastal sediments, marine salterns, coral reefs, dry and hot deserts and Antarctic lakes (Pierson 1992). Most locations where microbial mats develop are characterized by extreme conditions of temperature, salinity, moisture or pH. Such conditions are responsible for the absence of efficient grazers and seem to be a prerequisite for mat expansion (Castenholz 1994). Microbial mats can cover areas of several square kilometers (Des Marais et al. 1992) and reach a thickness of more than one meter (Stal and Caumette 1994). They represent the world's smallest ecosystem (Karsten and Kühl 1996) and are modern analogues of 3-3.5 billion year old stromatolites, laminated carbonate build-ups, that preserve evidence of the Earth´s earliest biosphere (Reid et al. 2000). To this day a few cyanobacterial mats still form stromatolites in a dynamic balance between sedimentation and intermittent lithification.

There are categorical differences between modern microbial mats (e.g. between marine/saline mats and hot spring mats (Castenholz 1994)) but most of them are characteristically dominated by a few functional groups of microorganisms (van Gemerden 1993). In most cases oxygenic phototrophs like cyanobacteria and diatoms, chemolithotrophic colorless sulfur bacteria, purple sulfur bacteria (anoxygenic phototrophs) and anaerobic sulfate-reducing bacteria are present (Fig. 1). Up to now, less attention has been paid to nitrifying/denitrifying bacteria, methanogens and aerobic/anaerobic heterotrophs in mats, although the latter group especially may play an important role in the foodweb of the mats (van Gemerden 1993).

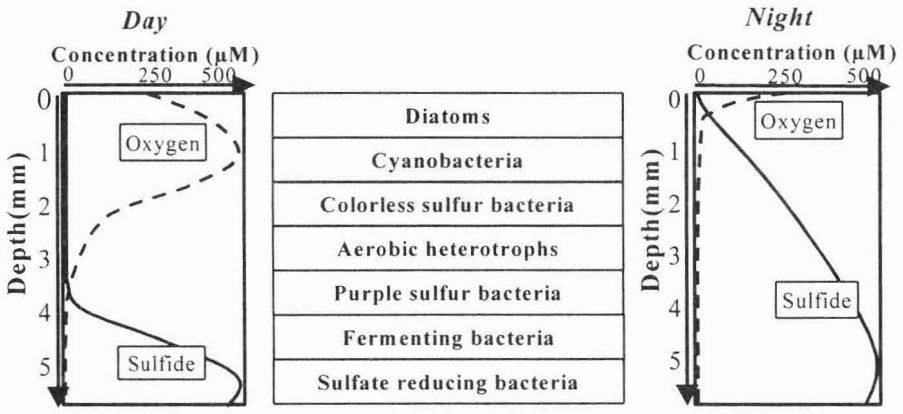

Figure 1: Schematic composition of a microbial mat in which the different functional groups of microorganisms appear to be vertically layered. Oxygen and sulfide concentrations fluctuate due to the bacterial activity in response to changing phototrophic conditions (van Gemerden 1993, modified by Jonkers 1999).

Filamentous cyanobacteria play a substantial role in the colonization of new sites. They stabilize the sediment by trapping sand grains in excreted exopolymers (Krumbein 1994). Representatives of this group can be found in a large number of microbial mats, often dominating the upper few millimeters (Jørgensen et al. 1983, Stal 1995). Diatoms (algae) can also occur in the upper layer of microbial mats, but eukaryotic organisms are often excluded from the extreme environments where mats are found because of their limited spectrum of metabolic capabilities as compared to prokaryotes (Stal 2000). By contrast, the prokaryotic cyanobacteria not only perform oxygenic photosynthesis, but are also capable of photoheterotrophy, sulfide-dependent anoxygenic photosynthesis and fermentation. This wide spectrum of metabolic capacities enables them to survive changing environmental conditions. An important function of cyanobacteria in microbial mats is the production of oxygen and organic carbon by photosynthetic activity. The carbon fixed by cyanobacteria during photosynthesis becomes available to the other bacteria in the mat community by excretion of glycolate, fermentation products and extracellular polymeric substances (EPS). Death and lysis of cyanobacteria is an additional source of reduced organic matter in microbial mats (Stal 1995). During these processes nitrogen compounds are also released into the porewater of the mats, especially by those cyanobacterial species which are able to overcome nitrogen limitation by fixing nitrogen from air. Excreted organic carbon is respired by heterotrophic bacteria under oxic conditions,

concomitant with O_2 consumption and the production of CO_2. Under anoxic conditions, which prevail in the deeper parts of the mats and during dark periods, when no oxygen production by photosynthesis occurs, organic substrates are fermented by cyanobacteria and heterotrophic microorganisms. The products of these processes can be used as substrates by sulfate-reducing bacteria. Dissimilatory sulfate reduction is the most important anaerobic process of carbon mineralization in hypersaline mats (Jørgensen et al. 1992, Canfield and Des Marais 1993) and often occurs because of the large amounts of sulfate contained in marine habitats. The sulfide produced is chemolithotrophically oxidized by colorless sulfur bacteria in the presence of molecular oxygen in the oxic zone of the mats. At the interface between the oxic and the anoxic zones, low oxygen concentrations can lead to incomplete sulfide oxidation. This favors purple sulfur bacteria, which are able to use sulfide as electron donor for photosynthetic carbon assimilation.

Due to high remineralization rates, the annual biomass accretion in microbial mats is low. The complex interactions between the different physiological groups cause almost closed substrate cycles (Fig. 2). Steep physical and chemical gradients occur due to the metabolic activity of the mat-inhabiting organisms, especially during periods of illumination. In the light, oxygen is produced by photosynthesis in the upper layer of the mat, often leading to oxygen concentrations far above saturation (Fig. 1). Photosynthetic carbon fixation causes CO_2 depletion, thereby increasing pH in that region. In the dark, oxygen is respired and often diffuses less than a millimeter into the mat, CO_2 is released and the pH decreases. Under such anoxic conditions sulfide accumulates and nearly reaches the surface of the mat. It is clear that microorganisms must be adapted to such extreme diurnal changes in order to survive in this environment. In addition to sulfide tolerance and metabolic versatility, UV resistance and motility are known adaptation strategies (Castenholz 1994).

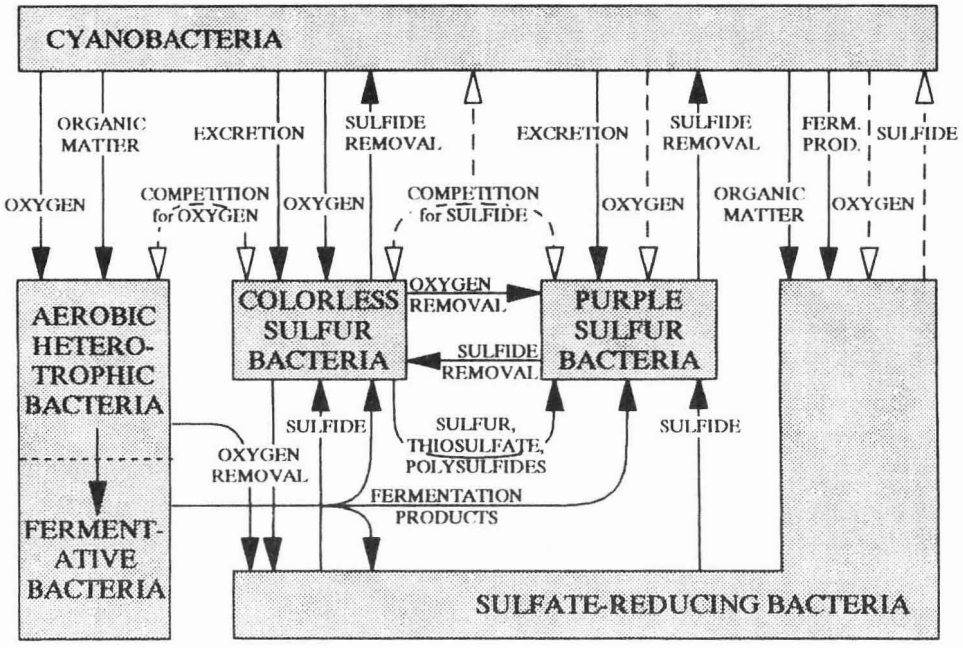

Figure 2: Schematic drawing of the interactions between the different functional groups of microorganisms in cyanobacterial mats. Positive interactions are indicated by black arrows, negative interactions are indicated by white arrows (from van den Ende and van Gemerden 1994).

DEGRADATION OF POLLUTANTS BY MICROBIAL MATS

The conditions inside microbial mats are highly suitable for biodegradation of organic pollutants . A glut of oxygen is provided in the upper layers of microbial mats by the activity of oxygenic phototrophs (diatoms and cyanobacteria) during the day (Fig. 1). O_2 not only serves as electron acceptor in the process of aerobic degradation of organic pollutants, but is often directly inserted into the substrate molecule by the activity of mono- and dioxygenases. The ability of many cyanobacteria to supply the mat with utilizable nitrogen compounds by fixation of atmospheric N_2 could represent an additional advantage for bioremediation approaches: the availability of oxygen and a nitrogen source are prerequisites for a successful aerobic degradation (Rosenberg et al. 1992). Furthermore, conditions suitable for anaerobic degradation exist in the lower parts of the mats. Not only fermentation products are used as substrates by sulfate-reducing bacteria but also organic pollutants can be mineralized under denitrifying or

sulfate-reducing conditions (Rabus et al. 1993, Rueter et al. 1994, Wilkes et al. 1995, Harms et al. 1999, Wilkes et al. 2000).

Many halogenated compounds are completely degraded only by a combination of anaerobic and aerobic steps (Fathepure and Vogel 1991, Gerritse and Gottschal 1992, Gerritse et al. 1997). Highly substituted compounds in particular require anaerobic degradation. Aerobic degradation, including O_2 insertion, is initiated by an electrophilic attack, which demands a certain electron density at the substrate site (Reineke and Schlömann 1997). This electron density is often decreased by electron-withdrawing substitutes (e.g. Cl, F, NO_2). By contrast, reductive reactions under anaerobic conditions are often nucleophilic and can be enhanced by electron-withdrawing substitutes. The less substituted the metabolites of anaerobic degradation processes become, the higher their degradability under aerobic conditions (Fig. 3). Since in microbial mats periods of oxygen oversaturation alternate with anoxic intervals within 24 h, they represent a promising environment for bioremediation of halogen-substituted hydrocarbons.

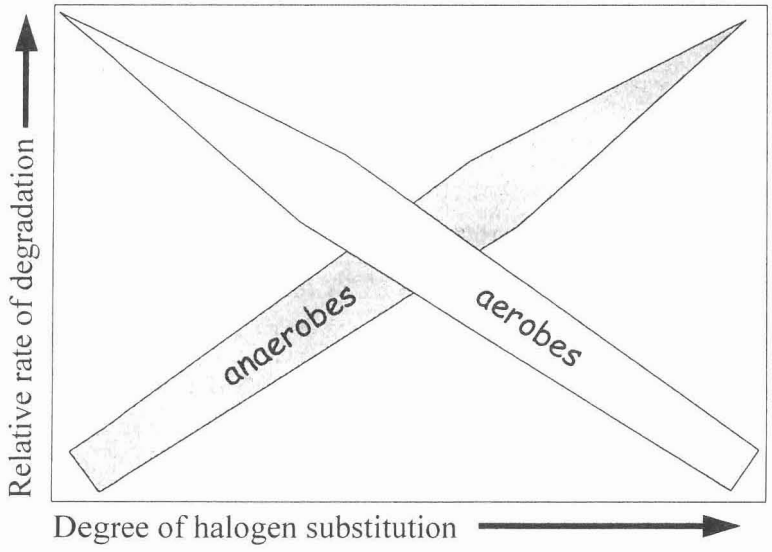

Figure 3: Trends in the degradation of halogenated compounds. Poly-halogenated compounds are best degraded by anaerobic bacteria, whereas compounds containing only few halogens are most readily mineralized by aerobes (Gerritse et al. 1992, modified).

Indirect evidence for the biodegradative capacities of microbial mats emerges from the metabolic features of their community members. Representatives from most of the physiological groups forming microbial mats (Fig. 1) were reported to contribute to microbial degradation of pollutants. Among the heterotrophic bacteria, e.g. the genera *Vibrio* and *Pseudomonas* possess a wide range of degradation abilities (Grimberg et al. 1996, Berardesco et al. 1998, Bugg et al. 2000). The non-sulfur purple bacterium *Rhodopseudomonas palustris* degraded halogenated benzoic acids (van der Woude et al. 1994, Krooneman et al. 1999). Under anaerobic conditions, degradation is often performed by sulfate-reducing bacteria. Isolates from a North Sea oil tank and from sulfide-rich marine sediments could oxidize aromatic and aliphatic crude oil hydrocarbons anaerobically (Rabus et al. 1993, Rueter et al. 1994, Wilkes et al. 1995, Harms et al. 1999, Wilkes et al. 2000). Several species of cyanobacteria were also reported to degrade organic pollutants. *Oscillatoria* sp. JCM was able to oxidize biphenyl to 4-hydroxybiphenyl (Cerniglia et al. 1980) and naphthalene to 1-naphthol (Narro et al. 1992a). *Agmenellum quadruplicatum* PR-6 converted phenanthrene into phenanthrene-trans-9,10-dihydrodiol (Narro et al. 1992b). The highly toxic chlorinated pesticide lindane (y-HCH) could be degraded by fifteen strains of cyanobacteria that belong to three taxonomic groups (Kuritz 1999). This metabolic feature was first reported for *Anabaena* sp. and *Nostoc ellipsosporum* (Kuritz and Wolk 1995).

First observations of a possible role for microbial mats in bioremediation were made after the Persian Gulf war of 1990/91. In total, 770 km of the Saudi Arabian coast were polluted by approximately 500,000 tons of crude oil released from the oil fields in Kuwait (Sorkhoh et al. 1992). The threat to marine life was particularly severe in intertidal zones, which are known to be the areas most sensitive to oil spills (Nybakken 1993). A few years after the spill, though, cyanobacterial mats were reported to have colonized polluted beaches and hypersaline enclosures ("Sabkhas"). Cyanobacterial mats, which commonly develop in coastal and hypersaline environments in temperate and arid regions, are the most important primary producers in the Persian Gulf (Golubic 1992, Sheppard et al. 1992, Al Thukair and Al Hinai 1993). Cyanobacterial mats dominated by *Microcoleus chthonoplastes* and *Lyngbya aestuarii* grew on weathered oil residues which were covered with sediment (Hoffmann 1994, Hoffmann 1996). Other mats, dominated by *Microcoleus* sp. and

Phormidium sp., developed directly on tar (Höpner et al. 1994, Höpner et al. 1996), which could indicate a connection between community composition and the specific biodegradative capabilities of the mats. Such cyanobacterial mats had not been observed before the oil spill and afterwards were found exclusively in polluted regions. Besides an apparent tolerance for hazardous oil components, the mats were directly involved in the disappearance of the oil (Höpner et al. 1996) by both biodegradation and physical removal. Shrinking, dried out mats which developed on tar, exposed the tar layer to sunlight (photooxidation), wind (erosion) and colonization by the next cyanobacterial mat layer. The ability to degrade crude oil hydrocarbons was found in two cyanobacterial strains from such mats and the oil-polluted sediment of the Gulf (Al Hasan et al. 1994, Al Hasan et al. 1998). *Microcoleus chthonoplastes* and *Phormidium corium* produced more biomass and biliprotein in the presence of crude oil or individual n-alkanes than in their absence.

In the present work, the ability of pristine cyanobacterial mats to degrade organic pollutants was investigated. The microbial mats were subjected to changing environmental conditions as well as additions of substrates and pollutants. The metabolic and functional reactions of the mats were analyzed in depth. Physiological reactions were investigated by use of microelectrodes measuring oxygen, photosynthesis, sulfide, pH and light. Additionally, the community structure of the mats was studied by use of polymerase chain reaction (PCR) in combination with denaturing gradient gel electrophoresis (DGGE). Chemical analyses of the water phase and of the mat material were done by means of HPLC and GC/GC-MS. The application of PCR and DGGE as well as of GC/GC-MS was contributed by co-authors of the individual studies (chapter 2, 4 and 5).

MICROSENSORS

Since the combination of limited transport of dissolved substances by diffusion and high microbial activity create steep physico-chemical gradients in microbial mats, it is necessary to investigate such processes with fine-scale analytical methods. Microsensors have proven highly useful in studies of microbial mats, biofilms and

microbial activity in general (Jørgensen and Revsbech 1985, Revsbech and Jørgensen 1986, Kühl and Jørgensen 1992). They allow the simultaneous measurement of several chemical or physical parameters with high spatial resolution and negligible disturbance of the samples. Three main groups can be distinguished: electrochemical microsensors, optical microsensors and bio-microsensors. In the present work, electrochemical microsensors and optical microsensors have been used.

Electrochemical microsensors are characterized by the conversion of a chemical into an electrical signal and can be divided into simple Ag/Ag+ half cells, ion-exchanger based electrodes, simple anodes or cathodes with or without polarization and Clark-type gas sensors with ion-impermeable membranes (Kühl and Revsbech 2001). Optical microsensors are based on the collection and direction of light signals through single strand fiber optics between the tip and the electronical measuring system (Holst et al. 2000). Optical microsensors comprise probes which sense light from the tip surroundings and microoptodes, which measure physicochemical variables via optical changes. Finally, microbiosensors are combinations of electrochemical microsensors and enzymes or entire cultures of microorganisms. The enzymes or microorganisms are immobilized in the sensor tip behind a membrane and transform a substance into a different chemical species. The product is subsequently detected by the inner sensor system.

O_2 microelectrodes are Clark-type sensors with a gold-coated cathode situated behind a silicon membrane and immersed in an electrolyte solution (Fig. 4). The measuring principle is based on the reduction of O_2 at the cathode. With tip sizes of 1-100 µm and a detection limit of 0.1 µM the sensor is very stable, insensitive to calcium and magnesium ions and pH independent. The lifetime of the sensors can be longer than a year. Background signals are reduced by a guard cathode which prevents diffusion of O_2 from behind to the measuring cathode (Revsbech 1989). Simultaneous measurements of oxygen concentration and volumetric gross oxygen production rate are possible with fast O_2 microsensors (90 % response time $t_{90} < 0.5$ s) and the light-dark-shift method (Revsbech and Jørgensen 1983). The method is based on the determination of the gross oxygen production rate by the decrease in O_2 concentration during a one second dark period. By flux calculations, it is possible to estimate the oxygen production and consumption in different layers of microbial mats like the

photic and aphotic zone (Jensen and Revsbech 1989, Kühl et al. 1996). Additional details can be found in chapters 2 and 3.

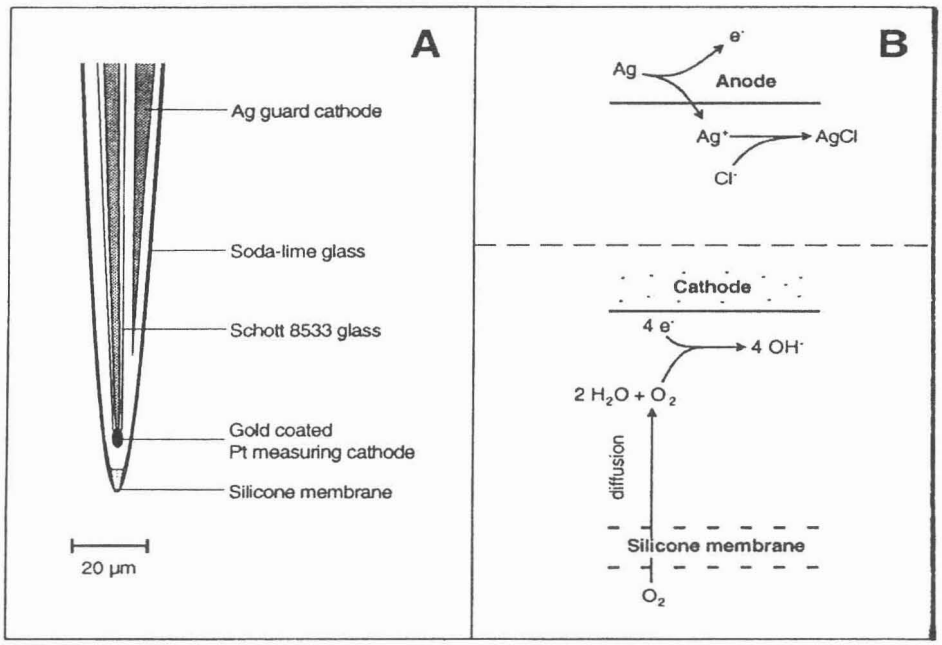

Figure 4: Clark-type O_2 microsensor. A: Detailed view of the sensor tip. B: Schematic drawing of the measuring principle (from Kühl and Revsbech 2001).

H_2S microelectrodes are also Clark-type sensors with a platinum anode behind a silicon membrane (Fig. 5), tip sizes of 10-100 µm and a detection limit of 1 µM H_2S. The lifetime of the sensors is comparatively short (weeks). Since the membrane is impermeable to ions, sulfide diffuses into the sensor only in the form of H_2S, and is oxidized to sulfur by ferricyanide. The ferrocyanide formed is subsequently re-oxidized at the platinum anode. Since the dissociation of H_2S depends on the pH, the H_3O^+ concentration has to be considered for the total dissolved sulfide determination (Kühl et al. 1998). Under conditions of pH < 9, the total sulfide concentration can be calculated by

$$S^{2-}_{tot} = [H_2S] (1 + K_1/[H_3O^+])$$

with K_1 as dissociation constant of the first dissociation stage of H_2S.

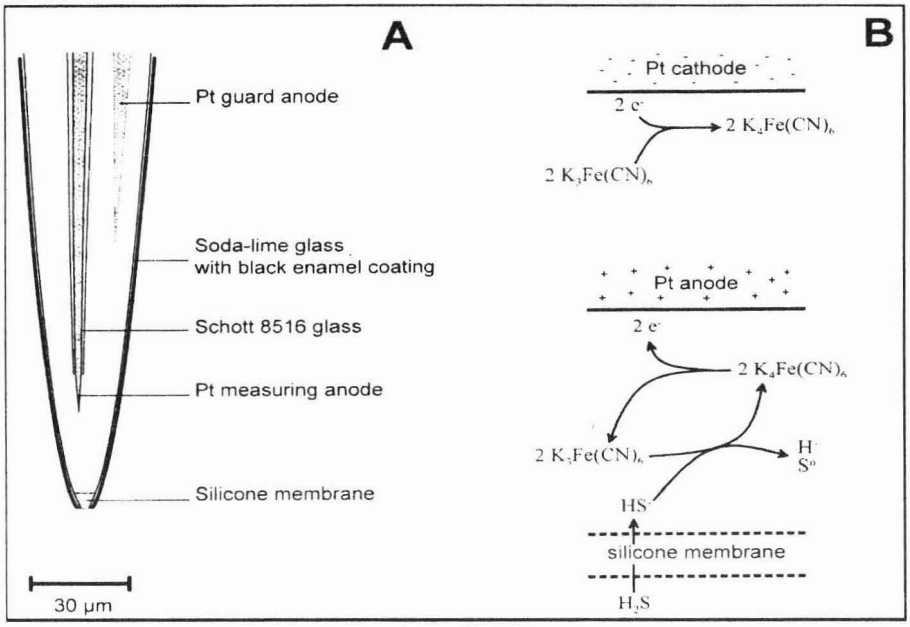

Figure 5: Clark-type H_2S microsensor. A: Detailed view of the sensor tip. B: Schematic drawing of the measuring principle (from Kühl et al. 1998).

pH microelectrodes are small versions of the well known commercial pH electrodes with tip sizes of 20-200 µm and a detection limit between pH 1 and 14. The measuring principle is based on the electrical potential at a pH-sensitive glass membrane (Hinke 1969, Thomas 1978). The electrode is the only glass microsensor relevant in microbial ecology and has a comparatively long lifetime.

Field radiance microprobes are simple tapered optical fibres which collect light from a certain angle. The tip size is 5-140 µm and the detection limit for UVB-NIR light is lower than 1 µmol photons/m^2s. The microprobes were designed to measure microscale spectral light distributions in sediments and biofilms. They have beeb applied to study zonations and migrations of photosynthetic microorganisms (Kühl and Revsbech 2001).

Scalar irradiance microprobes are tapered optical fibres with small light-scattering spheres at the tip. The probes sense the incident quantum flux from almost all directions (Lassen et al. 1992). With such scalar irradiance microprobes it is possible to measure the light available for photosynthetic organisms in benthic environments, which may experience irradiance from all directions.

THESIS OUTLINE

The main objective of the present work was to investigate changes in physiology, chemistry and community structure of hypersaline cyanobacterial mats upon addition of organic pollutants. Hypersaline mats represent ideal models for functional investigations of ecosystem reactions to pollution because of their nearly closed substrate cycles (Fig. 2). In the first phase, physiological changes in the mats under changing environmental conditions in general were investigated. In the second phase, direct degradation experiments were performed.

How does an additional carbon and energy source affect the internal carbon turnover in microbial mats?

To date there are no reports about the effects of the addition of non-toxic substrates on photosynthesis and respiration in intact microbial mat communities. It was hypothesized that upon substrate addition respiratory activity would increase, leading to higher photosynthetic rates, if the mats were CO_2 limited. Acetate, glycolate and glucose were added to hypersaline cyanobacterial mats from Eilat (Israel). The results of this study are presented in Chapter 2.

Assuming that the addition of a usable carbon source would lead to the depletion of oxygen in the microbial mats due to increased respiratory activity, what would be the feedback effect of the lower O_2 level on the phototrophic organisms?

CO_2 is fixed in photoautotrophs via the reductive pentose phosphate pathway (Calvin cycle) (Stal 2000). The carboxylation yield depends on the ratio of O_2 to CO_2 at the reaction centers of the key enzyme, ribulose-1,5-bisphosphate-carboxylase/ogygenase (Rubisco) (Falkowski and Raven 1997). In a laboratory study, the effects of oxygen concentration on respiration and photosynthesis in two hypersaline cyanobacterial mats from Eilat (Israel) and Mallorca (Spain) were investigated. The results of this study are presented in Chapter 3.

Do microbial mats represent an efficient system for the bioremediation of oil pollutants?

The ability of pristine hypersaline cyanobacterial mats to degrade four hydrophobic petroleum model compounds was investigated. In a four month laboratory study the impact of contamination with n-octadecane, pristane, phenanthrene and dibenzothiophene on the metabolism and community structure of mats from Eilat (Israel) was studied. The results of this study are presented in Chapter 4.

Are water-soluble chlorinated hydrocarbons degraded by microbial mats?

The degradability of a compound often depends on the abundance of similar chemical structures in nature; substances formed in the environment like e.g. petroleum hydrocarbons are usually degraded better than artificial structures like e.g. most haloorganic compounds (Reineke and Schlömann 1997). In a laboratory study, the degradation of the systemic herbicide 2,4-Dichlorophenoxyacetic acid (2,4-D) by hypersaline cyanobacterial mats from Guerrero Negro (Mexico) was investigated. The results of this study are presented in Chapter 5.

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Chapter 2

Metabolic shifts in hypersaline cyanobacterial mats upon addition of organic substrates

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ABSTRACT

Hypersaline cyanobacterial mats are typically closed ecosystems with respect to the carbon and sulfur cycles. As photosynthesis and respiration are closely coupled, net accretion of biomass is low. In this study the effects on respiratory and photosynthetic rates in such mats on the addition of acetate, glycolate or glucose were investigated using microsensors for oxygen, pH and sulfide. Changes in the community structure of the mat induced by the external carbon sources were investigated with molecular techniques. The addition of external carbon sources resulted in different physiological responses depending on the type of organic compound added. Acetate inhibited respiration in the photic zone, stimulated respiration in the aphotic zone, and had no effect on gross photosynthesis. Glycolate addition strongly increased both respiration and gross photosynthesis in the photic zone. Thus, glycolate and acetate were probably consumed in those regions of the mat where these substrates are usually formed. Moreover, photosynthesis was only stimulated by increased respiration and concomitant CO_2 production in the photic zone, supporting the hypothesis that respiration and photosynthesis are closely coupled. The photosynthetic and respiratory populations must be present in close proximity to each other. Glucose, a substrate which has not been reported to be present in the pore water of microbial mats, had an unexpected negative effect on the microbial community, strongly inhibiting both respiration and gross photosynthesis within hours after addition. After four days, oxygen profiles in the light were equal to those measured in the dark. After replacing the water phase with unamended water, photosynthesis and respiration recovered within a week. None of the physiological changes were accompanied by detectable shifts in the cyanobacterial or the overall microbial community. The mat recovered quickly from shifts induced by external organic compounds that are normally formed in the mats. However, glucose, an easily metabolizable compound that is not normally formed in the mats, strongly disturbed the ecological equilibrium. The mechanism of inhibition of photosynthesis by glucose requires further investigation.

INTRODUCTION

Hypersaline cyanobacterial mats are dense, stratified microbial agglomerations which consist of different physiological groups of microorganisms (Stal 1995). Primary production is accomplished by various phototrophs (cyanobacteria, diatoms, purple sulfur bacteria), while remineralization is mediated by the activities of aerobic and anaerobic heterotrophs (van Gemerden 1993). Microbial mats are distributed almost all over the world but develop predominantly under extreme conditions (e.g. high salinity or temperature) that limit grazing (Krumbein et al. 1977, Bauld 1984, Cohen 1989, D´Amelio et al. 1989). During the day, photoautotrophic organisms supply the mat with oxygen and organic photosynthates (Revsbech and Jørgensen 1983, Revsbech et al. 1989). Despite high photosynthetic rates, net biomass production is low due to high mineralization rates. Thus, the mats represent complete ecosystems characterized by internal element cycles (Karsten and Kühl 1996). In these closed systems the different metabolic processes are interdependent, e.g. respiration is limited by the presence of photosynthates, while photosynthesis may be limited by CO_2, a product of respiration. We hypothesized that upon addition of easily degradable organics respiration would increase, in turn leading to increased photosynthetic rates.

The effect of several organics (glucose, fructose, ribose, sucrose, glycerol) on individual cyanobacterial strains has been investigated previously (Rippka et al. 1979), showing that 75 out of 140 isolates could grow photoheterotrophically (Smith 1982). Observations on single species or uni-algal cultures cannot easily be extrapolated for cyanobacterial mats, because of the complexity of such microbial communities. The addition of glycolate, lactate, ethanol, acetate and glycine betaine to a cyanobacterial mat have been investigated, with emphasis on the effects on sulfate reduction activities (Fründ and Cohen 1992). Although the responses of sulfate-reducing bacteria to the additions were significantly different in the discrete layers of the mat, the effects on photosynthesis and respiration have not been studied. To analyze these metabolic functions and detect any changes in community structure in cyanobacterial mats, we applied a combination of microsensor techniques and molecular tools.

MATERIALS AND METHODS

Mat samples and incubation. Cyanobacterial mat samples were collected from a hypersaline experimental pond at the Interuniversity Institute IUI, Eilat (Israel). The pond had been inoculated several times with a total of 10 m^2 of mat material collected from Solar Lake, Egypt, between 1995 and 1998. During this period, the original mats spread, eventually covering an area of 48 m^2 with a thickness of 2 cm (1999). In April 1999, two pieces of such mat material (10 x 20 cm) were transferred to the Max Planck Institute for Marine Microbiology in Bremen (Germany), and pre-incubated in an aerated aquarium filled with artificial seawater (ASW). The salinity was 7.5 %, the temperature 27 °C and the pH 8.2. The samples were illuminated with a downwelling irradiance of 580 µmol photons/m^2s, 12 h light/12 h dark. Between June and November 1999 rectangular subsamples (8 x 5 x 2.5 cm; l/w/h) were embedded in agar (1.5 %, ASW) in a flowcell similar to the one described previously by Lorenzen et al. (1995). 1 l of aerated ASW was constantly cycled over the mat surface. The treatments were performed in the same salinity, temperature, pH and illumination as during the pre-incubation.

The mats were left to acclimate for at least one day in the flowcell before the measurements started. After an initial determination of net and gross photosynthesis on day 0 of each treatment, an organic substrate (acetate, glycolate or glucose) was added to the overlying water to a final concentration of 5 mM. One treatment with 15 mM acetate and an incubation with 5 mM formate were performed for three days. The glucose addition treatment was repeated twice in the laboratory and on site in Eilat. The control mat was maintained without addition. Regular measurements were done: 1) water samples were taken and frozen at –20° C for subsequent HPLC analyses; 2) small cores (~ 0.5 cm in diameter) of mat material were collected for molecular analyses and frozen at –80° C; 3) metabolic activities were measured with microsensors. In case of glucose addition, the entire water phase was replaced by fresh ASW after 12 days and the treatment was extended to 25 days.

Microsensors. Oxygen concentrations and gross photosynthetic rates were determined with fast-responding Clark-type O_2 sensors with guard cathodes (Revsbech 1989) using the light-dark-shift method (Revsbech and Jørgensen 1983). The O_2 sensors had tip diameters of less than 6 µm, a stirring sensitivity of less than 2 %, and a 90 % response time of $t_{90} < 0.5$ s. Measurements of pH values were performed with potentiometric glass microelectrodes (Revsbech et al. 1983), which had a tip diameter of less than 10 µm with the pH sensitive glass at the very tip not longer than 100 µm. The electrodes showed a 90 % response time of $t_{90} < 10$ s. The H_2S sensors (Kühl et al. 1998) had a tip diameter of less than 20 µm. All electrodes were calibrated as described elsewhere (Revsbech and Jørgensen 1986, Revsbech 1989, Santegoeds et al. 1998).

From oxygen concentration profiles and profiles of gross photosynthetic rates we determined respiration rates based on the mass balance

net oxygen production = gross oxygen production – oxygen consumption
(photosynthesis) (respiration)

From steady-state oxygen profiles, local areal fluxes of oxygen (i.e. fluxes through each depth layer J_z) were calculated using Fick´s first law of one-dimensional diffusion (Jørgensen and Revsbech 1985, Kühl et al. 1996)

$$J_z = D_e * dC_z/dz$$

with D_e as the effective diffusion coefficient and dC_z/dz as the concentration gradient at depth z.

Based on diffusion measurements in similar mats (Wieland et al. 2001), D_e was assumed to be 60 % of the free solution molecular diffusion coefficient for oxygen (D_0) (Broecker and Peng 1974, Li and Gregory 1974). Oxygen solubility values for different temperatures and salinities were calculated using equations from Garcia and Gordon (1992).

Local net oxygen production rates were calculated from local areal fluxes. Local respiration rates were calculated by subtracting local net oxygen production

rates from local gross photosynthetic rates. To determine areal rates for the entire mat, the local rates were integrated over depth. Areal fluxes for the entire mat as well as for the photic zone and the aphotic zone were also calculated from concentration gradients above the mats and below the photic zone (Jensen and Revsbech 1989, Glud et al. 1992, Kühl et al. 1996). We define the photic zone as the layer in which gross photosynthetic activity could be detected by use of a 1-s dark period (Glud et al. 1992). All data were plotted as an average of three measurements.

For statistical analyses the so-called "moving average" was used, a powerful statistical tool which provides trend information related to those data which the process under consideration would mask. Based on the average value of the variables over a specific number of proceeding periods, values in the forecast period are projected based on

$$F_{t=1} = \frac{1}{N}\sum_{j=1}^{N} A_{t-j+1}$$

with N being the number of prior periods to be included in the moving average, whereas A_j and F_j are the actual value and the forecasted value at time j, respectively. Experimental data from three individual microsensor profiles were taken and plotted along with the deviations of the profiles from the moving average

Molecular analyses. From mat cores obtained from all treatments (glucose, acetate and glycolate) nucleic acid extraction, polymerase chain reaction (PCR) and denaturing gradient gel electrophoresis (DGGE) were performed as described previously (Abed and Garcia-Pichel 2001). From mats treated with acetate or glycolate, the upper 0-2 and 2-4 mm layers of each core were used for the analyses, while from the mat treated with glucose the upper 0-10 mm layer was taken. Total RNA was extracted from the samples and 16S rRNA amplified by reverse transcription and PCR (RT-PCR). Two sets of oligonucleotide primers were used: CYA359F (with a 40 nucleotide GC clamp at the 5′ end) and CYA781R for cyanobacteria (Nübel et al. 1997), and GM5F with a GC-clamp in combination with the universal 907R primer for total bacteria. Approximately 10 ng of DNA was used as

template for the PCR. A hot start program was performed for the cyanobacterial-specific primers as described by Nübel et al. (1997), whereas in case of bacterial universal primers (GM5 and 907R), a hot-start touch-down program was used to minimize non-specific amplification (Santegoeds et al. 1998).

HPLC analyses. The centrifuged (15 min at 14.000 rpm) and acidified (7 mM H_2SO_4) water samples were analyzed with an HPLC system (Waters 600 E, USA) equipped with a Vertex column (Eurosphere-100 C18, 5µm) and UV-detector (Waters 991 PDA). The eluent was 20 mM NaH_2PO_4 (pH 2.8), the pressure 1900 psi and the flow 1 ml/min. A standard mixture of seven short chain fatty acids was used for calibration. All samples were screened for more than 40 individual substances, mainly fatty acids and amino acids. Glucose analyses was performed with a Dionex DX 500 HPLC system equipped with a CarboPac PA-10 column (4 x 250 mm; eluent: 45 mM NaOH solution) and a Dionex ED 40 electrochemical detector operated in pulsed-amperometry mode. The instrument was calibrated with 100 nM lyxose.

RESULTS

The mat samples consisted of a dense structure in which laminations could be observed macroscopically. The mat surface was fairly flat with some microtopography, soft and gelatinous, with a dark green colour and some light green patches. For all experimental conditions the effective diffusive boundary layer (DBL) (Jørgensen and Revsbech 1985) was 300-500 µm thick, as measured with oxygen microelectrodes.

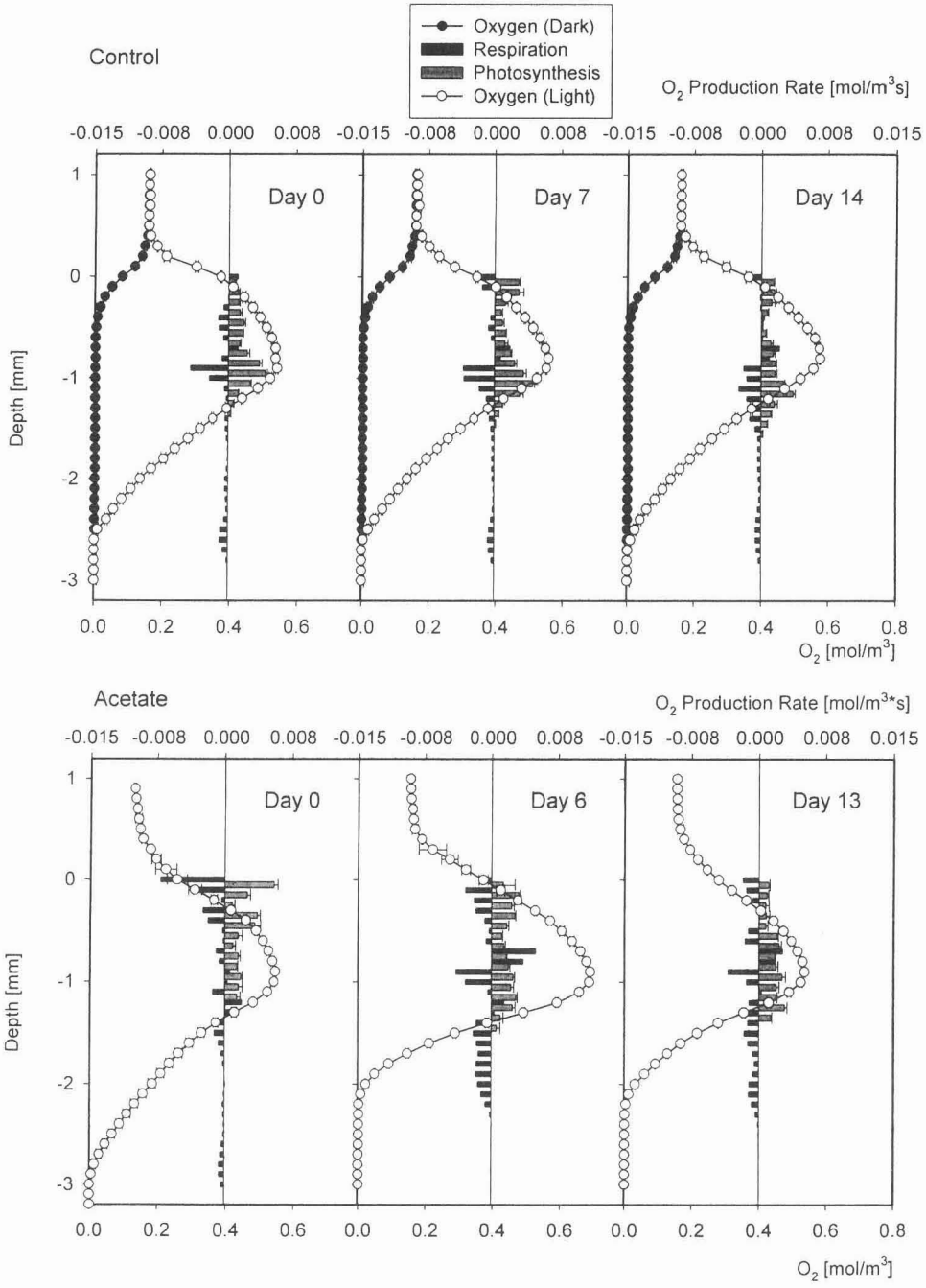

Figure 1: Top: Oxygen concentrations measured in the light and in the dark (means ± standard deviations, n = 3), volumetric gross photosynthetic rates (means ± standard deviations, n = 3) and calculated volumetric respiration rates in a cyanobacterial mat from Eilat during 14 days of incubation without substrate addition. Bottom: Oxygen concentrations (means ± standard deviations, n = 3),volumetric gross photosynthetic rates (means ± standard deviations, n = 3) and calculated volumetric respiration rates in the light in a cyanobacterial mat from Eilat during 13 days of incubation with 5 mM acetate (addition after measurement on day 0).

Control. Oxygen profiles in the light and in the dark as well as gross photosynthesis profiles and light respiration profiles are presented in Fig. 1 (top). Microprofiles measured in the light at different locations showed only small variations. The distribution of gross photosynthetic rates and respiration rates in the light varied slightly. Areal net oxygen production rates and areal respiration rates in the photic and aphotic zone of the mat (Fig. 5, A+B), remained constant during the incubation of 14 days. Thus, the mats remained in constant physiological condition in this experimental set-up, and any changes were caused by the experimental treatments. No organic compounds could be detected in the overlying water. There were no variations in the oxygen profiles during the dark periods within two weeks (Fig. 1, top). Nearly identical dark profiles were observed in all treatments (data not shown).

Acetate addition. Acetate addition had no immediate effect on gross photosynthesis and respiration. After one day the respiration rates below the photic zone increased, leading to a reduced oxygen penetration depth (Fig. 1, bottom). The respiration rates in the photic zone decreased, leading to an increase in the oxygen peak at 1 mm depth. The gross photosynthetic rates remained the same. The respiration rates in the aphotic zone increased and remained elevated throughout the rest of the experiment (Fig. 5, D). However, due to strongly inhibited areal respiration rates in the photic zone , the areal net oxygen production rate increased to more than 200 % of the initial values (Fig. 5, C). During the subsequent experimental exposure, the areal net oxygen production rates in the entire mat gradually decreased to the original levels. The acetate concentration in the overlying water decreased continuously to 0.5 mM at the end (Fig. 5, C). No other organic substances were detected. Upon exposure to 15 mM acetate in an additional treatment similar effects were observed: areal gross photosynthetic rates did not change, O_2 penetration depth decreased by 1 mm, areal respiration rates for the entire mat did not change (data not shown).

Glycolate addition. The addition of glycolate had no immediate effect on the mat (Fig. 6, A+B). After 48 h, both areal respiration rates and areal gross photosynthetic rates increased strongly. Areal respiration rates increased especially in the photic zone, reaching 5 times the initial values on day 6 (Fig. 6, B). Although the areal gross photosynthetic rates increased 3 fold in the top 300 µm of the mat, the areal net oxygen production increased only slightly (Fig. 6, A). The oxygen penetration depth and the maximum oxygen concentration (c_{max}) decreased, because of the strongly increased respiration rates in the upper 2 mm of the mat (Fig. 2). During the subsequent 13 days of the glycolate exposure, the gross photosynthetic rates and respiration rates gradually returned to almost the original values. Glycolate gradually decreased in the course of the treatment to 50 % of its initial concentration (Fig. 6, A), while lactate gradually accumulated to a concentration of 50 µM. No other organic compounds were detected.

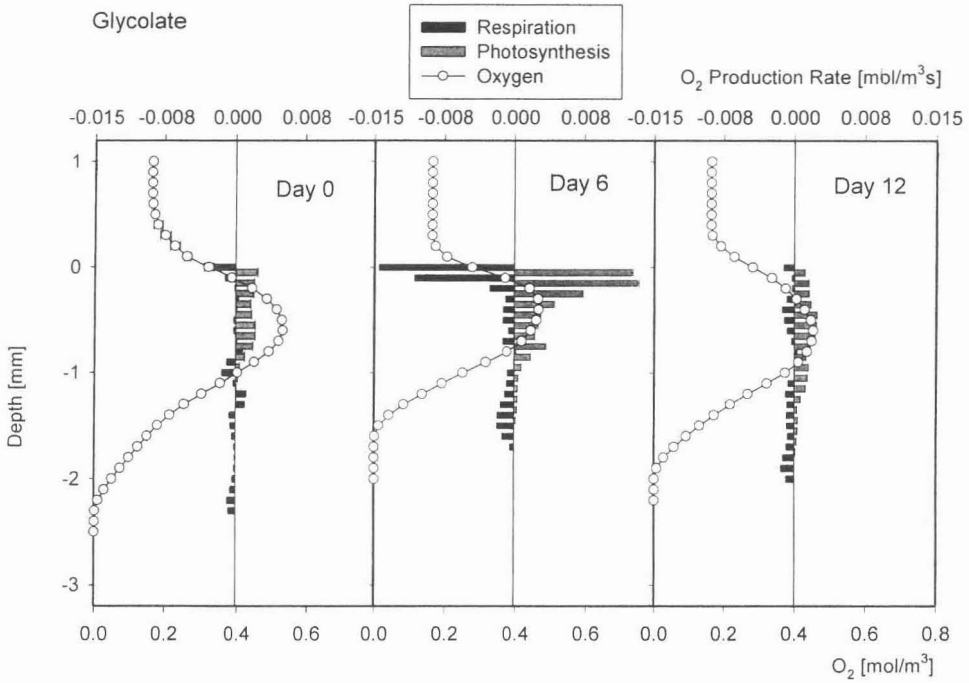

Figure 2: Oxygen concentrations (means ± standard deviations, n = 3), volumetric gross photosynthetic rates (means ± standard deviations, n = 3) and calculated volumetric respiration rates in the light in a cyanobacterial mat from Eilat during 12 days of incubation with 5 mM glycolate (addition after measurement on day 0).

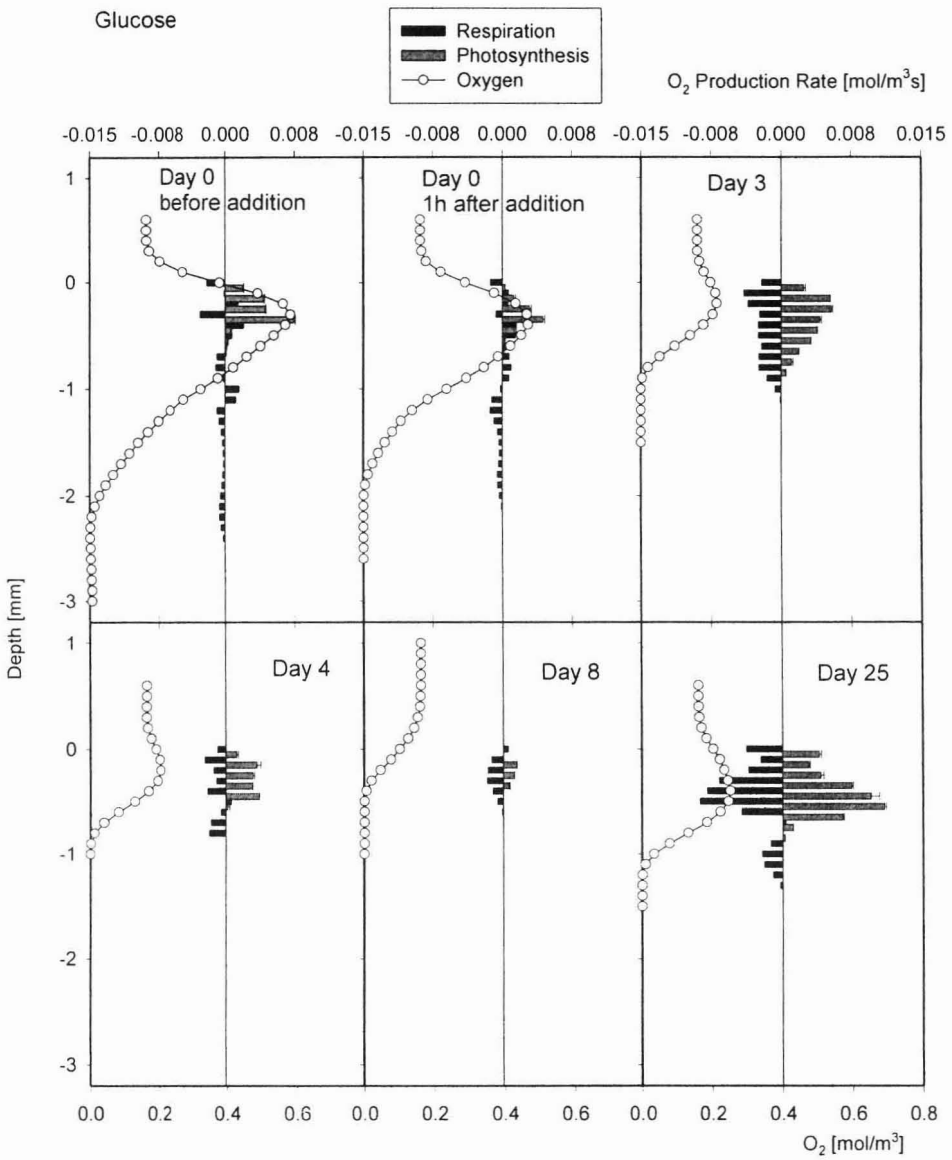

Figure 3: Oxygen concentrations (means ± standard deviations, n = 3), volumetric gross photosynthetic rates (means ± standard deviations, n = 3) and calculated volumetric respiration rates in the light in a cyanobacterial mat from Eilat during 25 days of incubation with 5 mM glucose (addition after measurement on day 0). On day 12 the water phase was replaced with fresh artificial seawater without substrate.

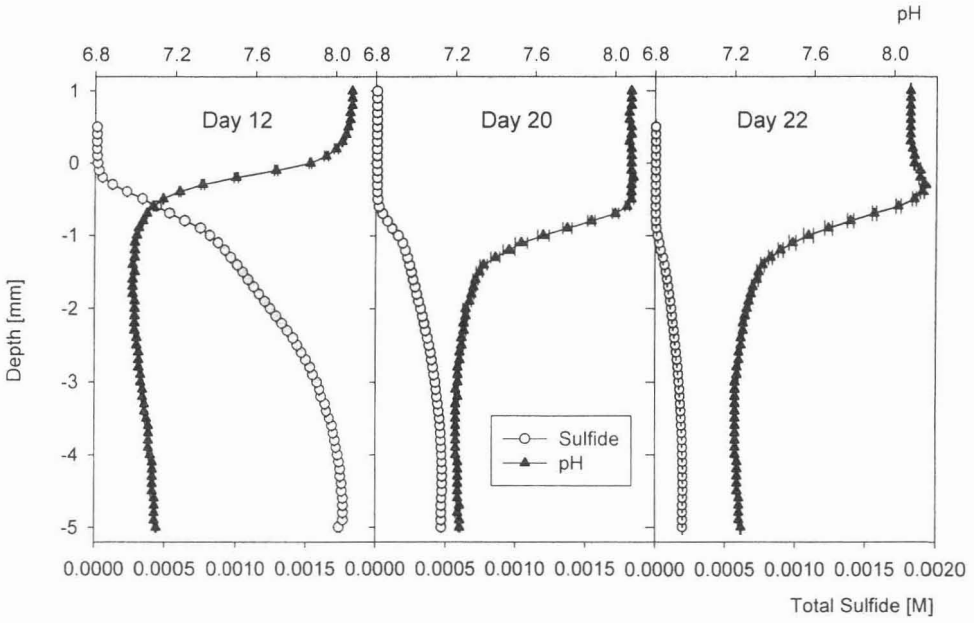

Figure 4: Total sulfide concentrations (means ± standard deviations, n = 3) and pH values (means ± standard deviations, n = 3) in a cyanobacterial mat from Eilat during incubation with 5 mM glucose. On day 12 the water phase was replaced with fresh artificial seawater without substrate.

Glucose addition. Upon glucose addition a complicated pattern of metabolic responses occurred. Immediately after glucose addition, gross photosynthetic rates decreased strongly (Fig. 3, top). Oxygen concentrations decreased within 5 minutes of glucose addition (data not shown). Simultaneously, respiration rates, oxygen penetration depth and c_{max} decreased. Subsequently, the thickness of the photic zone and the gross photosynthetic rates increased. While areal respiration rates in the photic zone increased by over 1000 % in the following days (Fig. 6, D), the oxygen concentration and areal net oxygen production rates decreased. After day 4 the mat consumed oxygen from the water phase (negative areal net oxygen production rates, Fig. 6, C). On day 8, the light oxygen profiles were identical to dark oxygen profiles, although some photosynthesis was still detectable. The overlying water was exchanged at day 12, which had no immediate effect on the oxygen profiles or photosynthetic rates. Sulfide was formed in the deeper layers of the mat and accumulated on day 12 to nearly 1.8 mM at 5 mm depth and 0.8 mM at 1 mm. The pH at 1 mm was 7 (Fig. 4). In the subsequent days the sulfide concentrations decreased and the mat became more

alkaline. On day 18 a first sign of a recovery was indicated by an increase in oxygen concentration. Thereafter, gross photosynthetic rates and respiration rates increased rapidly. Finally, areal gross photosynthetic rates were nearly 3 times higher and areal respiration rates were 5 times higher in the entire mat than before the addition. The glucose concentration decreased to 0.5 mM after 12 days (Fig. 6, C). During the same period, a variety of other organic substances were detected in the supernatant. Formate was detected with a maximum concentration of nearly 2 mM, the acetate concentration was over 1 mM and the α-ketoglutarate concentration was 2 µM at the end of the treatment. In summary, upon glucose addition respiration and photosynthesis were quickly inhibited, followed by a recovery to higher levels than before. A second decrease in both processes followed, from which the mats only recovered after replacement of the overlying water. The second recovery also resulted in higher respiration and photosynthesis rates than before.

In a short-term treatment, 5 mM formate inhibited areal gross photosynthetic rates by 17 % and areal respiration rates by 18 % within two hours. After one day, areal gross photosynthetic rates were 20 % lower and areal respiration rates 42 % lower. The inhibitory effect of 5 mM glucose was reproducible in several field and laboratory experiments. Under field conditions, the mats recovered within 8 days from the decrease in gross photosynthetic rates (64 %) and respiration rates (22 %).

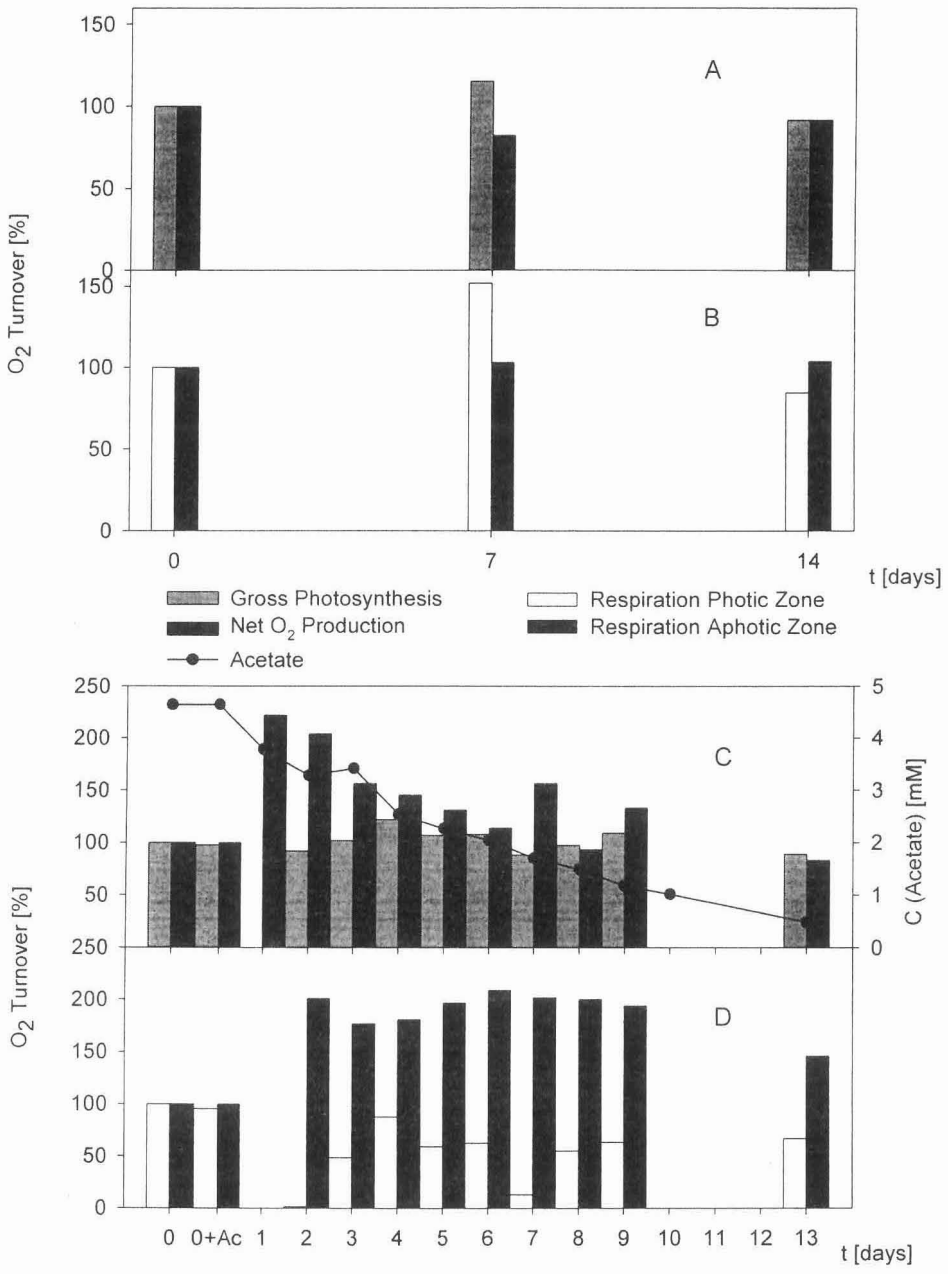

Figure 5: A: Areal gross and net oxygen production rates in the light in a cyanobacterial mat from Eilat during 14 days of light/dark incubation without substrate addition. Rates are normalized to 100 % for the start of the experiment. B: Areal oxygen consumption rates in the photic and aphotic zone of the same mat. C: Areal gross and net oxygen production rates in the light and acetate concentrations in a cyanobacterial mat from Eilat during 13 days of light/dark incubation after addition of 5 mM acetate (0+Ac). Rates are normalized to 100 % for the start of the experiment before addition of acetate. D: Areal oxygen consumption rates in the photic and aphotic zone of the same mat.

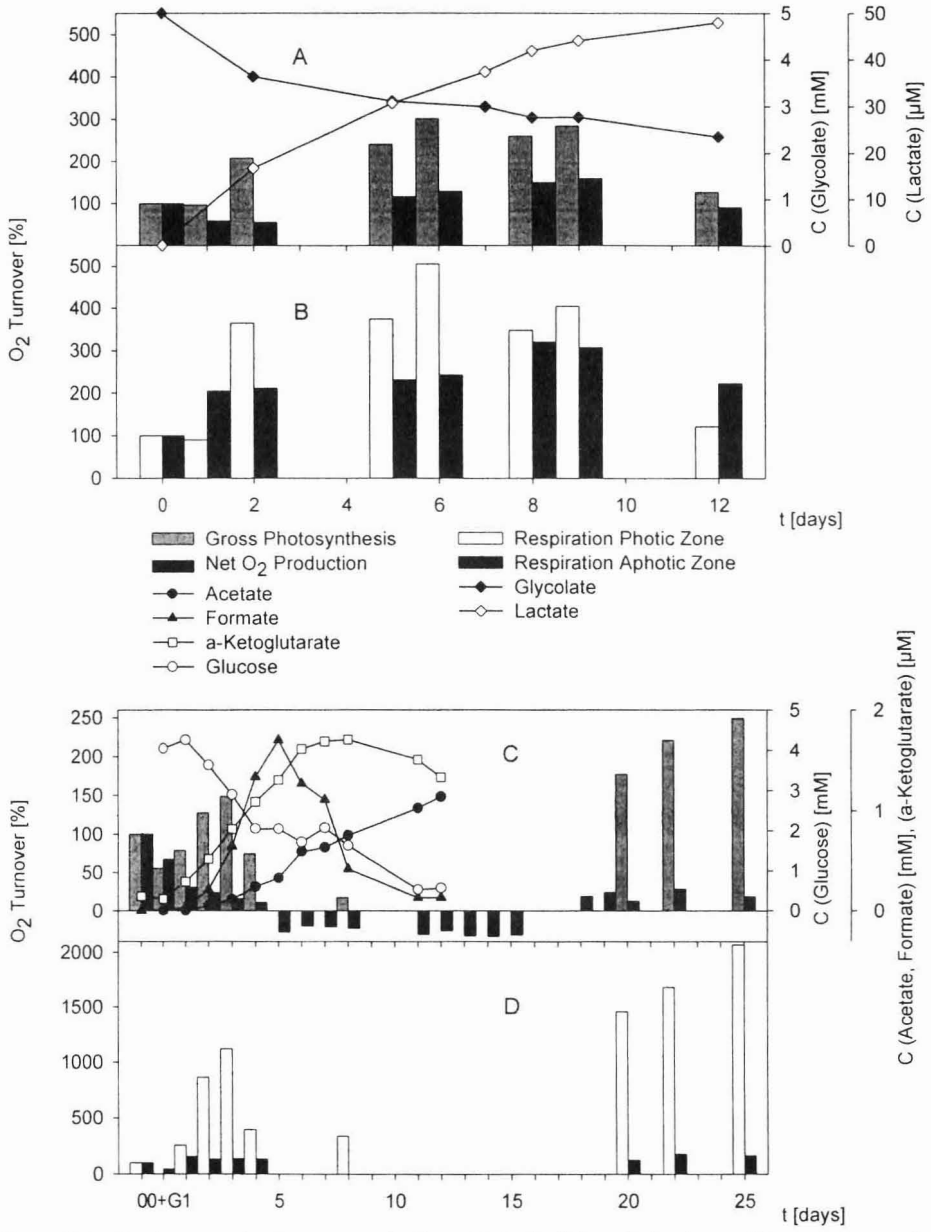

Figure 6: A: Areal gross and net oxygen production rates in the light and glycolate and lactate concentrations in a cyanobacterial mat from Eilat during 12 days of light/dark incubation after addition of 5 mM glycolate. Rates are normalized to 100 % for the start of the experiment before addition of glycolate. B: Areal oxygen consumption rates in the photic and aphotic zone of the same mat. C: Areal gross and net oxygen production rates in the light and glucose, acetate, formate and α-ketoglutarate concentrations in a cyanobacterial mat from Eilat during 25 days of light/dark incubation after addition of 5 mM glucose (0+G). Rates are normalized to 100 % for the start of the experiment before addition of glucose. On day 12 the water phase was replaced with fresh artificial seawater without substrate. D: Areal oxygen consumption rates in the photic and aphotic zone of the same mat.

Statistical analyses. Individual oxygen profiles and moving averages on the last day of each addition are shown in Fig. 7, A1-C1 (A: acetate, B: glycolate, C: glucose). The deviations of the individual profiles from the corresponding calculated moving averages are plotted in Fig. 7, A2-C2, to demonstrate the quality of the measurements on representative examples. The deviations are at least one order of magnitude smaller than the measured values, which shows a good agreement of the individual profiles and the corresponding calculated moving averages.

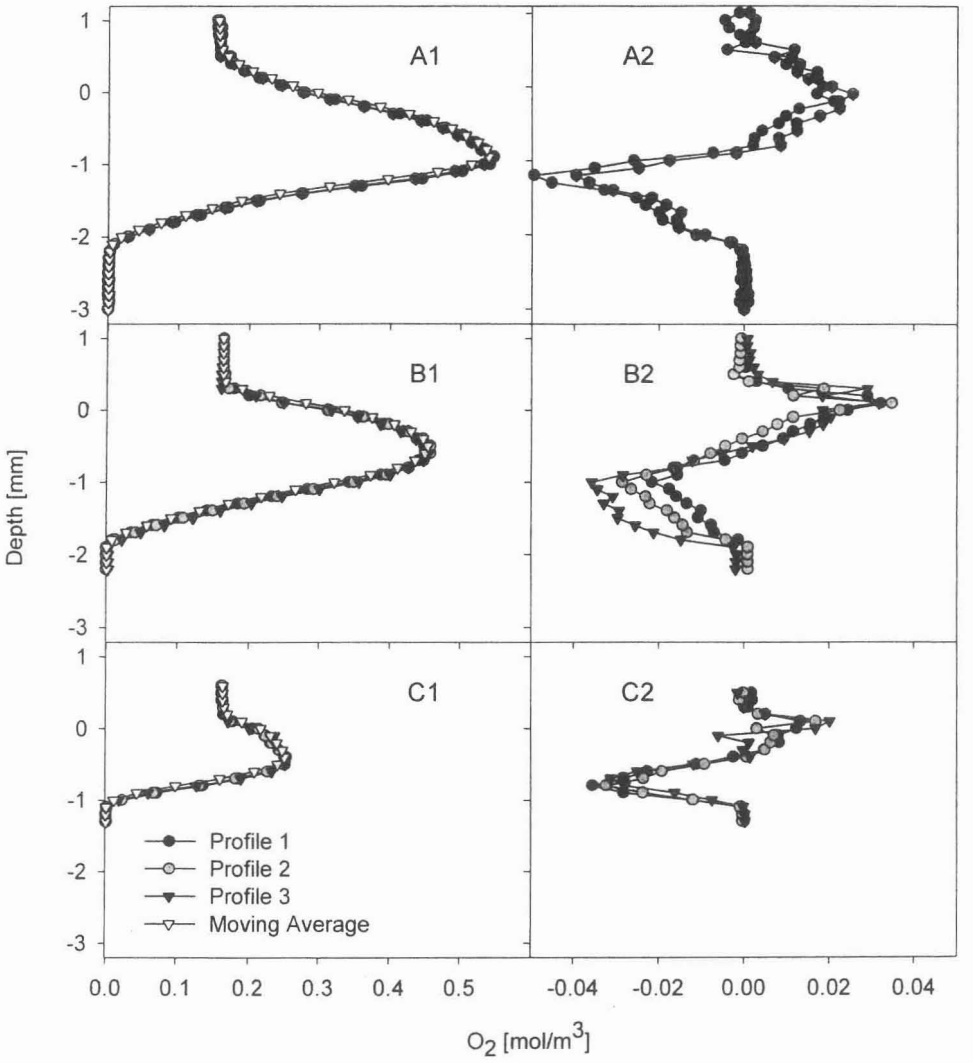

Figure 7: Single oxygen profiles with moving average (A1 –C1) and deviations of the profiles from the moving average (A2-C2) on the last day of each addition experiment; A : acetate, B: glycolate, C: glucose.

Molecular community analyses. The results from the DGGE analyses of mats treated with glucose are presented in Fig. 8 and 9. Fig. 8 shows the development of the cyanobacterial community in the upper 1 cm of mat from day 0 to day 22. The same six dominant bands were observed in the course of the treatment. Using eubacterial primers, the banding pattern also remained the same during the treatment (Fig. 9). The same results were obtained with mats treated with acetate or glycolate. Thus, none of the treatments resulted in detectable changes in the cyanobacterial or the eubacterial community.

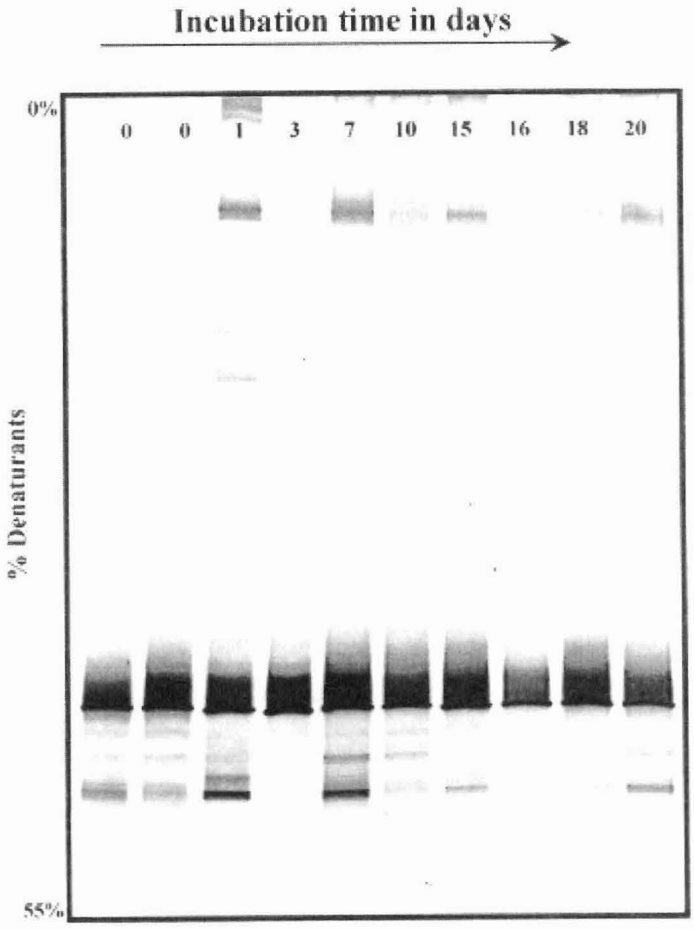

Figure 8: DGGE banding patterns of PCR-amplified 16S rRNA fragments obtained from microbial mats exposed to 5 mM glucose at different time intervals. The amplification was carried out using cyanobacterial specific primers (CYA359F and CYA805R).

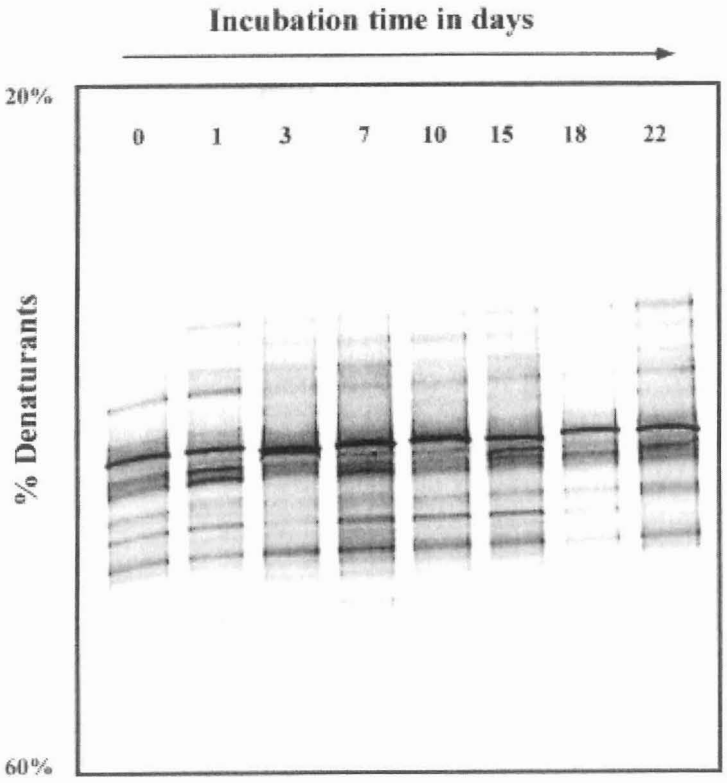

Figure 9: DGGE banding patterns of PCR-amplified 16S rRNA fragments obtained from microbial mats exposed to 5 mM glucose at different time intervals. The amplification was carried out using universal primers (GM5F and 907R).

DISCUSSION

In this study we investigated how externally supplied organic substrates affect oxygen production and consumption in hypersaline cyanobacterial mats. The effects on community structure were also investigated. The organic compounds were chosen because of their relevance in these environments. Acetate and glycolate are intermediates in the internal carbon cycle of the mats and have been detected in their porewater (Bateson and Ward 1988, van Gemerden 1993, Stal and Moezelaar 1997). Glucose is not present in the porewater of cyanobacterial mats, but can serve as a carbon and energy source for a wide range of micro-organisms and is one of the most abundant carbohydrates in many environments (Smith 1982).

We hypothesized that the addition of organic compounds would stimulate respiration and that the concomitant CO_2 formation would in turn lead to increased photosynthesis. The different treatments showed that this was not generally true. Instead, the effect on photosynthesis and respiration depended on the type of organic compound.

Although the changes in gross photosynthetic rates and respiration rates were significant (e.g. Fig. 3), we could not detect changes in the DGGE patterns, neither with cyanobacterial nor eubacterial primers (Fig. 8 and 9). The primer sets used in this study have been proven suitable for the detection of community changes in other environments (Santegoeds et al. 1998, Abed and Garcia-Pichel 2001). It is well documented that DGGE can detect populations, which account for 1 % or more of the total community (Muyzer et al. 1998). Therefore, we conclude that the dominant populations in the mats persisted throughout the experiment. Moreover, the addition of glucose, acetate or glycolate did not lead to development of a new dominant (> 1 %) population. All changes in photosynthesis and respiration were likely therefore physiological reactions and not due to changes in the community composition.

Glucose addition. Glucose had the most pronounced effect, leading to strong inhibition of gross photosynthesis and respiration. The first inhibitory effect of glucose was detected within 1 h (Fig. 3), suggests that the substrate itself and not a metabolite was causing the inhibition. The enzymatic equipment for photoheterotrophy is found in more than half of the cyanobacteria tested so far (Tandeau de Marsac and Houmard 1993) and many cyanobacteria with the ability to grow heterotrophically are able to take up glucose by active transport (Smith 1982). An indication of the direct uptake and metabolization of glucose by cyanobacteria is the formation of α-ketoglutarate. Many cyanobacteria lack α-ketoglutarate-dehydrogenase, the enzyme which turns this intermediate of the tricarboxylic acid cycle (TCA) over to succinyl-CoA (Beardall and Raven 1990), while eucaryotic microalgae possess the enzymes of a complete TCA cycle. Thus, although cyanobacteria are able to consume glucose, its addition had an acute inhibitory effect on the photosynthesis in the mats. The mechanism of this inhibition requires further investigation.

The two phases of inhibition suggest that it was a result of more than one process. This is supported by the appearance and subsequent degradation of α-ketoglutarate and formate in the water phase (Fig. 6, C). The observed sulfide concentrations have been reported before for hypersaline cyanobacterial mats at high temperatures in connection with decreased photosynthetic rates (Wieland and Kühl 2000). On the other hand, *Microcoleus chthonoplastes* can still carry out oxygenic photosynthesis at a sulfide concentration of 6 mM (Cohen 1984). Oxygenic photosynthesis is constitutive and preserved even after a longer period under 3 mM of H_2S. In our experiment we found an increase in photosynthetic rates and respiration rates after the water exchange on day 12 (Fig. 3, bottom right; Fig. 6, C+D) together with decreasing sulfide concentrations (Fig. 4). Therefore we assume that the observed high sulfide concentrations are one reason for the inhibition of photosynthesis.

Acetate detected in the supernatant was well below inhibitory amounts (Lasko et al. 2000). Acetate is a fermentation product of cyanobacteria during the dark phase, e.g. *Microcoleus chthonoplastes* produces ethanol, acetate and formate under dark anoxic conditions (Moezelaar et al. 1996). The concentration of formate was nearly 2 mM in the supernatant on day 5 (Fig 6, C), and its concentration was likely higher

inside the mat. Since the inhibition of photosynthesis and respiration by formate was observed in additional treatments, we conclude that the accumulation of formate could have contributed to the second inhibition phase occurring after three days.

Oxygen was absent in the photic zone of the mat for two weeks. This is an expression of a drastic physiological effect. Therefore it is remarkable that photosynthesis and respiration could recover to above the initial values (Fig. 6, C+D). This increase in activity was probably caused by the consumption of metabolites within the mat which remained after the water exchange. The inhibition by glucose and the ability of the mat to recover from weeks of anoxic conditions could be reproduced at least twice in other experiments. If the mats were sampled directly from the field and immediately incubated, the inhibition phase lasted only 2-4 days and a recovery took place without changing the incubation conditions. This is an indication that in highly active mats in the field (e.g. c_{max} = 1.1 mol/m^3 O_2), added substrates are turned over more rapidly than under laboratory conditions.

Acetate addition. The increase in net oxygen production upon acetate addition was the result of decreased respiration in the photic zone and increased respiration in the aphotic zone (Fig. 5, D). Gross photosynthesis was not affected (Fig. 5, C). Thus, acetate was not used by the cyanobacteria nor by the heterotrophic bacteria in the photic zone, presumably because it is not a common substrate in that region of the mat. This result is surprising since many heterotrophs, and even some cyanobacteria are known to be able to grow on acetate (Smith 1982). Respiration rates in the photic zone decreased within 48 h after acetate addition and remained below the initial level throughout the experiment. Thus, the community in the photic zone was unable to adapt to acetate, which is reported to be a prerequisite for acetate tolerance (Lasko et al. 2000). Indeed the DGGE analyses showed no community changes.

In the aphotic zone, respiration rates increased upon acetate addition (Fig. 1, bottom). Acetate is a fermentation product formed under anoxic conditions and was detected in the porewater of untreated mats during the dark period (Dr. Henk Jonkers, personal communication). The increase in respiration in the aphotic zone after acetate addition indicated that the ambient community in that layer was adapted to acetate and was able to consume the substrate. The results of the acetate addition suggest that the

heterotrophic community in the photic zone differed from the one in the aphotic zone. This is supported by the DGGE analyses, which showed that eubacterial diversity in the lower layer (2-4 mm depth) was higher than in the upper layer (0-2 mm depth) of the mat. At least three additional bands were observed in the lower layer (data not shown).

Glycolate addition. After glycolate addition, both gross photosynthesis and respiration increased (Fig. 6, A+B), with a smaller effect on the respiration in the aphotic zone. Glycolate is formed and consumed in the upper layers of microbial mats. Glycolate is excreted by cyanobacteria as a product of photorespiration (Renström and Bergmann 1989), as well as by other autotrophic organisms such as *Rhodospirillum rubrum* (Storrø and McFadden 1981) or *Thiobacillus neapolitanus* (Cohen et al. 1979). Glycolate excretion can be boosted under high oxygen concentrations. In a hot spring microbial mat, up to 58 % of excreted photosynthate was glycolate, which was taken up by two kinds of associated filamentous bacteria (Bateson and Ward 1988). Nevertheless, glycolate was detected neither in the control treatment nor in the porewater of uncontaminated mats (Dr. Henk Jonkers, personal communication). This indicates that the potential glycolate consumption rate is much higher than its production rate. Indeed, glycolate addition resulted in increased respiration rates, likely due to increased glycolate oxidation in the photic zone. The resultant increased CO_2 production was likely responsible for the increased photosynthesis rates measured (Fig. 2). The increased activity of the phototrophic organisms and concomitant accumulation of storage compounds is supported by the appearance of lactate in the supernatant. Lactate (a C_3-compound) produced during the experiment cannot be a degradation product of glycolate (a C_2-compound). It has been shown to be a fermentation product of many cyanobacteria from external carbon sources or from reserve material (Oren and Shilo 1979, Richardson and Castenholz 1987, Heyer et al. 1989, Moezelaar et al. 1996, Moezelaar and Stal 1997).

Our data on photosynthesis and respiration show that the addition of external carbon sources caused different physiological reactions in the different layers of cyanobacterial mats. This phenomenon has also been observed for sulfate reduction (Fründ and Cohen 1992). In a hypersaline cyanobacterial mat of the same origin as the mats used in our experiment, glycolate enhanced sulfate reduction only in the cyanobacterial region, where high oxygen levels occurred. The sulfate reduction in the region of the anoxygenic phototrophic bacteria (chemocline zone) was enhanced only by the addition of ethanol, while both ethanol and lactate enhanced sulfate reduction in the permanently reduced zone. Acetate decreased the sulfate-reducing activity there.

The connection between glycolate addition and increased photosynthesis in our experiment shows that increased respiration stimulates photosynthesis only when both processes occur in close proximity. Increased respiration in the aphotic zone (e.g. after acetate addition) does not increase photosynthesis. This is surprising, as inorganic carbon produced by respiration in the aphotic zone would eventually diffuse into the photic zone. Possibly, respiration and photosynthesis are coupled more efficiently through CO_2 than through HCO_3^- and CO_3^{2-}. CO_2 is the direct product of respiration, and its interconversion with HCO_3^- is slow (Falkowski and Raven 1997). The uncatalyzed reaction takes ~20 seconds to reach equilibration (Lehman 1978, Aizawa and Miyachi 1986). It has been suggested previously that enhanced respiration in a hypersaline cyanobacterial mat from Um-El-Yums (Egypt) was caused by increased photosynthate excretion by the phototrophic community at higher light intensities (Glud et al. 1999). These processes were observed in close vicinity to each other with planar optodes with high spatial resolution. Whatever the mechanism, our data support the view of a close spatial coupling of respiration and photosynthesis.

CONCLUSIONS

Microbial mats are in a dynamic fragile physiological equilibrium, with high turnover rates. No organic compounds could be detected in the water phase of the control, demonstrating that the mats efficiently remineralized photosynthates by respiration and thus that their carbon cycle is largely a closed system. We hypothesized that upon addition of easily degradable organics, respiration would increase, leading in turn to increased photosynthetic rates. However, organic substrates added to the mats had different effects on photosynthesis and respiration, apparently depending on their role in the metabolism of the mats. The substrates were degraded in those layers of the mats where they also occur under natural conditions. Moreover, respiration and photosynthesis seem to be closely coupled, since photosynthesis was stimulated by increased respiration in the photic zone but not by respiration in the aphotic zone. The photosynthetic and respiratory populations therefore must be present in close proximity to each other. The mats recovered quickly from additions of acetate and glycolate to the previous physiological state.

However, if an unusual but easily usable substance like glucose was added to the mat, the reaction was complex and led to total inhibition of net oxygen production. Inhibition of photosynthesis could be separated into fast (hours) and slow (days) processes, each with short-term (days) and long-term (weeks) recovery periods. Surprisingly, both photosynthesis and respiration were much higher after recovery than initially. Even after the strong changes in environmental conditions caused by the substrate addition, the community structure showed no detectable alteration.

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Chapter 3

Effects of oxygen concentration on photosynthesis and respiration in two hypersaline cyanobacterial mats

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ABSTRACT

The effects of oxygen concentration on photosynthesis and respiration in two hypersaline cyanobacterial mats were investigated. We carried out experiments on mats from two different locations: a mat from Eilat (Israel) with moderate photosynthetic activity, and a mat from Mallorca (Spain) with high photosynthetic activity. The oxygen concentration in the water overlying the mats was increased stepwise from 0 % to 100 % O_2. Subsequent changes in oxygen concentration, gross photosynthetic rates and pH values inside the mats were measured with microelectrodes. According to published reports on the regulation of ribulose-1,5-bisphosphate-carboxylase/oxygenase (Rubisco), the key enzyme in the CO_2-fixation pathway of phototrophs, we expected photosynthetic activity to decrease with increasing oxygen concentrations.

Gross photosynthetic and total respiration rates in both mats were highest at 0 % O_2 in the overlying water. Net oxygen production rates under these conditions were the same as under air saturation (21 % O_2), while gross photosynthetic and respiration rates were lowest at air saturation. In both mats, gross photosynthetic and respiration rates increased upon increasing the oxygen concentration in the overlying water gradually from 21 % to 100 %. These results were contradictory to expectations that photosynthesis would decrease with increasing oxygen concentration. Increased photosynthetic rates at oxygen concentrations above 21 % were probably caused by enhanced oxidation of organic matter and concomitant CO_2 production due to the increased oxygen availability. The cause of the high respiration rates at 0 % O_2 in the overlying water was presumably the enhanced excretion of photosynthetic products during increased photosynthesis. We conclude that the effect of the O_2/CO_2 concentration ratio on the activity of Rubisco as demonstrated *in vitro* on enzyme extracts cannot be extrapolated to the situation in intact microbial mats, because the close coupling of the activity of primary producers and heterotrophic bacteria plays a major role in this ecosystem.

INTRODUCTION

Microbial mats are dense, structured biofilms of up to several cm thickness with diurnal cycles in pH, oxygen and sulfide profiles (van Gemerden 1993). In terms of biomass, cyanobacteria are often the dominant representatives within the group of photoautotrophic microorganisms in such mats (Stal 1995, Stal 2000). In the light, oxygen is produced by photosynthesis, usually leading to several times oxygen saturation in the top of the mat (Jørgensen et al. 1979, Revsbech and Ward 1984, Epping and Jørgensen 1996). Concomitantly, CO_2 is fixed by cyanobacteria through the reductive pentose phosphate pathway (Calvin cycle) (Stal 2000). The key enzyme of this pathway is ribulose-1,5-bisphosphate-carboxylase/oxygenase (Rubisco). It possesses both carboxylase and oxygenase activities and the yield of carboxylation depends on the ratio of O_2 and CO_2 concentrations at the reaction centers of the enzyme (Falkowski and Raven 1997). This dependency varies among species: for example, the affinity constant $K_m(CO_2)$ of Rubisco enzyme extracts in air-equilibrated solutions ranges from 16 µM in *Polypodium aureum* to 129 µM in *Porphyridium cruentum*. The ratios of oxygenase to carboxylase activity range from 0.154 (*P. cruentum*) to 0.561 (*Synechococcus* sp.) (Falkowski and Raven 1997).

Rubisco is concentrated as tightly packed crystals in the carboxysomes (Brock et al. 2000). These are polyhedral bodies of about 100 nm in diameter surrounded by a protein membrane (Falkowski and Raven 1997) representing the sites of carbon fixation (McKay et al. 1993). The carboxysomes contain the highest Rubisco density in the cyanobacterial cell (Aizawa and Miyachi 1986, Bhaya et al. 2000).

Diffusion is the main transport process in microbial mats. Since diffusion is a slow process, steep gradients of substrates (CO_2) and products (O_2) of photosynthesis develop in highly active communities. The CO_2 concentration is probably close to zero in the porewater of the photosynthetic zone during illumination (de Beer 2001, de Beer et al. 1997). If CO_2 is the only source of dissolved inorganic carbon (DIC), growth rates can be limited by CO_2 diffusion, as reported for marine phytoplankton (Riebesell et al. 1993). Several phototrophs can overcome CO_2 limitation by utilization of HCO_3^-. Such organisms use carbonic anhydrase (CA), an enzyme which catalyzes the reaction

$CO_2 \Leftrightarrow HCO_3^-$. This ability has been reported for rhodophytes (*Porphyridium cruentum*), chlorophytes (*Nannochloris atomus and N. maculata*) (Huertas et al. 2000), diatoms (*Asterionella, Nitzchia, Rhizosolenia*) (Torell et al. 1997), cyanobacteria (*Anabaena variabilis, Coccochloris peniocystis, Synechococcus* sp.) and several algae (Aizawa and Miyachi 1986, Huertas et al. 2000, Torell et al. 1997). Additionally, the intracellular DIC concentration can be increased via active transport of CO_2 or HCO_3^- by a carbon concentrating mechanism (CCM). *Chlamydomonas reinhardtii* takes up CO_2 derived from HCO_3^- by means of external CA and active transport (Williams and Turpin 1987). In *P. cruentum, N. atomus and N. maculata* an internal CA and an active transport mechanism have been demonstrated (Huertas et al. 2000). *Nannochloris* and *Nannochloropsis* use dissolved inorganic carbon (DIC) only in the form of HCO_3^- (Huertas and Lubian 1998), while the cyanobacterium *Anabaena variabilis* ATCC29413 uses only CO_2 (Yagawa et al. 1984, Shiraiwa and Miyachi 1985). Several phototrophic microorganisms use both CO_2 and HCO_3^-, including the cyanobacteria *A. variabilis* M-3 (Kaplan et al. 1980, Volokita et al. 1984) and *Synechococcus* sp. (Badger and Andrews 1982, Badger et al. 1985) and the diatoms *Asterionella, Nitzchia* and *Rhizosolenia* (Torell et al. 1997), *Skeletonema costatum, Ditylum brightwellii* and *Chaetoceros calcitrans* (Korb et al. 1997), *Phaeodactylum tricornutum* and *Cyclotella* sp.(Rotatore et al. 1995). Both features, CA activity and CCM, increase the level of CO_2 in the carboxysomes, making the cells largely independent of low CO_2 levels in the medium.

Numerous investigations have been carried out concerning the influence of the O_2/CO_2 ratio on photosynthesis in individual organisms (Ingle and Colman 1975, Badger and Andrews 1982, Yagawa et al. 1984). In cell suspensions of microalgae grown under ambient O_2 and CO_2 levels, photorespiration, glycolate excretion and O_2 inhibition of photosynthesis were low compared to those in cells pre-cultured with CO_2-enriched air (2-4 % CO_2) and subsequent exposure to ambient conditions (Aizawa and Miyachi 1986). In an artificial diatom biofilm at a light intensity of 360 µmol photons/m^2s, elevated bicarbonate levels led to increased photosynthetic rates without a concurrent increase in respiration rates (Jensen and Revsbech 1989). In the same biofilm, respiration rates and, to some extent, photosynthetic rates increased with higher O_2 concentration. In cyanobacterial and freshwater diatom biofilms

illuminated at a light intensity of 85 µmol photons/m^2s, high O_2/CO_2 ratios caused increased respiration (Jensen and Revsbech 1989, Glud et al. 1992). In the same biofilms, the addition of bicarbonate lowered respiration but increased gross photosynthesis.

Very little is known about the effect of changing O_2/CO_2 levels on the O_2 production and consumption in intact microbial mats from extreme environments under saturating light intensities. In this study, we investigated how the photosynthetic activity of the primary producer community was affected by changes in the O_2/CO_2 ratio in dense hypersaline cyanobacterial mats. Laboratory measurements of oxygen concentrations, gross photosynthetic rates and pH were performed with microelectrodes.

MATERIALS AND METHODS

Mat samples were collected in May 1999 from a hypersaline experimental pond at the Interuniversity Institute in Eilat (Israel) and in June 2000 from a hypersaline ditch near the Salinas des Levantes on Mallorca (Spain). Both sites were characterized by similar salinities of 7-8 %. Sampled mats were transferred within 24 h after sampling to the Max Planck Institute for Marine Microbiology, Bremen (Germany). The mats were acclimated in aerated artificial seawater (ASW) containing 3 mM HCO_3^- with a salinity of 7.5 % and a temperature of 27° C, pH 8.2. The mats were illuminated for 12 h light/12 h dark at an irradiance of 600 µmol photons/m^2s. After several days acclimation to controlled lab conditions, mat subsamples (8 cm x 5 cm x 2.5 cm; l/w/h) were embedded in agar (1.5 %, ASW) and incubated in a flowcell system with a closed water cycle similar to one described previously by Lorenzen et al. (1995). All incubation parameters except aeration were kept constant. The oxygen concentration in the system was controlled by purging the water reservoir with air, N_2 gas, O_2 gas or a mixture of N_2/O_2 (both N_2 and O_2 gas contained 0.035 % CO_2). After steady-state conditions were reached (determined from oxygen microsensor profiles), control measurements were performed on consecutive days of the experiments at the same time of the day. Subsequently, the oxygen concentration in the overlying water was

changed and microsensor measurements were performed after new steady-state conditions were reached.

Oxygen concentrations in the mats were determined with fast-responding Clark-type O_2 sensors with guard cathodes (Revsbech 1989). Gross photosynthetic rates were determined with the same sensors by the light-dark-shift method (Revsbech and Jørgensen 1983). The applied O_2 sensors had tip diameters less than 6 µm, a stirring sensitivity of < 2 %, and a 90 % response time $t_{90} < 0.5$ s. pH measurements were performed with potentiometric glass microelectrodes (Revsbech et al. 1983), which had tip diameters of less than 10 µm with the pH sensitive glass at the very tip not longer than 100 µm. The electrodes showed a 90 % response time of $t_{90} < 10$ s. All electrodes were calibrated as described elsewhere (Revsbech and Jørgensen 1986, Revsbech 1989).

From profiles of oxygen concentration and gross photosynthetic rates, we determined respiration rates based on the mass balance

net oxygen production = gross oxygen production (photosynthesis) – oxygen consumption (respiration)

From steady-state oxygen profiles, local areal fluxes of oxygen (i.e. fluxes through each depth layer J_z) were calculated using Fick´s first law of one dimensional diffusion (Jørgensen and Revsbech 1985, Kühl et al. 1996)

$$J_z = D_e * dC_z/dz$$

with D_e as effective diffusion coefficient and dC_z/dz as concentration gradient at depth z. D_e was assumed to be 60 % of the free solution molecular diffusion coefficient for oxygen (D_0) (Broecker and Peng 1974, Li and Gregory 1974, Wieland et al. 2001). Oxygen solubility values at different temperatures and salinities were calculated from equations of Garcia and Gordon (1992).

Local net oxygen production rates were calculated from local areal fluxes (J). Local respiration rates were calculated from local net oxygen production rates and

local gross photosynthetic rates. The local rates were integrated over depth to determine areal rates for the entire mat. Areal fluxes for the entire mat as well as for the photic and the aphotic zone were also calculated from concentration gradients above the mats and below the photic zone (Jensen and Revsbech 1989, Glud et al. 1992, Kühl et al. 1996). We defined the photic zone as the layer in which gross photosynthetic activity could be detected by use of a one second dark period (Glud et al. 1992). All directly measured data were plotted as an average of three measurements.

RESULTS

During both series of experiments the mats from Eilat and Mallorca maintained the same appearance. The samples from Eilat contained 6 different cyanobacterial species and were dominated by a cyanobacterium described as "*Phormidium*-like" (Abed and Garcia-Pichel 2001). The Mallorca mats were dominated by *Microcoleus* sp. (Staats 2000). No significant changes in O_2 profiles were noticeable in the control measurements over the course of the experiments (Fig. 3, bottom right).

Photosynthetic activity and net oxygen production of the mat from Mallorca at ambient conditions (21 % O_2) were higher than those of the mat from Eilat. Although gross photosynthesis was restricted to the upper 0.6 mm in both mats (Fig. 1, center; Fig. 3, top center), the local volumetric rates were almost twice as high in the Mallorca mat compared to the Eilat mat. Since the range of local respiration rates was comparable in both mats, the higher photosynthetic rates in the Mallorca mat led to an oxygen concentration peak twice as high as in the Eilat mat.

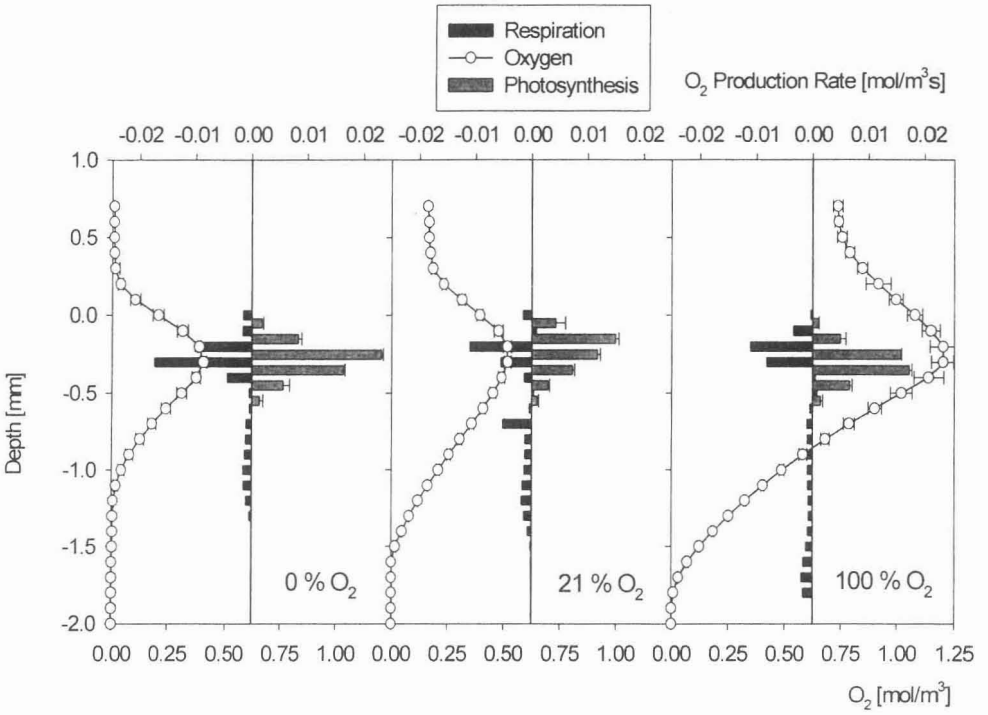

Figure 1: Oxygen concentrations (means ± standard deviations, n = 3), volumetric gross photosynthetic rates (means ± standard deviations, n = 3) and calculated volumetric respiration rates in a hypersaline cyanobacterial mat from Eilat with different O_2 concentrations in the overlying water.

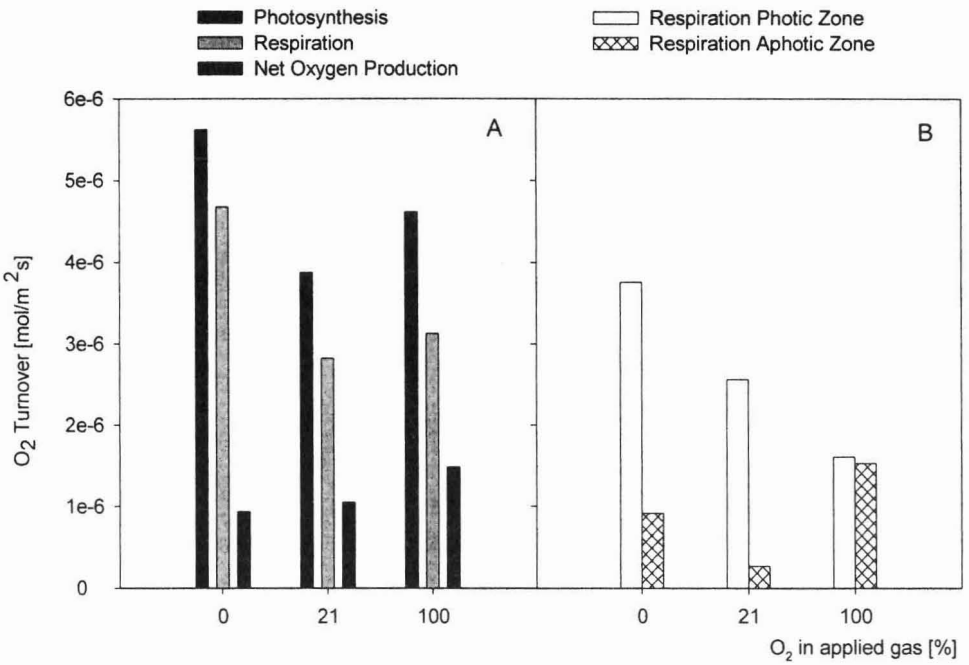

Figure 2: A) Areal gross photosynthetic rates, areal respiration rates and areal net oxygen production rates in a hypersaline cyanobacterial mat from Eilat with different O_2 concentrations in the overlying water. B) Areal respiration rates in the photic and aphotic zone of the same mat.

After removing all oxygen from the overlying water of the Eilat mat by gassing with N_2 (0 % O_2), photosynthetic activity led to an oxygen concentration peak of 2.7 fold air-saturation (Fig. 1, left), while oxygen penetrated to a depth of 1.1 mm. Areal photosynthetic rates and areal respiration rates in the photic and aphotic zone were the highest at 0 % O_2 (Fig. 2). At standard aeration (21 % O_2), gross photosynthetic and respiration rates in the photic zone decreased (Fig. 1, center), while the maximum oxygen concentration increased to 3.2 fold air-saturation and oxygen penetrated to 1.5 mm. The areal gross photosynthetic rate and the total areal respiration rate were lowest under this aeration regime (Fig. 2, A). At 100 % O_2 in the water phase (Fig. 1, right), the oxygen peak increased to 7.6 fold air-saturation and oxygen penetrated to a depth of 1.8 mm. The areal gross photosynthetic rate and the total areal respiration rate increased (Fig. 2, A). The areal respiration rate increased in the aphotic zone, but decreased in the photic zone (Fig. 2, B).

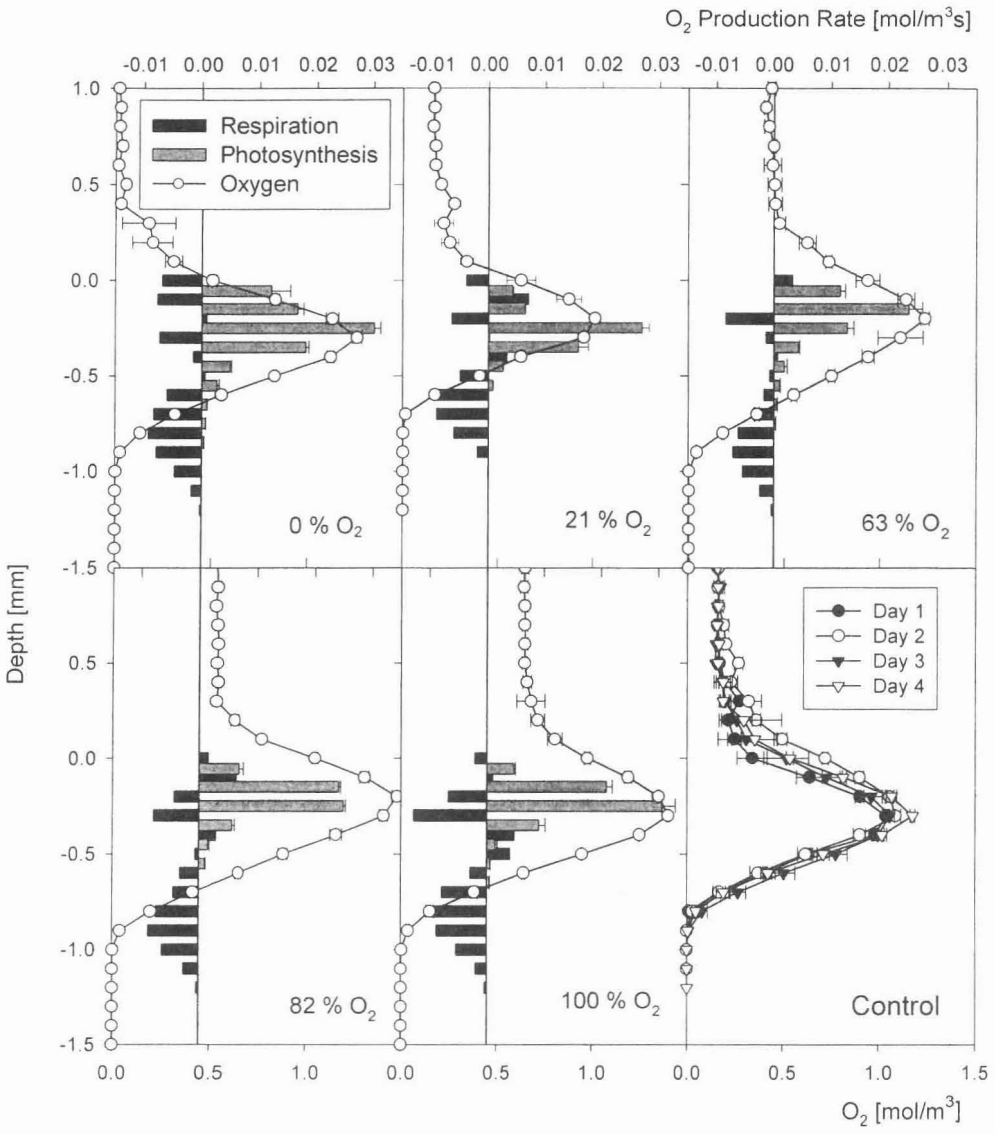

Figure 3: Oxygen concentrations (means ± standard deviations, n = 3), volumetric gross photosynthetic rates (means ± standard deviations, n = 3) and calculated volumetric respiration rates in a hypersaline cyanobacterial mat from Mallorca with different O_2 concentrations in the overlying water.

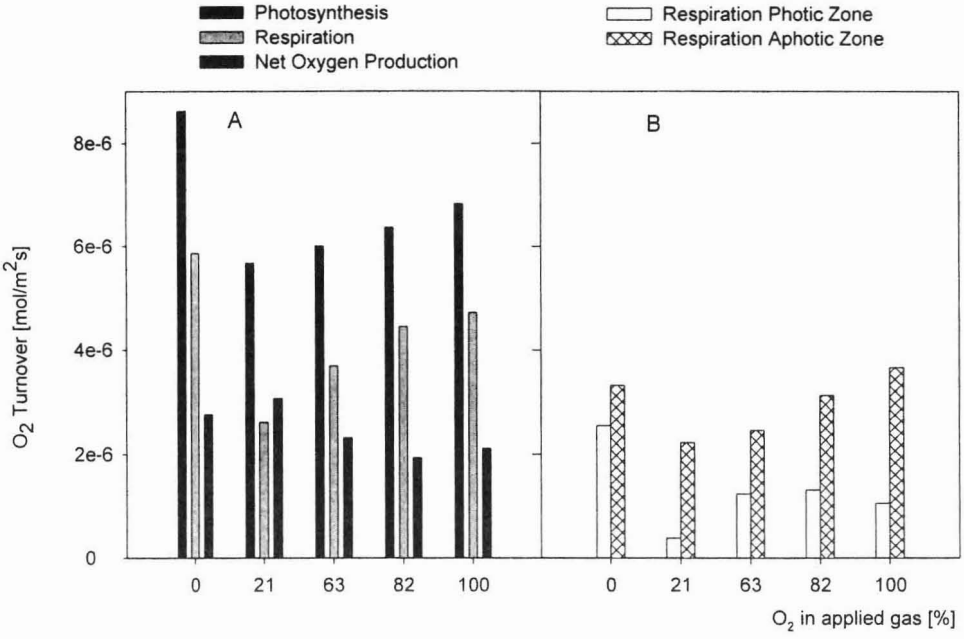

Figure 4: A) Areal photosynthetic rates, areal respiration rates and areal net oxygen production rates in a hypersaline cyanobacterial mat from Mallorca with different O_2 concentrations in the overlying water. B) Areal respiration rates in the photic and aphotic zone of the same mat.

In the mat from Mallorca, photosynthetic activity was detected in the upper 0.9 mm with 0 % O_2 in the overlying water (Fig. 3, top left). Oxygen penetrated to the same depth, and the profile showed a peak of 7.8 fold air-saturation. The areal photosynthetic rate, the total areal respiration rate (Fig. 4, A) and the areal respiration rate in the photic zone were found to be highest at 0 % O_2 (Fig. 4, B). Under standard aeration (21 % O_2) the oxygen peak decreased to 6.2 fold air-saturation and oxygen penetration decreased to 0.8 mm (Fig. 3, top center). The areal photosynthetic rate and the areal respiration rates in the photic and in the aphotic zone were lowest under these conditions (21 % O_2). When the O_2 concentration in the water phase was artificially increased (63 %, 82 % and 100 % O_2), gross photosynthetic rates increased concomitantly (Fig. 3), similar to the observations in the moderately active mat from Eilat. Areal gross photosynthesis and areal respiration in the aphotic zone increased gradually (Fig. 4) and the areal respiration in the photic zone was constantly higher than under standard aeration. In the upper first mm of the mat the highest pH values were measured at 100 % and 0 % O_2, the lowest at 21 % O_2 (Fig. 5), concomitant with

the trend in areal gross photosynthetic rates (Fig. 4, A). In the deeper layers of the mat, changing O_2 concentrations in the overlying water had no significant influence on the pH.

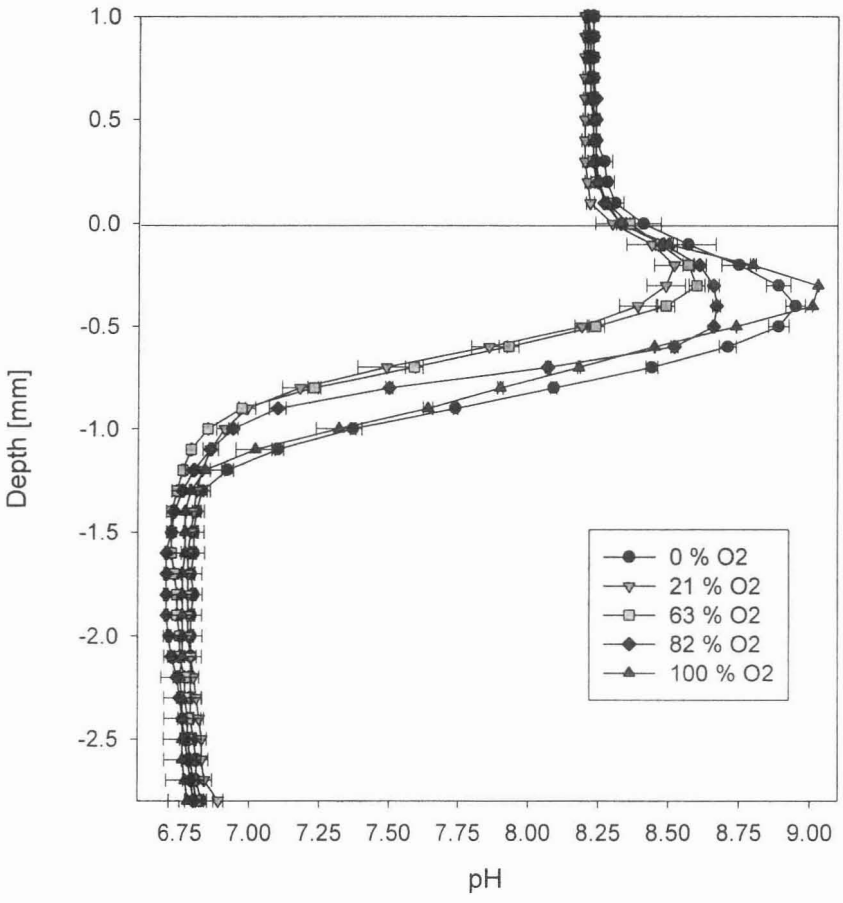

Figure 5: pH profiles (means ± standard deviations, n = 3) in a hypersaline cyanobacterial mat from Mallorca with different O_2 concentrations in the overlying water.

DISCUSSION

Our results show that in mats from both Eilat and Mallorca similar responses occurred upon changing O_2 concentrations in the overlying waterphase, although the mats differed in origin, species composition and activity. Gross photosynthetic rates and respiration rates were highest at 0 % O_2 in both mats and decreased significantly when oxygen was increased to 21 %, while areal net oxygen production rates did not change (Fig. 2 and 4). Upon further increase of O_2 to above air-saturation in the overlying water, gross photosynthetic rates and respiration rates increased again in both mats. These phenomena indicate that net oxygen production does not necessarily reflect gross photosynthetic activity in highly active mats. The dip in metabolic activities under air saturation shows oxygen dependence of at least two mechanisms affecting photosynthesis: the regulation of ribulose-1,5-bisphosphate-carboxylase/oxygenase (Rubisco) activity by the O_2/CO_2 concentration ratio and the close coupling of photosynthetic activity with respiratory processes in microbial mats.

Studies concerning photosynthetic and respiratory processes in pure cultures have often used net parameters: for example, uptake of radiolabeled carbon has been measured for the cyanobacteria *Anabaena variabilis* ATCC29413 (Shiraiwa and Miyachi 1985), *A. variabilis* M-3 (Volokita et al. 1984), *Coccochloris peniocystis* (Miller and Colman 1980), *Spirulina platensis* (Kaplan 1981) and the diatoms *Asterionella, Nitzchia* and *Rhizosolenia* (Torell et al. 1997), *Skeletonema costatum, Ditylum brightwellii* and *Chaetoceros calcitrans* (Korb et al. 1997). Gas exchange has been measured for the cyanobacterium *Synechococcus* sp. (Badger and Andrews 1982) and the diatoms *Phaeodactylum tricornutum* and *Cyclotella* sp. (Rotatore et al. 1995). The close coupling of autotrophic and heterotrophic processes in intact microbial mats demands more detailed determinations. Microsensor techniques allow measurements of volumetric photosynthetic rates, oxygen concentrations and pH values in the µm range, and have been successfully applied in connection with photosynthesis regulation by O_2/CO_2 (Jensen and Revsbech 1989, Glud et al. 1992).

Oxygen concentration changes in the overlying water directly affected oxygen concentration within the mats (Fig. 1, Fig. 3). Although the CO_2 concentrations in the

porewater of photosynthetic mats have been reported to be very low as measured with microelectrodes (de Beer et al. 1997, de Beer 2001), the CO_2 concentration within the cells remains unknown. Cyanobacteria and microalgae possess CA and CCM, allowing them to maintain high intracellular CO_2 levels (Aizawa and Miyachi 1986). In the present study, the DIC concentration in the water phase was not experimentally changed. Hence, we assume that the CO_2 concentration inside the cells was maintained and the O_2/CO_2 concentration ratio in the mats changed with the oxygen concentration in the overlying water.

A negative correlation was found between O_2/CO_2 ratios and gross photosynthetic rates for O_2 concentrations of 0 % and 21 %. This correlation is well known for pure cultures. O_2 and CO_2 are competitive substrates for Rubisco (Beardall and Raven 1990) since the enzyme has a very low affinity to CO_2 (Bhaya et al. 2000). The half-saturation constant for Rubisco is approximately 100 µM CO_2 in eukaryotic phytoplankton and even higher in cyanobacteria (Falkowski 1997). However, many photoautotrophic species including cyanobacteria and diatoms are reported to be able to overcome low ambient CO_2 levels by maintaining a high CO_2 concentration inside the cell (Miller and Colman 1980, Badger and Andrews 1982, Colman and Rotatore 1995, Rotatore et al. 1995, Korb et al. 1997, Torell et al. 1997, Huertas et al. 2000). In our experiments a low oxygen level was accompanied by a low O_2/CO_2 ratio but high gross photosynthetic rates. The regulation of Rubisco activity implies low photorespiration rates at low O_2/CO_2 concentration ratios. Since we measured maximum respiration rates in the photic zone of both mats at 0 % O_2, we conclude that the high respiration rates were due to heterotrophic activity and not due to photorespiration. Respiration by heterotrophic bacteria was probably stimulated by the excretion of photosynthates concurrent with the increase in gross photosynthesis. It has previously been observed that respiration in mats is not only controlled by O_2 availability but also by the supply of organic and inorganic substrates (Haack and McFeters 1982, Glud et al. 1992, Canfield and Des Marais 1993, Kühl et al. 1996, Epping et al. 1999).

When O_2 was increased from 21 % to 100 %, a positive correlation between O_2/CO_2 ratios and gross photosynthetic rates was observed. This effect is contradictory to the dependency of Rubisco activity on the O_2/CO_2 level in active cells. A similar

response has been observed in an artificial unialgal diatom biofilm (Jensen and Revsbech 1989). After increasing the O_2 concentration from 21 % to 100 % in the overlying water, areal gross photosynthetic rates increased by 9 %, areal respiration rates increased by 33 % and net oxygen production rates decreased by 29 %. In our experiments, an increase in the O_2/CO_2 ratio led to increased total areal respiration rates in both mats, although the $K_m(O_2)$ for oxygen consumption is only 10 µM (Epping et al. 1999). Respiration increased in the aphotic zone of both mats due to the increased O_2 penetration depth, thus extending the total zone of respiration. This has been observed earlier (Kühl et al. 1996).

We assume that the increase in gross photosynthetic rates in our experiments is due to increased CO_2 levels inside the mat concurrent with enhanced respiration. Gross photosynthesis in microbial mats has been demonstrated to be limited by DIC, based on HCO_3^- addition experiments (Jensen and Revsbech 1989). It has been shown that respiration and photosynthesis in mats are closely coupled if photosynthetic and respiratory populations are present in close proximity to each other (Glud et al. 1999, Groetzschel et al., submitted). Hence, the observed increased respiration rates in the aphotic zone are not likely to be the cause of enhanced photosynthetic rates in the photic zone. Photosynthesis could only have been increased by CO_2 production in the photic zone, not by CO_2 production in the aphotic zone. Due to the low affinity constant $K_m(O_2)$ for respiration, abiotic oxidation could play a role. Many reduced compounds can be oxidized under ambient conditions, such as sulfur, nitrite, many amino acids, peptides, proteins, ascorbate, quinones etc. Oxygen consumption has been measured to proceed, although less rapidly, in biofilms which were biologically inactivated by UV, mercury or pasteurization; in sediments biologically inactivated by radiation in a nuclear reactor, inhibitors or pasteurization; and in biologically inactivated cell suspensions (Dirk de Beer, unpublished results). Abiotic oxidation could be supported by the strong oxidant H_2O_2, which is produced in microbial mats during the light period. O_2^- generated by the Mehler reaction is disproportionated by superoxide dismutase (SOD), which occurs in the chloroplasts of algae and is present in all cyanobacteria (Tichy and Vermaas 1999).

Contrary to published reports about gross photosynthesis and respiration upon changing O_2 concentrations, the changes in areal net oxygen production rates were

different in both mats. This requires further investigation, but differences in species composition may help account for this discrepancy. For example, upon increasing O_2 concentration from 21 % to 100 %, a cyanobacterial biofilm showed a 14 % decrease in photosynthesis and no change in total respiration, while a diatom biofilm showed a 7 % decreased in photosynthesis and a 54 % decrease in total respiration (Glud et al. 1992). It has been suggested that the different ability of the dominating species to withstand high O_2/CO_2 ratios without photorespiration was responsible for the significant activity difference, which could also be the case in our experiments.

CONCLUSIONS

In two hypersaline cyanobacterial mats of different origin, similar responses to oxygen concentrations were observed. At 0 % O_2 in the overlying water, both mats showed the highest gross photosynthetic rates and respiration rates. This is in agreement with reports on pure cultures, where the Rubisco activity depended on the CO_2/O_2 concentration ratio in the water phase. The respiration rates in the mats were also high, probably due to consumption of excreted photosynthetic products and the close coupling of oxygen production and consumption by the autotrophic and the heterotrophic community in the mats.

Surprisingly, increased O_2 levels in the overlying water resulted in higher gross photosynthetic rates and respiration rates in both mats. These results are contradictory to the theory that CO_2 fixation, regulated by the activity of Rubisco, decreases with rising O_2 levels. Enhanced CO_2 production due to the oxidation of organic matter in the photic zone of the mats could explain the observed increase in photosynthesis. In conclusion, the straight causal relationship between the oxygenase and carboxylase functions of Rubisco, carbonic anhydrase activity and carbon concentrating mechanisms in the dominating phototrophs appears not to be valid in microbial mats due to the complex interspecies relations in such ecosystems.

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Chapter 4

Degradation of petroleum model compounds immobilized on clay by hypersaline cyanobacterial mats

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ABSTRACT

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

INTRODUCTION

Pollution of marine environments with oil and oil products became a world wide problem on the tide of industrialization. The sources of marine hydrocarbon pollution are mainly runoff from land and municipal/industrial wastes ($13.74x10^8$ l/a), routine ship maintenance like bilge cleaning ($5.19x10^8$ l/a), air pollution from cars and industry ($3.48x10^8$ l/a), natural seeps ($2.35x10^8$ l/a), tanker accidents ($1.4x10^8$ l/a) and offshore oil production ($0.57x10^8$ l/a) (NRC 1985, US Coast Guard 1990). At seawater salinity, microbial degradation can be a successful remediation approach even at low temperatures and after major catastrophes (Pritchard and Costa 1991). However, under hypersaline conditions microbial degradation of otherwise easily biodegradable contaminants is often more difficult, because salt stress is superimposed on pollution stress (Oren et al. 1992). For example, at a salinity of 20 % in Great Salt Lake brines (Utah, USA) no degradation of long chain alkanes was observed (Ward and Brook 1978). Nevertheless, when biofilms are formed, tolerance to hydrocarbons or degradation is possible, even in hypersaline environments. After the 1991 Gulf War, tar-covered beaches and hypersaline enclosures ("Sabkhas") at salinities higher than 5 % were recolonized by cyanobacterial mats (Hoffmann 1996, Höpner et al. 1996). Since it is essential for successful biodegradation that the microorganisms involved come in direct contact with the pollutants (Rosenberg et al. 1992), the immobilization of the mat cells in biofilms may have been an advantage.

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

In our laboratory experiment, we used five substances as representative petroleum components for straight-chain, branched and cyclic alkanes, as well as

aromatic and sulfur-aromatic compounds. To establish direct contact between these model compounds and benthic cyanobacterial mats, a modified clay mineral was used as carrier material. Over a period of four months, changes in oxygen and sulfide concentration, pH, photosynthetic rates and light fields were followed with microsensors. The community structure was analyzed with PCR and DGGE, and degradation of the model compounds was followed with GC/GC-MS.

MATERIALS AND METHODS

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

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

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

Contamination experiment. After five days of pre-incubation, 10 x 10 cm mat pieces 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 put into a basin containing water for temperature regulation. After five additional days of incubation, the contamination experiment was started:

1) Two mat samples were contaminated by direct contact with the OCC: 0.6 g OCC (containing 0.12 g petroleum model compound mixture) were suspended in a small amount of the aquarium water. The suspension was poured under gentle stirring into a high plasticized glass frame that confined the OCC cover to the surface of the mats only. Within minutes the OCC settled to the mat surface and the frame was removed.

2) Two mat samples were covered with 4,8 mg/cm^2 of the hydrophobic clay as controls for the effect of the carrier material

3) The hydrophobic petroleum model compounds were directly applied to the water phase of two aquaria with mat samples: 0.12 g of a mixture of the petroleum model compounds was dissolved in 1 ml dichloromethane and transferred to a glass slide. After evaporation of the solvent one slide was placed in each of the two aquaria.

4) One aquarium contained nothing but ASW and agar with OCC on the surface as a control for physical removal of the petroleum model compounds from the OCC.

5) Two mat samples were left undisturbed as an untreated control.

Microsensor analyses. Oxygen concentrations were determined with fast responding Clark-type O_2 sensors with guard cathodes (Revsbech 1989). Gross photosynthetic rates were determined with the same sensors by the light/dark-shift method (Revsbech

and Jørgensen 1983). The O_2 sensors had tip diameters of less than 6 µm, a stirring sensitivity < 2 %, and a 90 % response time of t_{90} < 0.5 s. Measurements of pH values were performed with potentiometric glass microelectrodes (Revsbech et al. 1983), which had a tip diameter of less than 10 µm with the pH sensitive glass at the very tip not longer than 100 µm. The electrodes showed a 90 % response time of t_{90} < 10 s. The H_2S sensors (Kühl et al. 1998) had a tip diameter of less than 20 µm. All electrodes were calibrated as described elsewhere (Revsbech and Jørgensen 1986, Revsbech 1989, Santegoeds et al. 1998). Fiberoptic microprobes for field radiance and scalar irradiance (Kühl et al. 1994) were applied at the beginning and at the end of the experiment after 18 weeks to compare the light fields of the mats.

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

We defined the photic zone as the layer in which gross photosynthetic activity could be detected by use of a one second dark period (Glud et al. 1992). Calculations and evaluation procedures for the obtained data are described elsewhere (Groetzschel et al., submitted). All directly measured data were plotted as an average of three measurements.

Chemical analyses. Mat samples collected in week 0, 5, 9, 13 and 18 (cores of ~7 mm in diameter, ~2.5 g wet weight) were extracted ultrasonically with a 1:0.5:0.4 (v/v/v) mixture of methanol (MeOH), dichloromethane (DCM), and water (modified after Bligh 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 a MeOH:DCM:H_2O ratio of 1:1:0.9 (v/v/v) resulting in phase separation. The DCM layer was collected and the methanol-water

phase was washed three times with DCM. The solvent of the combined DCM phase was removed in a rotary evaporator and the extract was diluted to concentrations appropriate for gas chromatography analyses.

The extracts were analyzed using a Hewlett Packard 6890 gas chromatograph equipped with a Gerstel KAS3 temperature-programmable injector, a flame ionization detector and a fused silica column (J & W DB-5HT, 30 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 minutes. For gas chromatography-mass spectrometry (GC-MS), an HP 5890 gas chromatograph (GC conditions as above) with a DB-5 HT (J & W) fused silica column was connected to a Finnigan SSQ 710B mass spectrometer as detector (operated at 70 eV, cycle time 1 scan/s). Model compounds were quantified by integration of the FID signals and comparison with an internal standard (squalane), which was added directly after extraction. The calculated initial amount of each model compound in 2 ml sample was 66,67 µg. Data on the abundance of the model compounds are presented relative to this value.

Molecular analyses. Regularly collected mat cores (see chemical analyses) were subjected to nucleic acid extraction, polymerase chain reaction (PCR) amplification and denaturing gradient gel electrophoresis (DGGE) as described previously (Abed and Garcia-Pichel 2001). The PCR was carried out for the amplification of 16S rRNA using two sets of oligonucleotide primers. CYA359F (with a 40 nucleotide GC clamp at the 5´ end) and CYA781R were used as specific primers for cyanobacteria (Nübel et al. 1997) and GM5F with a GC-clamp in combination with the universal primer 907R for all bacteria. A hot start program was performed for the cyanobacteria-specific primers, as described by Nübel et al. (1997). In case of bacterial universal primers (GM5 and 907R), a hot-start touchdown program was used to minimize nonspecific amplifications (Santegoeds et al. 1998). The DGGE was performed at 60°C and a constant voltage of 200 V for 3.5 hours.

RESULTS

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

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

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

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

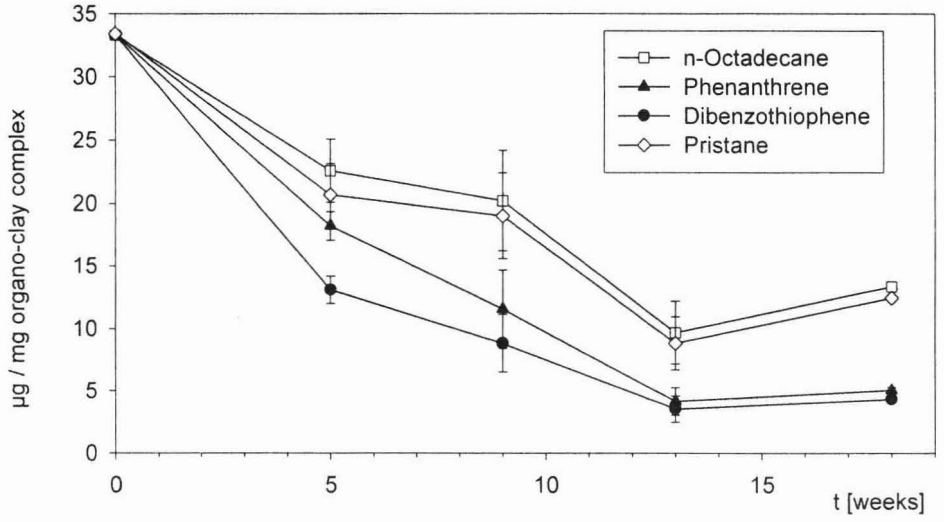

Figure 1: Concentrations of petroleum model compounds (means ± standard deviations, n = 3) in the OCC-contaminated microbial mats during 18 weeks.

At the end of the degradation experiment, pieces of the mats contaminated with OCC and the reference mats were sectioned into layers of 1 or 2 mm thickness in order to analyze the vertical distribution of the petroleum model compounds and the lipid composition of the individual layers. In the OCC-treated mats, the major portion of the petroleum model compounds was found in the top layer (Fig. 2). This layer was characterized by filamentous cyanobacteria which penetrated the applied organo-clay complex cover. 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. The aromatic and aliphatic compounds were at nearly equal concentrations in the 1-2 mm interval, with the aromatic compounds prevailing below that.

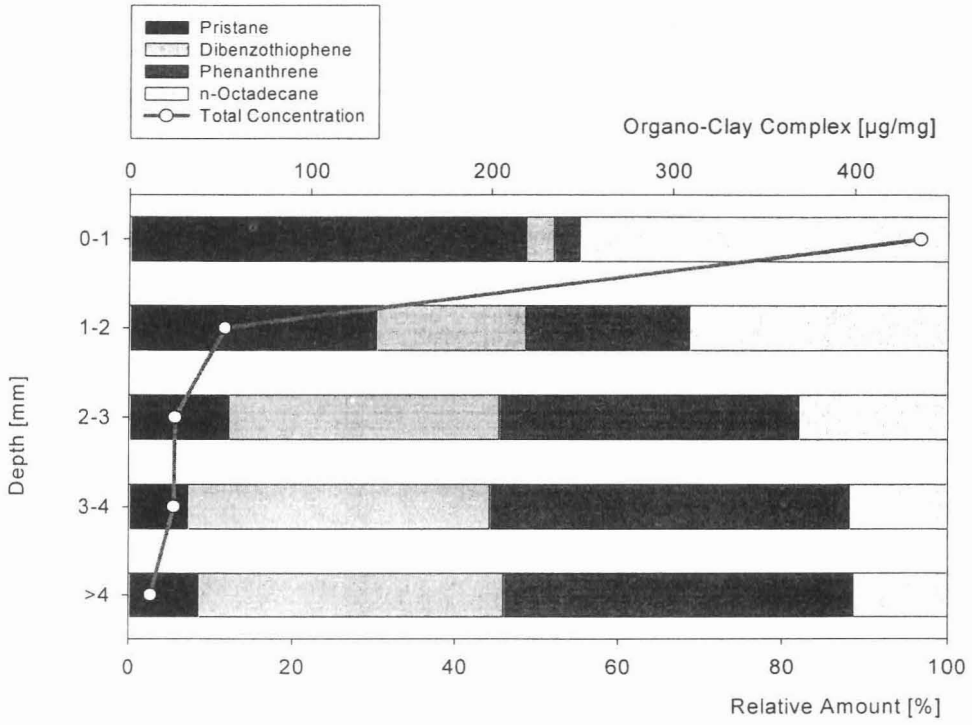

Figure 2: Relative composition of the petroleum model compounds present in different layers of the OCC-contaminated microbial mats (means ± standard deviations, n = 3) at the end of the experiment (normalized to 100 %, bottom horizontal axis) and the total concentrations (top horizontal axis).

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

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

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

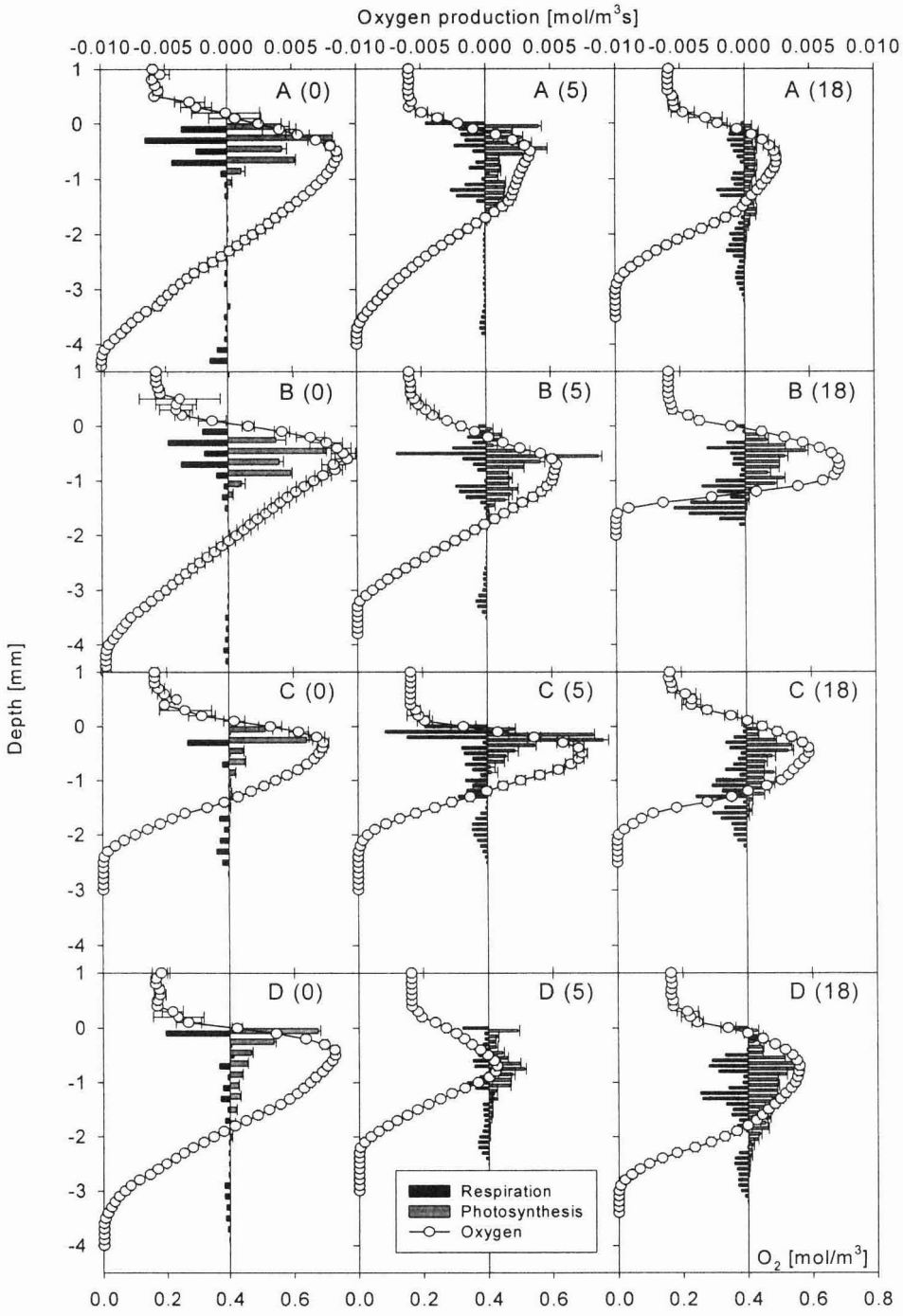

Figure 3: Profiles of oxygen concentrations (means ± standard deviations, n = 3), volumetric gross photosynthetic rates (means ± standard deviations, n = 3) and calculated volumetric respiration rates in microbial mats with different additions in week 0, 5 and 18. A: control; B: hydrophobic clay on the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

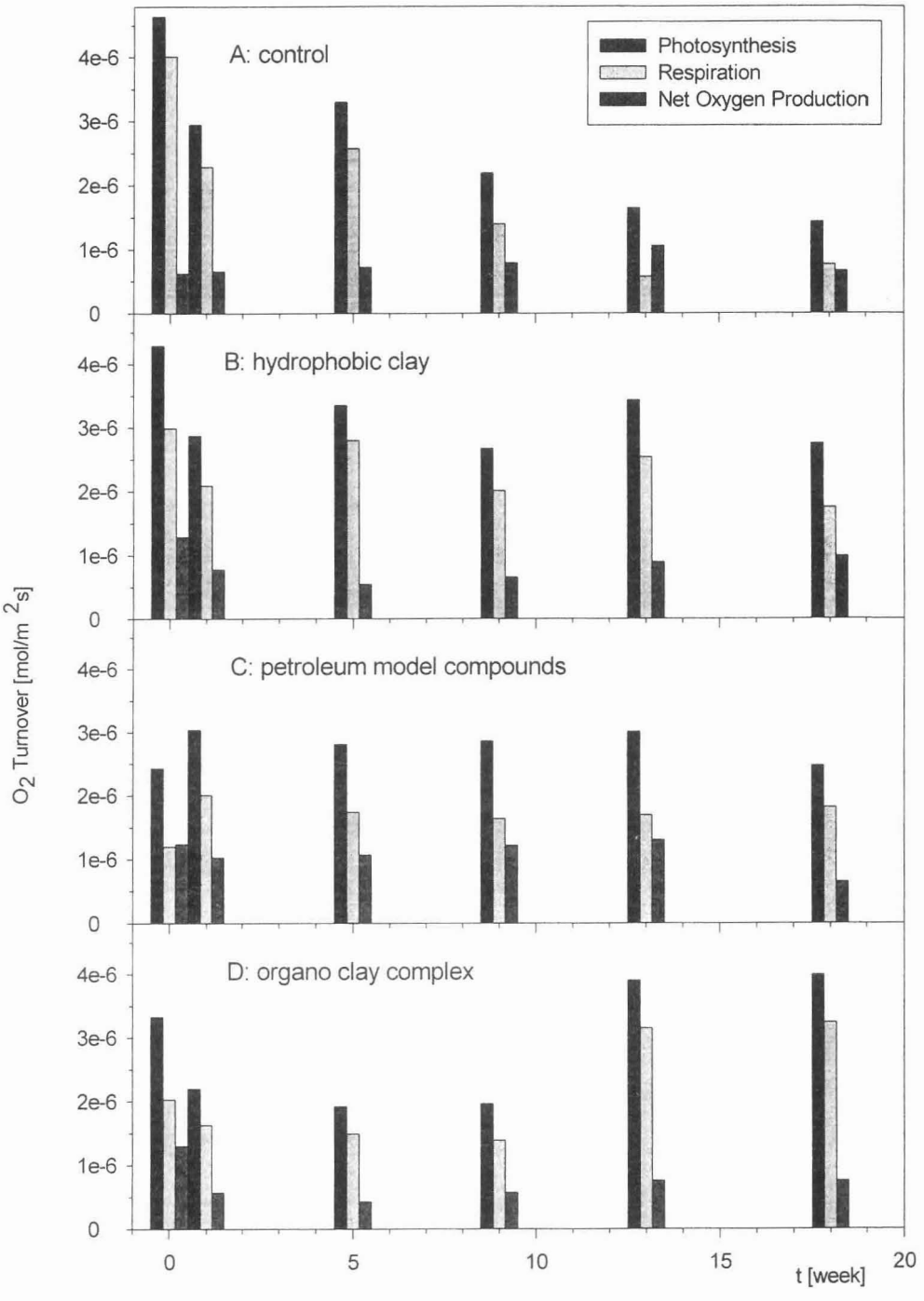

Figure 4: Areal photosynthetic rates, areal respiration rates and areal net oxygen production rates in microbial mats during 18 weeks calculated from oxygen and photosynthesis profiles. A: control; B: hydrophobic clay on the surface of the mats; C: petroleum model compounds added to the water phase;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

At the beginning of the experiment (Fig. 3, A 0-D 0), the thickness of the photic zone varied from 1.3 mm in the control mat (A 0) and the mat with the hydrophobic clay (B 0) to 2 mm in the OCC mat (D 0), with similar maximum gross photosynthetic rates. Oxygen penetration depth varied between 4.1 mm (A 0) and 2.4 mm (C 0). After 5 weeks, the oxygen penetration depth decreased in all of the mats. In week 18 the thickness of the photic zone increased in all of the mats, but the oxygen penetration depth was further reduced, except for the OCC mat (Fig. 3, D 18), in which it increased by 0.8 mm. This was the only treatment in which an increase in gross photosynthetic and respiration rates in the photic zone could be observed after 13 weeks (Fig. 4, D). After a decrease in areal gross photosynthetic rates and respiration rates in this mat in the first 9 weeks of the experiment, the rates more than doubled in the following months. Areal rates in the photic zone of the OCC mat showed the same trends as in the entire mat. On average 73 % of the areal respiration took place in the photic zone (data not shown). This indicates a concentration of the respiratory activity in the photic zone. In all other mats areal photosynthetic rates and respiration rates decreased continuously (Fig. 4, A) or remained largely constant after an initial increase (Fig. 4, C) or decrease (Fig. 4, B) in the first week. Sulfide concentration profiles and pH profiles showed similar trends in the different mats. Initially, the light intensities as measured with fiberoptic microprobes in the OCC and the hydrophobic clay treatments were higher than in the other treatments; at the end of the experiment the light fields in all mats were equal (data not shown).

Molecular analyses. The community response of both cyanobacteria and bacteria to the incubation with the four model compounds is shown in Fig. 5. In case of the cyanobacteria, the community composition at the beginning of the experiment was identical in all mat pieces, as assessed by DGGE patterns. A detailed description of the cyanobacteria inhabiting these mats is given elsewhere (Abed and Garcia-Pichel 2001). The community was dominated by two cyanobacterial populations; *Microcoleus chthonoplastes* and a small unicellular cyanobacterium (Picobenthos), as revealed by subsequent sequencing and phylogenetic analyses of the two bands displayed on the gel. The same community members persisted to the end of the experiment, with the development of one additional population represented by band D.

Phylogenetic analyses of this band revealed that its 16S rRNA sequence belonged to a diatom plastid. In the treatment in which the model compounds were applied directly to the mats without a carrier, the development of an extra population represented by band O1 was observed. As the chemical analyses showed that the model compounds did not dissolve in the water phase and did not reach the mats, this change in the community can not be attributed to degradation activities.

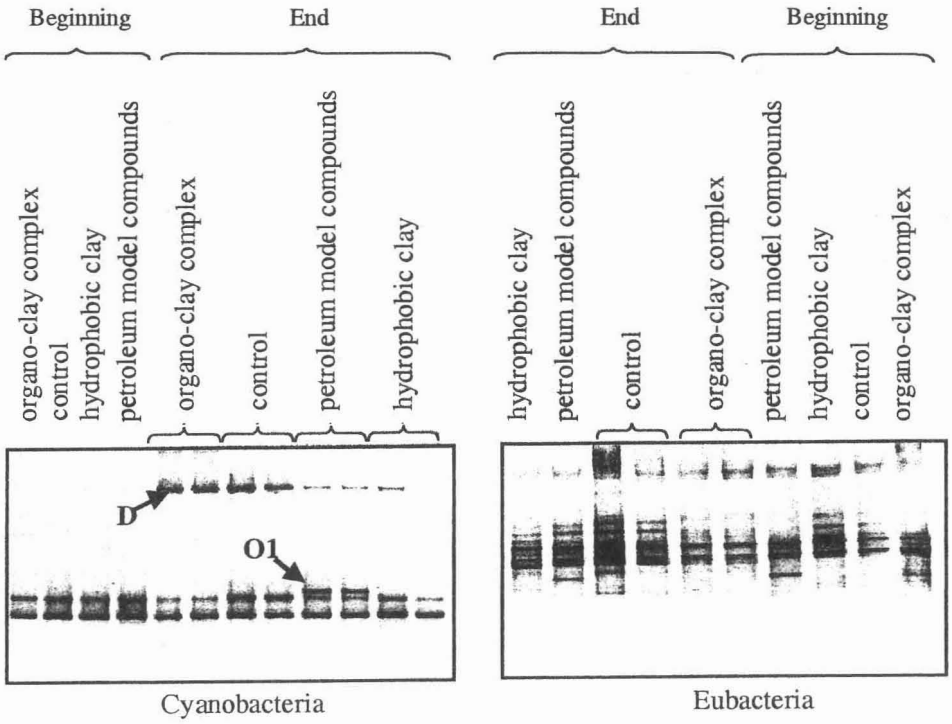

Figure 5: DGGE banding patterns of PCR-amplified 16S rRNA fragments obtained from microbial mats with different additions at the beginning and at the end of the experiment. The amplification was carried out using cyanobacterial specific primers (CYA359F and CYA805R, left) and eubacterial primers (GM5F and 907R, right).

Using the universal bacterial primers, the banding pattern was more complex. This is not surprising, as this primer set amplifies 16S rRNA of all bacteria. The banding pattern at the beginning of the experiment suggested a slight difference in the community composition in case of the mats subjected directly to the petroleum model compounds. At the end of the experiment, as in the case of cyanobacteria, the bacterial community did not show any significant response attributable to the incubation with

the model compounds. The total number of bands at the end of the experiment was higher than the number at the beginning of the experiment. The mats exposed to the organo-clay complexes exhibited a lower number of bands in comparison to other mats.

DISCUSSION

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

The biodegradation of the petroleum model compounds in the present study is considered to be slow and incomplete, since after 18 weeks a residue of 13-40 % was left, depending on the type of compound (Tab. 1). A comparison of the microbial degradation in our experiment with literature data is possible only to a limited extent since there are important differences between slurry or culture approaches and the investigation of intact mats. *Rhodococcus erythropolis* was reported to desulfurize alkylated dibenzothiophenes at a much faster rate of 0.115-0.547 µmol/d*mg (dry cell weight) (Folsom et al. 1999), while *Rhodococcus* sp. 5F degraded dibenzothiophene at a rate of 240 µg/d*mg dry biomass and phenanthrene at a rate of 288 µg/d*mg dry

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

The apparently accelerated disappearance of aliphatic model compounds between week 9 and 13 (Fig. 1) coincided with enhanced photosynthetic rates and respiration rates as measured by microsensors (Fig. 4, D). After 13 weeks, no further degradation was observed. The concentrations of the petroleum model compounds, as

well as the photosynthetic and respiratory activities, remained approximately constant. This agreement in the chemical and the microsensor data seems to indicate an apparent connection between higher activity rates in the mats and increased alkane degradation. The total amount of C added to the 10 x 10 cm mats in the form of the four petroleum model compounds was 1.9 mmol. Calculating with a net oxygen production rate of 5×10^{-7} mol/m^{2}s (Fig. 4, D, first week), a total stoichiometric oxidation of the added C to CO_2 would have taken 4.4 days, if all the oxygen produced by the mats was used for that purpose. For the total oxidation of the added C during the treatment, only 3.5 % of the net oxygen production would have been needed. Since even after 18 weeks a considerable amount of the petroleum model compounds was detected, we conclude that the model compounds did not significantly affect the internal carbon cycle in the mats and that their degradation was instead a side effect of the usual high metabolic activity. The shifts in metabolic activity in the OCC-treated mats, as revealed by the microsensor measurements, did not reflect the degradation activity, since major shifts in metabolic activity could also be measured in the OCC-untreated mats (Fig. 3, Fig. 4).

The molecular analyses in our experiment did not indicate that the degradation of the petroleum model compounds affected the structure of the mat community, as neither cyanobacterial nor bacterial populations showed any changes specific to the OCC-treated mats (Fig. 5). This suggests that the addition of petroleum compounds to these mats did not favor the growth of certain microorganisms over others. This is supported by the observation of only small changes in the lipid composition of the mats. At the end of the experiment, the control mat and the mat exposed to the organo-clay complex showed very similar profiles of their major lipid components (data not shown). The only community changes detected before and after the experiment could be attributed to experimental settings. Such changes have been observed earlier when mats were maintained under laboratory conditions (Abed and Garcia-Pichel 2001). In the presence of organo-clay complexes, we observed a decrease in the intensity (Fig. 5, cyanobacteria, panel A) or in the number (Fig. 5, bacteria, panel B) of some bands. This may indicate that the petroleum compounds have a toxic effect on the growth of certain microorganisms. Many studies have demonstrated that crude oil contains

constituents which are inhibitory to cyanobacteria even at low concentrations (Radwan and Al-Hasan (2000) and references therein).

The chemical analyses of the mats at different depths showed that the major portion of the petroleum model compounds remained in the surface layer of the mat (Fig. 2). In the different treatments the same light fields were measured at the end of the experiment, which indicates that the top layer consisting of OCC or hydrophobic clay was penetrated by cyanobacteria and diatoms. This was also observed under the microscope. The depletion of aromatic compounds in the top layer corresponds to the observations made in the time series (Fig. 1). However, a minor transport of model compounds into the mats to at least 6 mm was found. The change in the relative composition implies that this transport occurs after desorption rather than by mixing of organo-clay complex with the microbial mat. Whether composition changes are due to different desorption, diffusion or transport rates of aromatic vs. aliphatic compounds, or whether the preferential degradation of the alkanes plays a role, needs further investigation.

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

CONCLUSIONS

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

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Chapter 5

Degradation of 2,4-Dichlorophenoxy acetic acid (2,4-D) by hypersaline cyanobacterial mats

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ABSTRACT

Microbial mats may possess degradation capacities for haloorganic pollutants because of their wide range of different functional groups of microorganisms, combined with their extreme diurnal changes in pH, oxygen and sulfide gradients. In this study, 20 mg/l of the chlorinated herbicide 2,4-Dichlorophenoxy acetic acid (2,4-D) were applied to a pristine hypersaline cyanobacterial mat from Guerrero Negro (Mexico), under light/dark incubation conditions. The depletion of 2,4-D due to photooxidation or adsorption was checked in control experiments. Within 13 days, the light/dark incubated mats degraded 97 % of the herbicide, while in dark incubations only 35 % was degraded. Adsorption of 2,4-D to the mat material, agar or glass walls was negligible (4.6 %), whereas 21 % of the herbicide was degraded photochemically. The half life of 2,4-D in the light/dark incubations was comparable to values reported for soils. 2,4-D addition permanently inhibited the photosynthetic activity in the mat by 17 % on average. Sulfate reduction in the entire mat and respiration in the photic zone were more strongly inhibited but returned eventually to their original levels. Since at the end of the experiment the photosynthetic and respiratory activity of the mats were almost as high as in the beginning, and 2,4-D had almost completely disappeared, we conclude that the mats examined represent a robust and effective system for the degradation of the herbicide.

INTRODUCTION

Halogen-substituted aromatic compounds belong to the most problematic anthropogenic substances in the environment. Notorious representatives are polychlorinated biphenyls (PCB), tetrachlorinated dibenzo-p-dioxins (TCCD) and dibenzofurans (TCDF). Quantitatively more important are chlorobenzenes and chlorophenols. The worldwide consumption of chlorobenzenes is 700,000 t/a (Reineke and Schlömann 1997), with emission of 400,000 t/a by production and usage (Koch 1989); 150,000 t chlorophenols are consumed each year (Reineke and Schlömann 1997). 2,4-Dichlorophenoxy acetic acid (2,4 D), a chlorophenol, was introduced in 1946. 2,4-D rapidly became the most widely used herbicide in the world, and remains so to date (Rhoades 2001). It is a selective herbicide with preferential activity against dicotyledons, used against broadleaf plants in cultivated agriculture, in pasture and rangeland applications, forest management, homes, gardens and to control aquatic vegetation. 2,4-D is used in different formulations as solid alkali salt concentrate, as a salt-based water-miscible solution or as an ester-based emulsifiable concentrate; the water solubility ranges from 0,3 g/l at pH 1 to 34,2 g/l at pH 9 (ARS 1995, WHO 1978). It is used in mixtures with other herbicides and represents a main constituent of the defoliant "Agent Orange" (Zhao et al. 1987). 2,4-D is slightly to moderately toxic to mammals and humans, but highly toxic in aquatic environments (Stevens and Sumner 1991, anonymous 1994, NLM 2001). The LC 50 for cutthroat trout ranges from 1 to 100 mg/l, depending on the formulation of the herbicide (EPA 1988). At 2,4-D concentrations between 1 and 10 µM, photosynthesis in aquatic microorganisms is inhibited; 10 µM can inhibit growth by as much as 50 %, depending on the species and environmental conditions (Koch 1989). When exposed to 2.21 mg/l 2,4-D, the algae *Chlorella pyrenoidosa, Chlorococcum* sp. and *Lyngbya* sp. and the cyanobacterium *Anabaena variabilis* were unaffected in terms of growth, respiration and photosynthesis (Hawxby et al. 1977). Growth and heterocyst formation of the cyanobacterium *Nostoc* was reduced by 10 mg/l 2,4-D, and growth stopped at concentrations above 1000 mg/l (Gangawane et al. 1980).

Mixed cultures from a 2,4-D manufacturing plant effluent or pure cultures of *Pseudomonas* sp. and *P. aeruginosa* grew on 2,4-D as the sole source of carbon and energy (Buenrostro-Zagal et al. 2000, Musarrat et al. 2000). 2,4-D was readily and rapidly degraded in soil at a concentration of 1.5 mg/kg (O´Connor et al. 1981). The half life of 2,4-D in cultures of soil microorganisms ranged from 5 to 8 days at a pH range from 5 to 8.5. It increased to 21 days at pH 4.5 and to 41 days at pH 4 (Torstensson 1975). At low concentrations, 2,4-D is also rapidly degraded in water. Most of 21 species of freshwater algae exposed to 0.01 mg/l 2,4-D butoxyethanol ester fully degraded the substance within two weeks, and seven isolates reduced 2,4-D to less than 20 % of the amount added (Butler et al. 1975). However, at higher concentrations, in saline environments or at higher temperatures, degradation can be more difficult. Organisms become more sensitive to 2,4-D as water temperature increases (WHO 1989). Concentrations higher than 1-5 mg/l can impede degradation because of enhanced toxic effects. Under hypersaline conditions, microbial degradation of otherwise easily biodegradable contaminants is often more difficult, because salt stress is superimposed on pollution stress (Oren et al. 1992). 2,4-D pollution in saline and hypersaline environments occurs where there is intense agricultural use in arid regions, e.g. in Gaza, Palestine (Safi, in press) or in salt lakes (Donald and Syrgiannis 1995, Maltseva et al. 1996). This encourages bioremediation studies with microbial mats which are able to grow under such inimical conditions.

Hypersaline microbial mats are dominated by several functional groups of microorganisms. The activities of cyanobacteria, diatoms, aerobic heterotrophs, colorless sulfur bacteria, purple sulfur bacteria and sulfate reducers lead to extreme gradients of pH, oxygen and sulfide concentrations (van Gemerden 1993). Cyanobacteria play a major role in such mats because they provide the biofilm with oxygen and photosynthates. Furthermore, many cyanobacteria have the ability to fix N_2 from air and supply the mat with a utilizable nitrogen source. The availability of oxygen and a nitrogen source are prerequisites for successful aerobic degradation (Rosenberg et al. 1992). In the permanently anaerobic region of the mats, sulfate serves as the terminal electron acceptor for degradative metabolism (Holliger and Schink 1997). Many chlorinated compounds are degraded completely only by a combination of anaerobic and aerobic steps (Fathepure and Vogel 1991, Gerritse and

Gottschal 1992, Gerritse et al. 1997). Since in microbial mats periods of oxygen oversaturation alternate with anoxia within 24 h, such mats represent a promising community for the bioremediation of halogen-substituted hydrocarbons.

In our experiments, hypersaline cyanobacterial mats from Guerrero Negro (Mexico) were contaminated with 2,4-D. Effects on microbial physiology were studied by measuring photosynthetic rates, pH, sulfide and oxygen concentrations with microelectrodes. The degradation process was monitored by gas chromatography.

MATERIALS AND METHODS

Mat samples were collected in May 2000 in Guerrero Negro (Mexico), and transferred directly to the laboratory. The mats were maintained for 6 months in an aquarium in artificial seawater (ASW) at a salinity of 10 % and a temperature of 22 °C. Five subsamples of the mats (8 x 6 x 4 cm, l/w/h) were embedded in agar (1.5 %, ASW) in small glass aquaria. Two of the subsamples had been killed in advance by heat treatment (3 min, 105 °C). This procedure did not change the physical structure of the samples. Two intact mats and one dead mat were incubated with a light regime of 12 h dark/12 h light. The mats were illuminated with a halogen lamp (150 W) at an intensity of 600 µmol photons/m^2s. One intact mat and one dead mat were incubated in permanent darkness. An overview of the different incubations is presented in Tab. 1. Prior to the degradation experiments the samples were in steady state, as measured with oxygen microelectrodes. To all the aquaria 20 mg/l of 2,4-D were added, except for one control in the light. The incubation was maintained for two weeks.

Table 1: Incubation of the microbial mats in different treatments

Treatment	Light	Dead	2,4 D	Effect
B	x			control
C	x		x	degradation in the light + photooxidation + adsorption
D	x	x	x	photooxidation + adsorption
A			x	degradation in the dark + adsorption
E		x	x	adsorption

Microsensor measurements of oxygen and sulfide concentrations, pH and photosynthetic rates were performed directly before the addition of 2,4-D and on day 1, 4, 7, 10 and 13 after contamination. Oxygen concentrations were determined with fast-responding Clark-type O_2 sensors with guard cathodes (Revsbech 1989). Gross photosynthetic rates were determined with the same sensors by the light-dark-shift method (Revsbech and Jørgensen 1983). We defined the photic zone as the layer in which gross photosynthetic activity could be detected by use of a 1-s dark period (Glud et al. 1992). The O_2 sensors used had tip diameters of less than 6 µm, a stirring sensitivity of less than 2 %, and a 90 % response time (t_{90}) of less than 0.5 s. pH measurements were performed with potentiometric glass microelectrodes (Revsbech et al. 1983), which had a tip diameter of less than 10 µm with the pH sensitive glass at the very tip not longer than 100 µm. t_{90} of the electrodes was less than 10 s. The H_2S sensors (Kühl et al. 1998) had a tip diameter of less than 20 µm. All electrodes were calibrated as described elsewhere (Revsbech and Jørgensen 1986, Revsbech et al. 1989, Santegoeds et al. 1998). Calculations and evaluation procedures for the data obtained are described elsewhere (Groetzschel et al., submitted). All directly measured data were plotted as an average of three measurements.

For the chemical analyses of 2,4-D, 5 ml samples of the overlying water were taken with a syringe after 1, 2, 4, 7, 10 and 13 days. The samples were acidified with HCl (pH < 2) and stored at –20 °C until analyses. The samples were transferred to a separatory funnel and NaCl was added to almost saturation. The samples were extracted four times with 3 ml dichloromethane (DCM) by vigorous shaking. The solvent was removed from the combined extracts by a rotary evaporator and the extract was diluted with DCM to a concentration suitable for gas chromatography

(GC). Squalane was added as an internal standard. The efficiency of the extraction procedure was tested with a 2,4-D standard mixture and yielded a recovery of more than 90 % of the 2,4-D.

For GC analyses, 50 µl aliquots of the extract were transferred into a microvial and derivatized with MSTFA (N-methyl-N-trimethylsilyltrifluoracetamid, 50 µl, 70 °C for 2 h). We used 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 of 2 min at 60 °C the oven was heated to 150 °C at a rate of 20 °C/min, and then to 310 °C at a rate of 3 °C/min, and maintained at that temperature for 15 min. The 2,4-D was quantified by integration of the FID signal and comparison with that of the internal standard. Values were corrected for the different FID response of the 2,4-D derivative compared to the internal standard.

RESULTS

The appearance of the mat samples did not change during the experiment, but the water phase above the dead mats became turbid after day 1. Concentrations of 2,4-D in the overlying water of the different experiments are presented in Fig. 1. In the aquarium with the dark incubated, active mat (treatment A) the 2,4-D concentration decreased continuously from the initially added 20 mg/l to 12.1 mg/l on day 13 (Fig. 1A). The 2,4-D removal rate was 0.6 mg/l*d. With active mats incubated in the light, 2,4-D was almost completely degraded after 13 days (Tab. 2).

Table 2: 2,4-D removal characteristics in the different treatments

Treatment	C	D	A	E
Effect	adsorption + photooxidation + degradation in the light	adsorption + photooxidation	adsorption + degradation in the dark	adsorption
Final 2,4-D Concentration [mg/l]	0.7 ± 0.6	14.9 ± 0.4	12.1 ± 0.1	19.1 ± 0.5
2,4-D Decrease [mg/l]	19.3 ± 0.6	5.1 ± 0.4	7.9 ± 0.1	0.9 ± 0.5
2,4-D Removal Rate [mg/l*d]	1.5 ± 0.05	0.4 ± 0.03	0.6 ± 0.01	0.1 ± 0.04
2,4-D Half Life [d]	6.7 ± 0.2	25.5 ± 1.8	16.5 ± 0.3	140.6 ± 47.9
2,4-D Removal [%]	96.6 ± 3.2	25.5 ± 2.0	39.3 ± 0.7	4.6 ± 2.4

In the water phase above the dead mat incubated in the dark, the 2,4-D concentration remained constant (Fig. 1B). In the aquarium with the dead mat incubated with light the concentration of 2,4-D slowly declined to a final concentration of 14.9 mg/l (Tab. 2). 2,4-D metabolites were not detected in any of the aquaria.

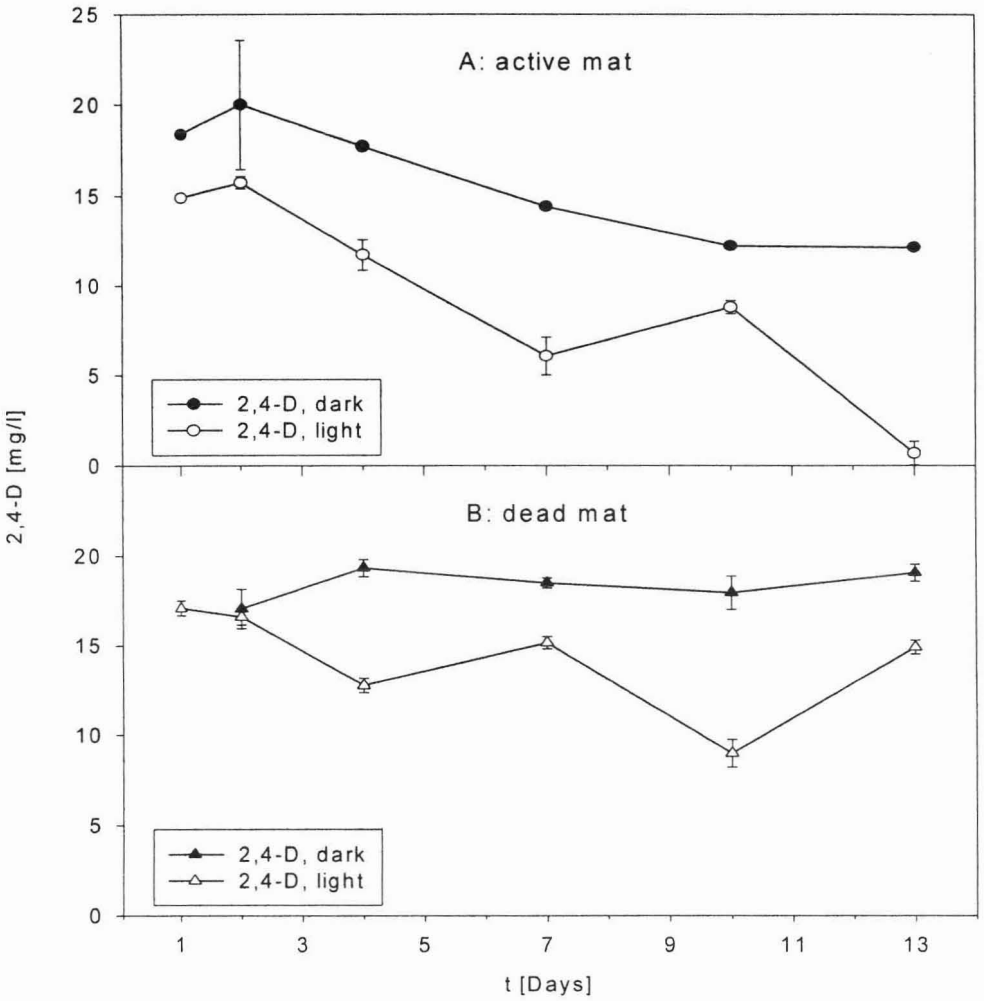

Figure 1: A: 2,4-D concentrations (means ± standard deviations, n = 3) in the overlying water of an active mat incubated in the dark and in 12h dark/12h light after addition of 20 mg/l 2,4-D at day 0. B: 2,4-D concentrations (means ± standard deviations, n = 3) in the overlying water of a dead mat incubated in the dark and in 12h dark/12h light after addition of 20 mg/l 2,4-D at day 0.

Profiles of oxygen concentrations in the light, photosynthetic rates and calculated respiration rates in the control mat are shown as an average of three measurements each on day 0, 5 and 10 (Fig. 2, top left). The small standard deviations demonstrate that only minor changes were detected throughout the experiment. Fig. 2 also shows average profiles of oxygen concentrations in the light and in the dark, gross photosynthetic rates and calculated respiration rates in the 2,4-D contaminated mat incubated in light/dark cycles. At the start of the experiment, profiles of gross

photosynthetic rates, respiration rates and oxygen concentrations were almost identical with the profiles of the control (Fig. 2, top middle). The oxygen profile in the light, with a major peak at 1.2 mm depth and a minor peak at 2.6 mm depth (Fig. 2, top middle), changed significantly after contamination. During the experiment the upper peak decreased and the lower peak increased, so that at the end only one broad maximum between 1.4 and 2.4 mm was detected (Fig. 2, bottom right). The profiles of gross photosynthetic rates showed a similar pattern, with a major peak at 1.6 mm and a minor peak at 2.4 mm on day 0 (Fig. 2, top middle) and one broad peak at the end (Fig. 2, bottom right). The areal gross photosynthetic rates were constant following an initial decline on day 1 (Fig. 3, A). The areal respiration rates in the photic zone (Fig. 3, B) decreased after 7 days by 47 % and increased later to the initial values. The areal respiration rates of the entire mat in the dark (Fig. 3, C) showed the same trend and decreased after 7 days by 49 % and increased thereafter to the initial rates. The areal respiration rates in the aphotic zone (Fig. 3, B) remained constant.

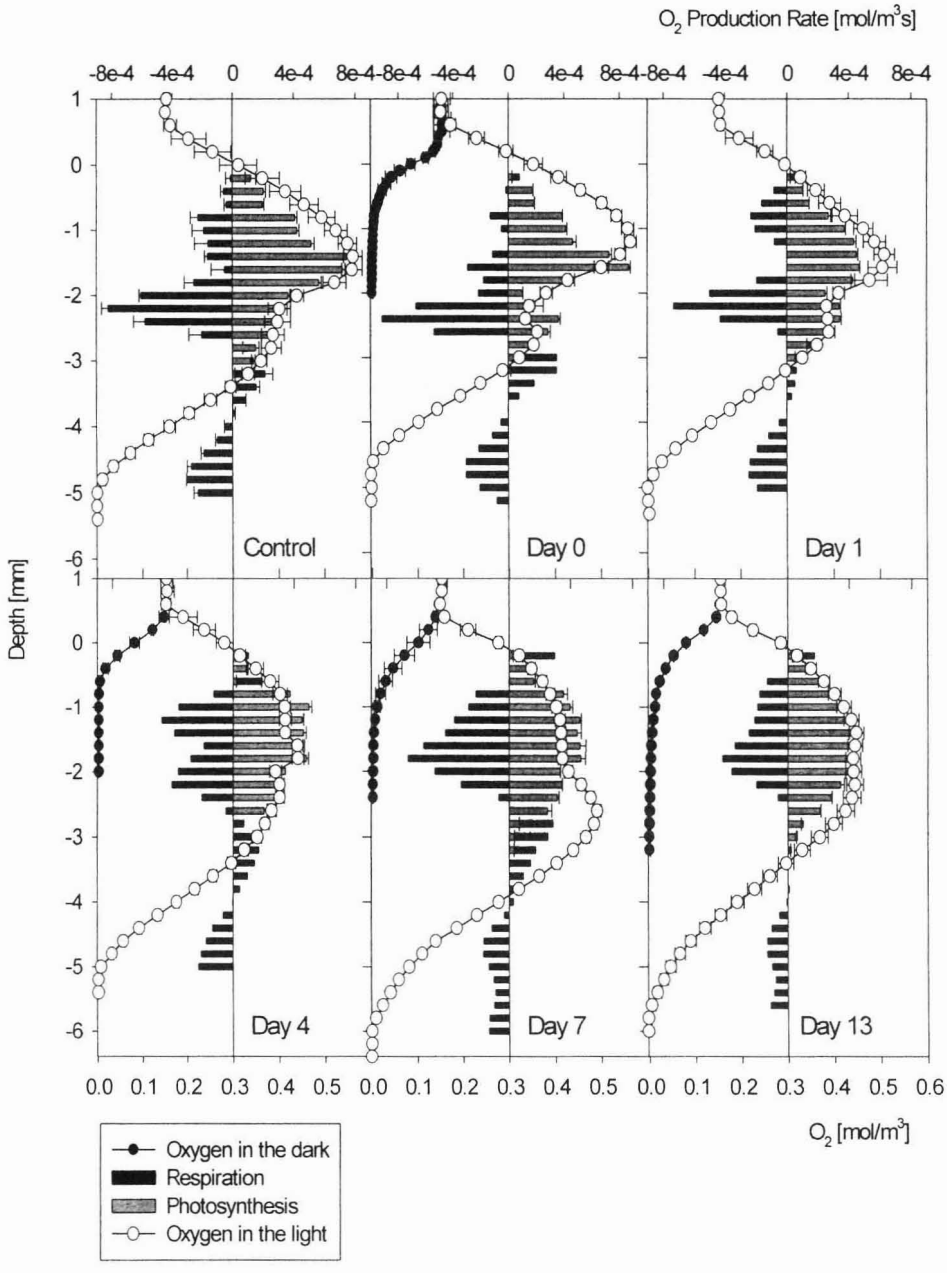

Figure 2: Control: Profiles of oxygen concentrations in the light, volumetric photosynthetic rates and volumetric respiration rates in an active mat incubated in 12h light/12h dark as means of 9 profiles from days 0-10 with standard deviations. Day 0- Day 13: Profiles of oxygen concentrations in the light and in the dark, volumetric photosynthetic rates and volumetric respiration rates in an active mat incubated in 12h light/12h dark with 20 mg/l 2,4-D as means of 3 profiles with standard deviations.

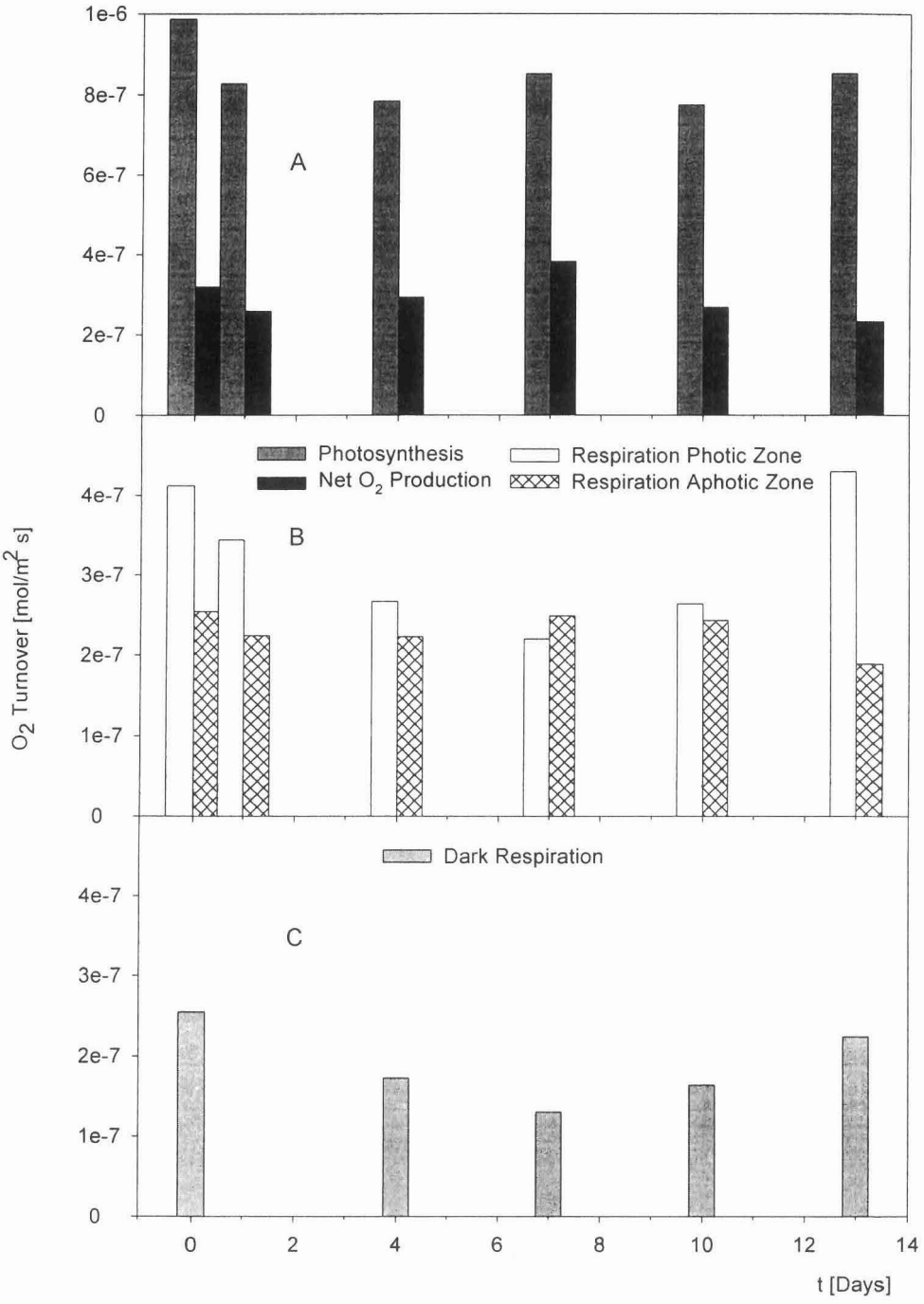

Figure 3: Activity rates as calculated from microsensor profiles in an active microbial mat incubated in 12h light/12h dark with 20 mg/l 2,4-D. A: Areal photosynthetic rates and areal net oxygen production rates in the light. B: Areal respiration rates in the photic zone and in the aphotic zone in the light. C: Areal respiration rates in the dark.

Profiles of total sulfide concentration in the dark, pH in the dark and pH in the light are presented in Fig. 4. The total sulfide concentration in the mat during the dark period declined in the first 7 days of the experiment and increased subsequently (Fig. 4, left). The pH values in the dark increased until day 7 and decreased thereafter (Fig. 4, middle). The same applies to the pH values in the light below 3.4 mm in the aphotic zone (Fig. 4, right). In the upper 1.2 mm the lowest pH values were measured on day 7.

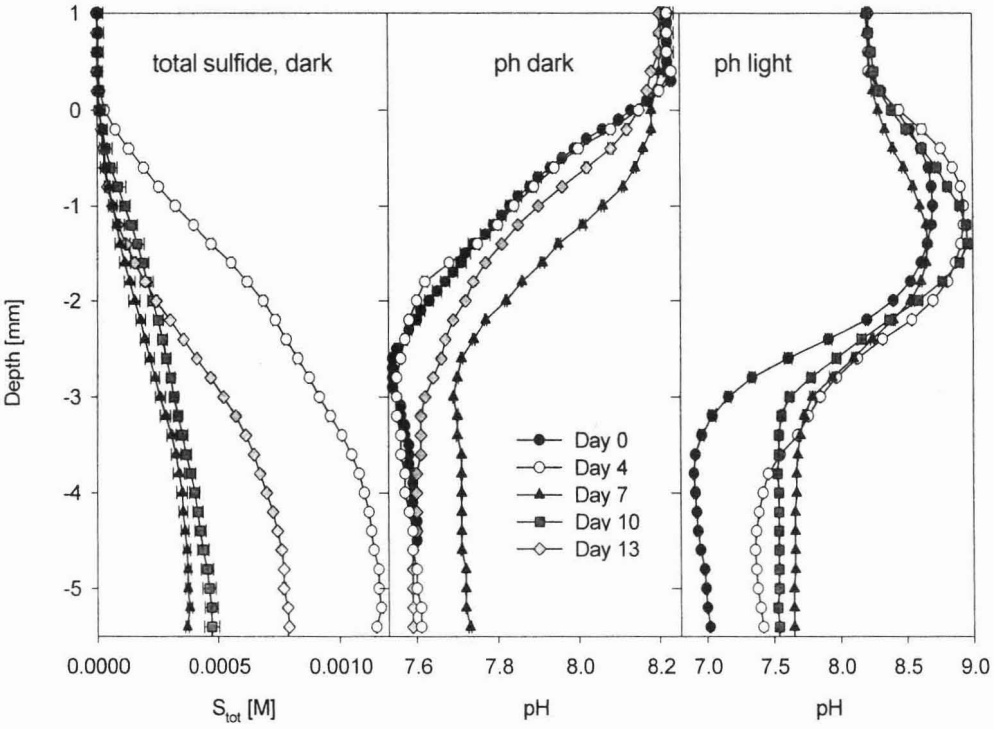

Figure 4: Profiles of total sulfide concentrations in the light, pH in the light and pH in the dark (means ± standard deviations, n = 3) in an active microbial mat incubated in 12h light/12h dark with 20 mg/l 2,4-D.

DISCUSSION

The constant concentration of 2,4-D in the aquarium with the dead mat incubated in the dark (Fig. 1B) indicates that 2,4-D was not adsorbed or absorbed in significant amounts by the mat material, the agar or the glass walls of the aquarium. The 2,4-D loss in that treatment (E) was only 4.6 ± 2.4 % (Tab. 2). This result is in agreement with column experiments using aquifer material, in which no sorption of 2,4-D could be detected (Tuxen et al. 2000). Our results also show that the dead mat was indeed inactive. It can be ruled out that the slight decrease in 2,4-D concentration in the aquarium with the dead mat incubated in the light (treatment D) was due to microbial activity or adhesion phenomena. Therefore we conclude that a small amount of the pesticide was degraded photochemically. Direct photolysis of 2,4-D was first reported in 1966 (Crosby and Tutass 1966). It follows first order kinetics with respect to the absorbed light but is independent of the 2,4-D concentration (Cabrera et al. 1997). Metabolites were not expected to be detected in our experiment since the two main photochemical reaction pathways, with the intermediates 2,4-Dichlorophenol (DCP) and chlorohydroquinone (CHQ), end in total mineralization of the compounds (Alfano et al. 2001).

The decrease of 2,4-D concentration in the aquarium with the active mat incubated in the dark (Fig. 1A) could only be a result of microbial degradative activity, since in the parallel treatment with the dead mat (Fig. 1B) the 2,4-D concentration remained constant. In the dark, the mats were almost exclusively anoxic. Anaerobic degradation of 2,4-D has previously been reported in an anoxic muck soil which was contaminated with 0.5-1 kg/ha of the herbicide (Cheah et al. 1998). The degradation followed first order kinetics and the half life was 9.3 days. In an anoxic sewage sludge incubated in the dark, 24 µg/g 2,4-D were completely removed after 17 days (Zipper et al. 1999). Compared to these literature data, the degradation in the dark under anaerobic conditions in the present experiment is slow, with an average degradation rate of 0.6 mg/l*d and a 2,4-D half life of 16.5 days (Tab. 2).

In the aquarium with the active mat incubated in 12 h light/12 h dark (treatment C), 2,4-D was almost completely removed (Fig. 1A). Since sorption

processes were negligible (treatment E), we conclude that a combination of photochemical degradation and biodegradation during the periods in the dark and in the light was causing this removal. The 2,4-D degradation in the light was 2.5 times faster than in permanent darkness (Tab. 2). In the light, up to 3.5-fold oxygen saturation occurred (Fig. 2), whereas the degradation in the dark took place under anoxic conditions. The degradation of 2,4-D was apparently more effective under aerobic than under anaerobic conditions. Faster degradation of 2,4-D under aerobic conditions has been reported for muck soil with a half life of 3.4 days (9.3 days anaerobically), but in a sandy loam the 2,4-D half life was 35.9 days (Cheah et al. 1998). Degradation rates depend on the type of matrix and on its grain size: the half life of 2,4-D differed considerably in loam soil (4 days), clay soil (6 days), sandy clay loam (7 days) and fine sand (23 days) of northern and southern Ontario (Thompson et al. 1984). The observed half life of 6.7 days in our experiment was in the same range.

From the decrease of 2,4-D in the different treatments, the relative importance of the degradative process could be calculated (Tab. 3). The major removal processes by far appeared to be the microbial degradation in the light and in the dark. Sorption was negligible, but photochemical removal of 2,4-D was significant, as determined in treatment D with an inactive mat in the light. Photochemical 2,4-D degradation may even be higher in the presence of an active mat: the rate of 2,4-D photolysis was reported to be 20 times higher in the presence of hydrogen peroxide (H_2O_2) (Alfano et al. 2001). In microbial mats, H_2O_2 is produced during the light period. O_2^- generated by the Mehler reaction is disproportionated by superoxide dismutase (SOD), which is found in the chloroplasts of algae and in all cyanobacteria studied to date (Tichy and Vermaas 1999).

Table 3: Calculated proportions of 2,4-D removal

Effect	Calculated 2,4-D Degradation [%]
degradation in the light	36.4
photooxidation	20.9
degradation in the dark	34.7
adsorption	4.6

The observed shifts in photosynthetic rates, respiration rates and oxygen concentrations (Fig. 2) were effects of the addition of 2,4-D to the water phase, since the rates in the control were almost constant. The initial decrease and the persistently lower areal photosynthesis rates after 2,4-D addition (Fig. 3A) indicate a constant inhibition of the phototrophic community in the mat. Respiration in the aphotic zone was not affected by 2,4-D and the areal rates remained constant (Fig. 3B). On the other hand, areal respiration rates in the photic zone decreased by 47 % in the first week and increased again to the initial values at the end of the experiment. The decrease of the respiration rates in the photic zone in the first week cannot be attributed solely to the inhibition of photorespiration concomitant with the inhibition of the phototrophic community, since the toxic effect on respiration was stronger. We conclude that the aerobic heterotrophs in the photic zone were directly affected by the 2,4-D addition. This is supported by the trends of the areal respiration rates during the dark periods (Fig. 3C), which are comparable to the rates in the photic zone in the light.

Trends in sulfate-reducing activity were similar to those for respiratory activity in the photic zone upon 2,4-D addition (Fig. 4, left). The decrease in sulfide concentrations from day 4 to day 7 caused the highest pH values below 3.2 mm both in the dark and in the light. It is not likely that the transient depression in respiratory activity in the photic zone and in sulfate reduction is linked to the degradation of 2,4-D, because the 2,4-D concentration decreased continuously in the dark treatment (linear decrease, $r^2 = 0.90$) as well as in the light treatment (linear decrease, $r^2 = 0.86$) (Fig. 1A). Although 35 % of the total 2,4-D decrease was due to biodegradation in the dark (Tab. 3) and the sulfide concentrations increased after an initial inhibition, it is not clear if sulfate-reducing bacteria were involved in the anaerobic removal of 2,4-D. As mentioned above, the degradation rates in the dark were comparatively low, which is in agreement with a report that no 2,4-D degradation was observed under sulfate-reducing conditions in a laboratory filter system with natural underground material, independent of 2,4-D concentration, time or nutrient concentrations (Kuhlmann et al. 1995). 2,4-D dechlorination by a microbial consortium in soil decreased under sulfate-reducing conditions (Chang et al. 1998). It is concluded that the decrease in sulfate-reducing activity in the first week was due to 2,4-D toxicity, which applies for the respiration in the photic zone as well.

The highest 2,4-D degradation yield was achieved by a combination of light and dark incubation, which resulted in alternate aerobic and anaerobic conditions. Together with the increased respiration rates in the photic zone in the second week, this could be an indication that aerobic heterotrophic bacteria were involved in the 2,4-D degradation.

CONCLUSIONS

Within 13 days, 20 mg/l of 2,4-D were removed by a pristine, hypersaline cyanobacterial mat from Guerrero Negro (Mexico) incubated in 12 h light/12 h dark. The major removal process appeared to be microbial mineralization, both in the light and in the dark, but photochemical degradation also occurred. Degradation of 2,4-D in permanent darkness was comparatively slow, whereas the 2,4-D half life in the light/dark incubations was similar to those reported for soils. The phototrophic community of the mat was permanently inhibited by 17 % with respect to photosynthetic rates. Respiration in the photic zone and sulfate reduction were inhibited more strongly, but only transiently. Since 97 % of the added 2,4-D was degraded during the incubation period, at salt concentrations as high as 10 %, we conclude that the examined mats represent a robust and efficient ecosystem for mineralization of the herbicide.

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Chapter 6

Summary

SUMMARY

Hypersaline microbial mats represent ideal model systems in which to study metabolic processes and their regulation, since their carbon and sulfur cycles are almost closed. The mats are characterized by steep, diurnally changing gradients of pH, oxygen and sulfide concentrations caused by the activity of a whole variety of different physiological groups of microorganisms. Such qualities, together with observations that a) many mat-inhabiting microorganisms posses biodegradation capacities, and b) cyanobacterial mats were able to tolerate and degrade petroleum hydrocarbons after an oil spill, suggest that microbial mats posses a pronounced potential for the degradation of organic pollutants. In this study several questions have been investigated concerning the application of organic substances to hypersaline microbial mats:

How does an additional carbon and energy source affect the internal carbon turnover in microbial mats (Chapter 2)?

After addition of acetate, glycolate or glucose to hypersaline cyanobacterial mats, it was found that there was no general physiological reaction pattern upon the addition of organic carbon. Rather, the effects on photosynthesis and respiration depended on the kind of compound added and its role in the metabolism of the mats. The substrates were degraded in those layers of the mat where they occur under natural conditions. Moreover, respiration and photosynthesis seem to be closely coupled. The populations responsible for photosynthesis and respiration must be present in close proximity of each other. If an unusual but easily usable substance like glucose was added to the mats, the reaction was complex and led to total inhibition of net oxygen production. Even after the strong changes in the environmental conditions caused by the substrate addition, the mats could recover and restore their activity. The community structure showed no detectable alterations, supporting the conclusion that the mat community is robust.

Assuming that the addition of a usable carbon source would lead to the depletion of oxygen in the microbial mats due to increased respiratory activity, what would be the feedback effect of the lower O_2 level on the phototrophic organisms (Chapter 3)?

The results of Chapter 2 showed that carbon addition does not always lead to O_2 depletion in microbial mats. In Chapter 3 the responses of two hypersaline cyanobacterial mats of different origin to changing oxygen concentrations were investigated. In both mats the highest rates of gross photosynthesis and respiration were found when no oxygen was present in the overlying water. This is in agreement with pure-culture studies, where the activity of the key enzyme in CO_2 fixation, ribulose-1,5-bisphosphate-carboxylase/ogygenase (Rubisco), depended on the CO_2/O_2 relation in the water phase. Nevertheless, high respiration rates were found in the mats as well, presumably due to excreted photosynthetic products and the close coupling of the autotrophic and the heterotrophic community in the mats. These interrelations between the community members of the microbial mats also led to higher photosynthetic and respiratory activity when the oxygen concentration in the overlying water was increased above the ambient level. This result is contradictory to the theory that CO_2 fixation, regulated by Rubisco, is decreased by high O_2 levels. It was concluded that due to the complex interspecies relations in the mats investigated, the proposed straight causal associations among the oxygenase and carboxylase activites of Rubisco, carbonic anhydrase activity and carbon concentrating mechanisms in individual phototrophic microorganisms are not applicable to microbial mats.

Do microbial mats represent an efficient system for the bioremediation of oil pollutants (Chapter 4)?

To increase the availability of selected hydrophobic petroleum compounds to submersed hypersaline cyanobacterial mats, a carrier material was successfully introduced. The microbial mats were able to degrade the petroleum compounds, albeit at low rates. The degradation rates depended on the type of compound: aromatic hydrocarbons were degraded faster than alkanes. The internal carbon cycling in the

mats was apparently not affected by the pollutants, but indications of slight toxic effects to some members of the microbial community were found. It was concluded that the enzymes required for the degradation of organic pollutants exist in the microbial mats investigated, but that the turnover occurs in addition to the usual high activity rates. The degradation activity did not significantly affect mat physiology or community structure.

Are water-soluble chlorinated hydrocarbons degraded by microbial mats (Chapter 5)?

Hypersaline cyanobacterial mats from Guerrero Negro (Mexico) incubated at 12 h light/12 h dark degraded 20 mg/l of 2,4-Dichlorophenoxy acetic acid (2,4-D) during 13 days. The major removal process was microbial mineralization in the light and in the dark, but photochemical degradation also occurred. 2,4-D degradation in permanent darkness was comparatively slow, whereas the 2,4-D half life in the light/dark incubations was in agreement with reported values in soil. The phototrophic community of the mat was slightly, but constantly inhibited by the 2,4-D addition. Sulfate reduction in the entire mat and respiration in the photic zone were inhibited to a larger extent, but the effect was reversible. Since 2,4-D was removed under salt concentrations as high as 10 %, it was concluded that the mats examined are a robust and effective system for degradation of the herbicide.

Despite the fact that the microbial mats studied represent pristine systems, they showed a pronounced degradative potential for organic pollutants, especially for a water-soluble chlorinated compound. An investigation of microbial mats from polluted sites with a similar combination of scientific methods could provide more detailed information about the applicability of such mats in bioremediation campaigns in the future.

ZUSAMMENFASSUNG

Hypersaline mikrobielle Matten stellen ideale Modellsysteme für die Erforschung metabolischer Prozesse und deren Regulation dar, da in diesen Ökosystemen annähernd geschlossene Kohlenstoff- und Schwefelkreisläufe vorherrschen. Die Matten sind durch sich im Tagesverlauf verändernde, steile Gradienten der Sauerstoff- und Sulfidkonzentration sowie des pH-Wertes gekennzeichnet, die auf die Aktivitäten einer ganzen Reihe unterschiedlicher physiologischer Gruppen von Mikroorganismen zurückzuführen sind. Zusammen mit Beobachtungen, daß a) viele Gruppen von Mikroorganismen, die an der Bildung o.g. Matten beteiligt sind, die Fähigkeit zur Mineralisierung von Schadstoffen besitzen und b) Cyanobakterienmatten nach einer Kontamination mit Rohöl in der Lage waren, Mineralöl-Kohlenwasserstoffe zu tolerieren und zu verwerten, lassen die genannten Merkmale darauf schließen, daß mikrobielle Matten über ein erhebliches Potential zum Abbau organischer Schadstoffe verfügen. In der vorliegenden Arbeit wurden mehrere Fragen im Zusammenhang mit der Verwertung organischer Substanzen durch hypersaline mikrobielle Matten untersucht:

Wie beeinflußt die Zugabe einer zusätzlichen Kohlenstoff- und Energiequelle den internen Kohlenstoffumsatz in mikrobiellen Matten (Kapitel 2)?

Nach Zugabe von Acetat, Glycolat oder Glucose zu hypersalinen Cyanobakterienmatten zeigte sich kein generelles physiologisches Reaktionsmuster im Zusammenhang mit der Konzentrationserhöhung der organischen Substanzen. Die Auswirkungen auf die Photosynthese- und Respirationsaktivität hingen im Gegenteil von der Art der zugegebenen Substanz und ihrer Rolle innerhalb des Metabolismus der Matten ab. Die Substrate wurden in den Schichten der Matten abgebaut, in denen sie auch unter natürlichen Bedingungen vorkommen. Außerdem scheinen Photosynthese und Respiration in enger Wechselwirkung zu stehen. Die Populationen, die für beide Prozesse verantwortlich sind, müssen in enger räumlicher Nähe zueinander existieren. Sobald den Matten eine im Porenwasser seltene, aber leicht verwertbare Substanz wie

Glucose hinzugefügt wurde, ergab sich eine komplexe Reihe von Reaktionen, die in der absoluten Hemmung der Netto-Sauerstoffproduktion gipfelte. Doch selbst nach diesen extremen Veränderungen der Umweltbedingungen innerhalb der Matte, die durch die Substratzugabe ausgelöst wurden, kam es zu einer Erholung und zur Wiederherstellung der mikrobiellen Aktivität. Die Populationsstruktur zeigte keine nachweisbaren Veränderungen auf, was den robusten Charakter der Matten bestätigt.

Welche Auswirkungen eines niedrigen Sauerstoffniveaus ergäben sich für die phototrophen Organismen innerhalb einer mikrobiellen Matte unter der Annahme, daß die Zugabe einer verwertbaren Kohlenstoffquelle zu einer Verminderung der Sauerstoffkonzentration aufgrund erhöhter Respirationsaktivität führt (Kapitel 3)?

Die Ergebnisse des 2. Kapitels haben gezeigt, daß eine Zugabe organischen Kohlenstoffs nicht zwangsläufig zu einer Verminderung der Sauerstoffkonzentration in mikrobiellen Matten führen muß. Im 3. Kapitel wurden die Reaktionen zweier hypersaliner Cyanobakterienmatten verschiedener Herkunft auf sich ändernde Sauerstoffkonzentrationen untersucht. In beiden Matten traten die höchsten Photosynthese- und Respirationsraten auf, wenn kein Sauerstoff im Überstandswasser vorhanden war. Dies stimmt mit Untersuchungen an Reinkulturen überein, bei denen die Aktivität des Schlüsselenzyms der CO_2-Fixierung, Ribulose-1,5-bisphosphat-Carboxylase/Oxygenase (Rubisco), vom Verhältnis CO_2/O_2 in der Wasserphase abhing. In den Matten wurden allerdings auch hohe Respirationsraten gemessen, vermutlich aufgrund des Zusammenhangs von ausgeschiedenen Photosyntheseprodukten und der engen Verflechtung der autotrophen und heterotrophen Gemeinschaften innerhalb der Matten. Diese Wechselwirkungen zwischen den verschiedenen Gruppen von Mikroorganismen führten zu verstärkten Photosynthese- und Respirationsaktivitäten, wenn die Sauerstoffkonzentration im Wasser über das natürliche Maß hinaus erhöht wurde. Dieses Ergebnis widerspricht der Theorie, daß sich die Fixierung von CO_2, die durch Rubisco reguliert wird, bei steigendem O_2 Gehalt vermindert. Daraus wurde geschlossen, daß sich der direkte kausale Zusammenhang zwischen Oxygenase- und Carboxylaseaktivität von Rubisco, Carboanhydraseaktivität und Kohlenstoffkonzentrationsmechanismen, der für einzelne

phototrophe Mikroorganismen vorgeschlagen wurde, wegen der komplexen Interaktionen zwischen den physiologischen Gruppen von Mikroorganismen innerhalb mikrobieller Matten nicht auf diese anwenden läßt.

Stellen mikrobielle Matten ein effizientes System zum Abbau von Ölbestandteilen dar (Kapitel 4)?

Um die Verfügbarkeit ausgewählter hydrophober Ölbestandteile für hypersaline Cyanobakterienmatten unter der Wasseroberfläche zu erhöhen, wurden Tonmineralien erfolgreich als Transportmaterial eingesetzt. Die mikrobiellen Matten waren in der Lage, die Ölbestandteile abzubauen, wenn auch z.T. recht langsam. Die Abbauraten hingen von der Substanzklasse ab; aromatische Kohlenwasserstoffe wurden schneller abgebaut als aliphatische. Der interne Kohlenstoffumsatz der Matten wurde anscheinend von der Schadstoffzugabe nicht beeinflußt, aber es traten leichte toxische Effekte bei einigen Mitgliedern der mikrobiellen Gemeinschaft auf. Daraus ergibt sich, daß in den untersuchten mikrobiellen Matten die Enzymausstattung für den Abbau organischer Schadstoffe vorhanden ist, der Umsatz aber vor dem Hintergrund der ohnehin hohen Aktivitätsraten erfolgt. Die Abbauaktivität hatte keinen signifikanten Einfluß auf die Physiologie oder die Populationsstruktur der Matten.

Werden wasserlösliche chlorierte Kohlenwasserstoffe von mikrobiellen Matten abgebaut (Kapitel 5)?

Eine mikrobielle Matte aus Guerrero Negro/Mexico, inkubiert bei 12 h Licht/12 h Dunkelheit, baute 20 mg/l 2,4-Dichlorphenoxyessigsäure (2,4-D) innerhalb von 13 Tagen ab. Der bei weitem wichtigste Abbauprozeß bestand hierbei in der mikrobiellen Mineralisation im Licht und im Dunkel, aber auch photochemischer Abbau trug zur Verminderung der 2,4-D-Konzentration bei. Der Umsatz von 2,4-D im Dunkel erfolgte relativ langsam, wohingegen, die 2,4-D-Halbwertszeit in der Licht/Dunkel inkubierten Matte mit Literaturdaten übereinstimmt, die für den Abbau in Böden angegeben wurden. Die phototrophe mikrobielle Gemeinschaft innerhalb der Matte wurde leicht, aber anhaltend gehemmt. Die Sulfatreduktion in der gesamten

Matte und die Respiration in der photischen Zone wurden in einem stärkeren Umfang gehemmt; dieser Effekt war allerdings reversibel. Da 2,4-D bei einer Salzkonzentration von 10 % abgebaut wurde, ergibt sich für die untersuchten Matten das Bild eines robusten und effektiven Systems für den Abbau dieses Herbizids.

Trotz des Umstands, daß es sich bei den untersuchten mikrobiellen Matten um pristine Systeme handelt, wiesen diese ein erhebliches Potential für den Abbau organischer Schadstoffe und besonders für die Verwertung einer wasserlöslichen chlorierten Substanz auf. Die Untersuchung mikrobieller Matten kontaminierter Standorte mit der angewendeten Kombination wissenschaftlicher Methoden könnte in der Zukunft noch detailliertere Informationen über die Verwendung solcher Matten im Rahmen von biologischen Dekontaminationsprogrammen erbringen.

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„Es ist nicht der Berg, den wir bezwingen, sondern uns selbst."

Edmund Percival Hillary, Bergsteiger

„Eine gefaßte Hypothese gibt uns Luchsaugen für alles sie Bestätigende und macht uns blind für alles ihr Widersprechende."

Arthur Schopenhauer, Philosoph

„Unrast ist kein Fleiß."

Kurt Tucholsky, Schriftsteller

„Die Welt beurteilt unser Verhalten nicht nach unseren Beweggründen, sondern nach unserem Erfolge. Was bleibt uns da übrig? Man muß Glück haben!"

Friedrich II, König

„Man erkennt nur das, was man kennt."

Georg Wilhelm Friedrich Hegel, Philosoph

„Und überhaupt,
trotz Tod und tausend Teufeln:
Solang auch nur noch Einer an Dich glaubt,
hast Du kein Recht, persönlich zu verzweifeln.
Das ist ganz einfach nicht erlaubt!"

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„Man versteht ein Gedicht nicht besser, wenn man eine Lupe nimmt und die Buchstaben näher betrachtet."

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„Mach Dein Ding, steh' dazu,
heul nicht rum, wenn andere lachen."

Die Ärzte, Kommerzpunkband

„Der Versuch ist der erste Schritt zum Mißerfolg."

Homer J. Simpson, Atomkraftwerksspezialist

LIST OF PUBLICATIONS

1. **Metabolic shifts in hypersaline cyanobacterial mats upon addition of organic substrates**

   S. Grötzschel, R.M.M. Abed and D. de Beer

   Concept by S. Grötzschel and D. de Beer, experimental work by S. Grötzschel (community analyses by R.M.M. Abed), writing by S. Grötzschel with editorial help by the co-authors.

2. **Effects of oxygen concentration on photosynthesis and respiration in two hypersaline cyanobacterial mats**

   S. Grötzschel and D. de Beer

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3. **Degradation of petroleum model compounds immobilized on clay by hypersaline cyanobacterial mats**

   S. Grötzschel, J. Köster, R.M.M. Abed and D. de Beer

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4. **Degradation of 2,4-Dichlorophenoxy acetic acid (2,4-D) by hypersaline cyanobacterial mats**

   S. Grötzschel, J. Köster and D. de Beer

   Concept by S. Grötzschel, experimental work by S. Grötzschel (chemical analyses by J. Köster), writing by S. Grötzschel with editorial help by the co-authors.

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