



**Diversity, salinity adaptation, and role in
carbon cycling of microbial communities
inhabiting the oxic layer of intertidal
hypersaline microbial mats**

Katharina Kohls

Title page shows hypersaline microbial mats from the upper intertidal flat of the Arabian Gulf coast at Abu Dhabi (UAE). The red mat is lying in a channel and is always covered with seawater, whereas the grey mat is completely dry and cracked into polygons.

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Table of contents

Summary.....	1
Zusammenfassung.....	3

Part I: Combined presentation of results

A Introduction.....	7
1. Microbial mats.....	7
2. Cyanobacteria and aerobic heterotrophic prokaryotes in microbial mats	
-Autotrophy and Heterotrophy-	9
3. Intertidal microbial mats of Abu Dhabi	12
4. Salinity adaptation strategies of microorganisms in microbial mats.....	15
5. Methods.....	16
5.1 Molecular tools.....	16
5.2 Microsensors.....	17
6. Objectives of this thesis.....	19
B Results and Discussion.....	21
1. Effects of salinity on microbial behavior, metabolism, and community	
structure.....	21
1.1 Salinity-driven migration of cyanobacteria – “Halotaxis”.....	21
1.2 Effect of salinity on oxygen consumption and photosynthesis	23
1.3 Effect of salinity on microbial diversity.....	24
2. Interaction between cyanobacteria and aerobic heterotrophs.....	26
2.1 Diversity and abundance of aerobic heterotrophic prokaryotes in	
hypersaline microbial mats.....	26
2.2 DNA-SIP to identify key aerobic heterotrophic prokaryotes	
involved in carbon cycling using an intact microbial mat	28
2.3 Cyanobacterial monocultures as model systems.....	29

C Final discussion.....	31
1. Adaptation strategies of microorganisms to extreme conditions.....	31
2. Aerobic heterotrophic prokaryotes in the Abu Dhabi mats.....	33
3. Microbial consortia in extreme environments: Interaction between aerobic heterotrophic prokaryotes and cyanobacteria.....	34
4. Relevance of studying microbial communities in extreme habitats.....	37
5. Conclusion and outlook.....	38
D References.....	39

Part II: Publications

A List of Publications.....	55
I. Publications presented in this thesis.....	55
II. Publications not presented in this thesis.....	56
B Publications.....	61
1. Halotaxis of cyanobacteria in an intertidal hypersaline microbial mat.....	61
2. Effect of salinity changes on the bacterial diversity, photosynthesis and oxygen consumption of cyanobacterial mats from an intertidal flat of the Arabian Gulf.....	71
3. Lipid biomarkers, pigments and cyanobacterial diversity of microbial mats across intertidal flats of the arid coast of the Arabian Gulf (Abu Dhabi, UAE).....	83
4. Abundance and community composition of Bacteria and Archaea in the oxic layer of a hypersaline intertidal cyanobacterial mat.....	99
5. Molecular identification of aerobic heterotrophic bacteria in hypersaline microbial mats and their interaction with associated cyanobacteria.....	129

Danksagung.....	157
Eidesstattliche Erklärung.....	159

Summary

The main objective of this thesis was to study the diversity, salinity adaption, and role in carbon cycling of microorganisms inhabiting the oxic layer of intertidal hypersaline microbial mats. For this purpose, mats from the Arabian Gulf coast of Abu Dhabi, United Arab Emirates (UAE), which are subjected to multiple harsh environmental conditions of temperature, UV and light intensity, salinity and salinity fluctuations, as well as desiccation, were investigated.

In the first study (publication 1), a new salinity-driven taxis of cyanobacteria in the upper tidal mat was discovered and termed as 'Halotaxis'. *Microcoleus chthonoplastes* filaments migrated up and down when salinity was decreased below or increased above 15%, respectively. The migration caused a color change of the mat's uppermost layer from red to green and vice versa. We assume that this migration has a protective function for cyanobacteria inhabiting environments that are exposed to strong salinity fluctuations (e.g. intertidal microbial mats), since the bacteria always migrated to lower salinities. Furthermore, a decrease of photosynthesis and oxygen consumption rates at salinities higher than 10% was shown in a low, middle, and upper tidal mat (publication 2). In the upper mat, which was exposed to the highest salinities as well as salinity fluctuations (i.e. from 6 to 20%), the extent of inhibition of these processes turned out to be lower, indicating a more efficient salt adaptation of the resident microorganisms. Interestingly, this mat possessed the highest bacterial diversity. Probably, the resistant fraction of the original community was not affected, whereas the growth of halophilic bacteria was promoted, leading to an increased biodiversity. A further study showed that the mats' microorganisms also possessed adaptation mechanisms to strong desiccation, high UV and light intensities, and high temperatures (publication 3).

The upper tidal mat was further investigated with respect to the composition and role in carbon cycling of aerobic heterotrophic prokaryotes (AHP). This mat hosted a novel and unique diversity of potential AHP. Sequences related to ones of *Chloroflexi*-like bacteria, *Bacteroidetes*, *Proteobacteria*, *Haloarchaea*, and *Crenarchaeota* dominated the clone libraries of the uppermost oxic part of this mat (publication 4). Among the

sequences, many extremophilic (mainly halophilic) bacterial and archaeal genera, such as e.g. *Deinococcus*, *Salinibacter*, and *Halobacteria* were found. Since cyanobacteria live in close proximity to aerobic heterotrophs, we further investigated the interactions between the two groups (publication 5). DNA-stable isotope probing revealed a higher activity of unicellular cyanobacteria with regard to CO₂ fixation, compared to filamentous cyanobacteria. A specific and clear ¹³C-labeling of aerobic heterotrophic bacteria (AHB) was not evident, most likely due to methodological artifacts. However, the results hinted to the potential importance of *Chloroflexi*-like bacteria, *Bacteroidetes*, and *Proteobacteria* in carbon cycling. Investigations of unialgal cyanobacterial cultures as model systems indicated that the community structure of associated AHB is species-specific and depends on the environment, from which the culture was obtained. Interestingly, a community of cyanobacteria-associated AHB or rather their released substances apparently stimulated growth of their natural host, but inhibited other cyanobacterial strains, thereby enhancing the host's competitiveness. The advantage for associated bacteria might be the supply with certain cyanobacterial exudates, vitamins, or growth factors. These interactions are manifold, very complex, highly specific, and provide high potential for biotechnological purposes, e.g. for the discovery of new bioactive substances.

Zusammenfassung

Das Hauptziel dieser Arbeit lag darin, die Diversität, Salzanpassung und Rolle im Kohlenstoffkreislauf von Mikroorganismen, welche die oxische Schicht hypersaliner mikrobieller Matten der Gezeitenzone bewohnen, zu untersuchen. Zu diesem Zweck wurden Matten der Küste des Arabischen Golfes von Abu Dhabi, Vereinigte Arabische Emirate (VAE), die multiplen extremen Umweltbedingungen, wie hoher Temperatur, UV- und Lichtintensität, hoher Salinität und starken Salinitätsschwankungen, sowie Austrocknung ausgesetzt sind, untersucht.

In der ersten Studie (Publikation 1) wurde eine salinitätsbedingte Taxis von Cyanobakterien in einer Matte aus der oberen Gezeitenzone entdeckt und als „Halotaxis“ bezeichnet. *Microcoleus chthonoplastes*-Filamente wanderten auf und ab, wenn die Salinität unter 15% erniedrigt beziehungsweise über 15% erhöht wurde. Diese Wanderung verursachte eine Farbänderung der obersten Mattenschicht von rot nach grün und umgekehrt. Wir nehmen an, dass es sich hierbei um eine Schutzfunktion der Cyanobakterien handelt, die extremen Salinitätsschwankungen ausgesetzt sind (wie z.B. mikrobielle Matten in Gezeitenzonen). Darüber hinaus sanken die Photosynthese- und Sauerstoffverbrauchsrate bei Salinitäten von mehr als 10% in Matten von verschiedenen Positionen der Gezeitenzone (niedrig, mittel und hoch). In der Matte aus der oberen Gezeitenzone, die den höchsten Salinitäten und Salinitätsschwankungen ausgesetzt ist (6 bis 20%), war die Inhibierung dieser Prozesse geringer, was auf eine effizientere Salzanpassung der anwesenden Mikroorganismen hindeutet (Publikation 2). Interessanterweise wies diese Matte auch die höchste bakterielle Diversität auf. Möglicherweise blieb die resistente Fraktion der ursprünglichen Gemeinschaft unbeeinflusst, während das Wachstum von halophilen Bakterien gefördert wurde, was zu einer Erhöhung der Biodiversität führte. Eine weitere Studie zeigte, dass die Mikroorganismen aus den Matten auch Anpassungsstrategien an starke Austrocknung, hohe UV und Lichtintensitäten, sowie hohe Temperaturen aufweisen (Publikation 3).

Die Matte aus der höheren Gezeitenzone wurde weitergehend im Hinblick auf die Zusammensetzung der aerob heterotrophen Prokaryoten (AHP) und deren Funktion im Kohlenstoffkreislauf untersucht (Publikation 4). Diese Matte wies eine neue und

einzigartige Diversität von potentiellen AHP auf. Sequenzen mit naher Verwandtschaft zu *Chloroflexi*, *Bacteroidetes*, *Proteobakterien*, *Haloarchaeen* und *Crenarchaeen* dominierten die 16S rRNA Genbanken des oxischen Teils der Matte. Unter den Sequenzen wurden viele Extremophile (hauptsächlich Halophile), wie z.B. *Deinococci*, *Salinibacter* und *Halobacterium* gefunden. Aufgrund der engen Nachbarschaft von Cyanobakterien und aerob Heterotrophen, haben wir des Weiteren die Beziehung zwischen den beiden Gruppen untersucht (Publikation 5). Inkubationsexperimente an einer intakten Matte mit stabilen C¹³-Isotopen (DNA-SIP) zeigten, dass einzellige Cyanobakterien im Vergleich zu filamentösen Cyanobakterien eine höhere CO₂-Fixierung aufwiesen. Eine eindeutige und spezifische C¹³-Markierung von aerob heterotrophen Bakterien (AHB) war aufgrund methodischer Probleme nicht möglich. Dennoch weisen die Ergebnisse darauf hin, dass *Chloroflexi*, *Proteobakterien* und *Bacteroidetes* im Kohlenstoffkreislauf involviert sind. Die Untersuchung von nicht-axenischen Cyanobakterienkulturen zeigte, dass die Struktur der AHB-Gemeinschaft artspezifisch ist und davon abhängt, aus welchem Habitat die Kultur isoliert wurde. Interessanterweise wurde auch gezeigt, dass Cyanobakterien-assoziierte AHB, die das Wachstum ihres cyanobakteriellen Wirtes zu stimulieren scheinen, das Wachstum von anderen Cyanobakterien komplett inhibieren, und so eine Verstärkung der Konkurrenzfähigkeit des Wirt-Cyanobakteriums bewirken. Der Vorteil für die AHB könnte in der Versorgung mit organischen Substanzen, Vitaminen oder Wachstumsfaktoren seitens der Cyanobakterien liegen. Die Interaktionen zwischen Cyanobakterien und AHB sind vielfältig, sehr komplex und extrem spezifisch, und bergen großes biotechnologisches Potential, z.B. für die Entdeckung neuer bioaktiver Substanzen.

Part I: Combined presentation of results

A Introduction

1. Microbial mats

Microbial mats are hot spots of bacterial diversity and constitute a rich reservoir of gene diversity for future studies of bacterial evolution and genomics (Ley et al., 2006). They became model systems for microbial ecology and represent the modern analogues to ancient life, and possibly extraterrestrial ecosystems (Des Marais, 2003). Microbial mats are considered to be the oldest ecosystems on Earth, dating back to 3.4 billion years (Tice and Lowe, 2004) and their lithified remains are known as stromatolites (Awramik, 1984; Knoll, 1989). Furthermore, they are thought to have significantly influenced the

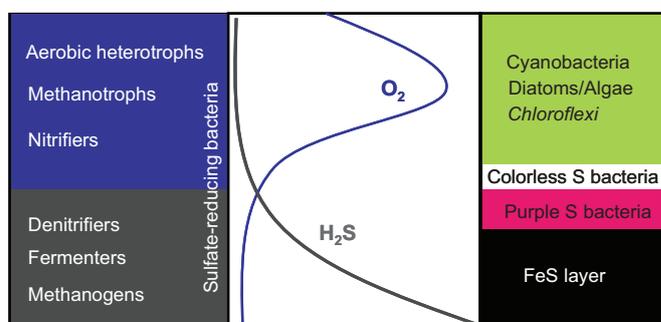


Figure 1. Schematic orientation of microorganism in a microbial mat, with respect to O_2 and H_2S gradients in the light. Organism that contribute to the visible lamination are shown in the right column (modified after Van Gemerden, 1993; Karsten and Köhl, 1996 and Ludwig 2004).

atmospheric composition of the early Earth, with production of O_2 , H_2 , and CH_4 (Hoehler et al., 2001). Microbial mats can extend over several square kilometers and their thickness can range from several millimetres to more than one meter (Stal and Caumette, 1994; Urmeneta and Navarrete, 2000). They occur in diverse habitats, mainly in coastal

zones and extreme environments (Urmeneta and Navarrete, 2000; Franks, 2009), where the abundance and activity of grazing organisms is limited (Javor and Castenholz, 1984; Cohen, 1989; Farmer, 1992). Biomass and extracellular polymeric substances (EPS) contribute to the formation of cohesive structures of microbial mats by embedding cells in a gelatinous matrix (Stal, 2000). Their stratified, thin-layered structure is the result of physicochemical gradients, which are produced by metabolic activity of inhabiting microorganisms and their interactions (Jørgensen et al., 1983; Franks, 2009). In these gradients, microorganisms need to find a niche with the most favourable conditions for optimal growth. Hence, the orientation of phototrophic colored microorganisms causes the visible lamination of microbial mats (Fig. 1). Characteristically, microbial mats are

dominated by a few functional groups of microbes, mainly cyanobacteria, diatoms, aerobic heterotrophic prokaryotes (AHP), green non-sulfur bacteria, colorless sulfur bacteria, purple sulfur bacteria, and sulfate-reducing bacteria. Their combined metabolic activities result in steep environmental gradients, particularly of oxygen and sulfide (Van Gemerden, 1993; Fig. 1). Oxygen gradients develop due to oxygen production by cyanobacterial photosynthesis and oxygen consumption by respiration and other oxidation processes (e.g. sulfide oxidation). Hydrogen sulfide (H_2S) production by sulfate reducing bacteria and the consumption of H_2S by colorless sulfur bacteria and anoxygenic phototrophs (i.e. green sulfur bacteria, purple sulfur bacteria, green non-sulfur bacteria), results in H_2S gradients. The major biogeochemical cycles that are driven within mats are shown in Fig. 2.

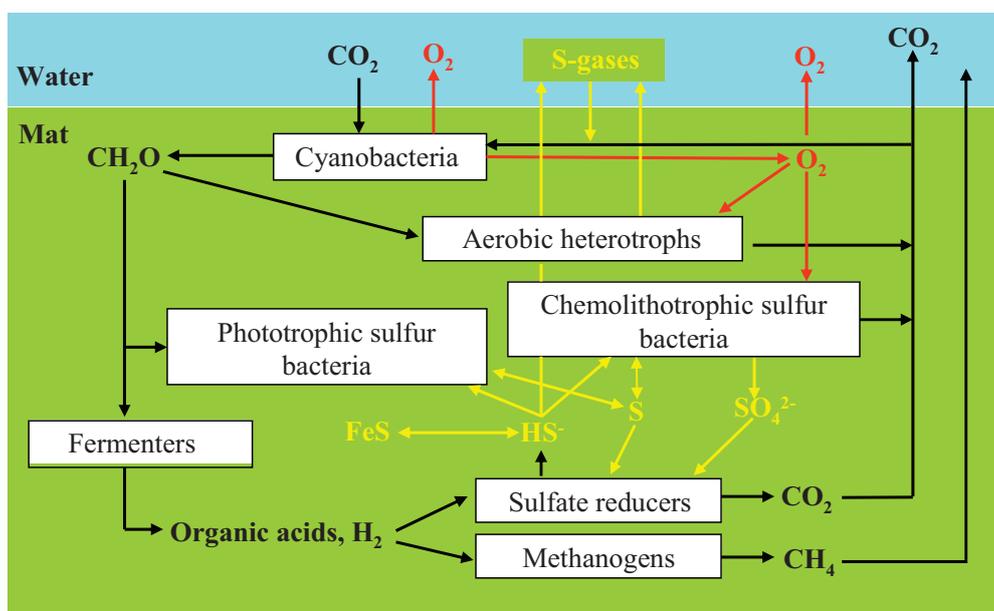


Figure 2. Biogeochemical cycling in a microbial mat (Fenchel and Findlay, 1995; modified)

The upper layer of hypersaline microbial mats are mostly populated by cyanobacteria and diatoms as the main primary producers, driving the activities of the remaining microbial community by performing photosynthesis, which results in the production of oxygen and organic carbon. AHP respire the excretion-, lysis-, and decomposition products of cyanobacteria, leading to oxygen depletion and regeneration of CO_2 , which is subsequently taken up by autotrophs.

Additionally, green non-sulfur bacteria (i.e. *Chloroflexi*) are often found in close neighborhood to cyanobacteria, which support photoheterotrophical growth due to the release of organics (Pierson and Castenholz, 1992). However, *Chloroflexi*-like bacteria are known as nutritionally versatile organisms, being also able to grow photoautotrophically, by performing anoxygenic photosynthesis, or chemoheterotrophically (Pierson and Castenholz, 1992, Van der Meer et al., 2003). Thus, they can also be found in deeper layers of microbial mats. Colorless and purple sulfur bacteria, which usually form the layer below the cyanobacteria, can reoxidize sulfide, which inhibits cyanobacteria, to sulfate. In the bottom layers, sulfate-reducing bacteria produce sulfide using residual organic compounds (e.g. fermentation products). However, several studies showed that sulfate-reducing bacteria can also be abundant and active in oxygen saturated zones of microbial mats (Canfield and Des Marais, 1993; Minz et al., 1999; Jonkers et al., 2005; Ley et al, 2006; Fourçans et al., 2008).

2. Cyanobacteria and aerobic heterotrophic prokaryotes in microbial mats- Autotrophy and Heterotrophy

Cyanobacteria possess the ability to synthesize chlorophyll *a* (Chl *a*) and perform oxygenic photosynthesis. Cyanobacteria also synthesize the phycobilin pigment phycocyanin, which gives them a bluish color and hence, they were earlier known as blue-green algae (Whitton and Potts, 2000). Cyanobacteria are morphologically diverse. They may be unicellular or filamentous and may occur as single cells or grouped in colonies (Whitton and Potts, 2000). As the first oxygenic phototrophs, being potentially responsible for the transition from anaerobic to aerobic life, cyanobacteria have had a profound biogeochemical impact on the earth's biosphere (Paerl, 1996). However, Ettwig and colleagues (2010) recently provided evidence for a new pathway of oxygen production, besides photosynthesis, chlorate respiration and detoxification of reactive oxygen species. They showed that *Methyloirabilis oxyfera* forms oxygen out of two molecules of nitrite without using light. The oxygen is further used to oxidize methane. These findings indicate that oxygen could have been available to microbial metabolism before the evolution of oxygenic photosynthesis (Ettwig et al., 2010).

Cyanobacteria are metabolically versatile, possessing the capability to switch from one mode to another (Stal, 1995). Many cyanobacteria are able to fix atmospheric nitrogen. In most well-oxygenated environments, this takes place inside the heterocyst (Wolk et al., 1994), a thick-walled cell without an oxygenic photosystem (Donze et al., 1972; Fay, 1992). Few cyanobacteria are able to fix nitrogen under well-oxygenated conditions even without a heterocyst, but this ability becomes widespread under micro-oxic conditions (Stal, 2000), since the enzyme needed for nitrogen fixation (i.e. nitrogenase) is inhibited by high oxygen concentrations. Furthermore, some cyanobacteria are also capable of photoheterotrophy, chemoorganotrophy, sulfide-

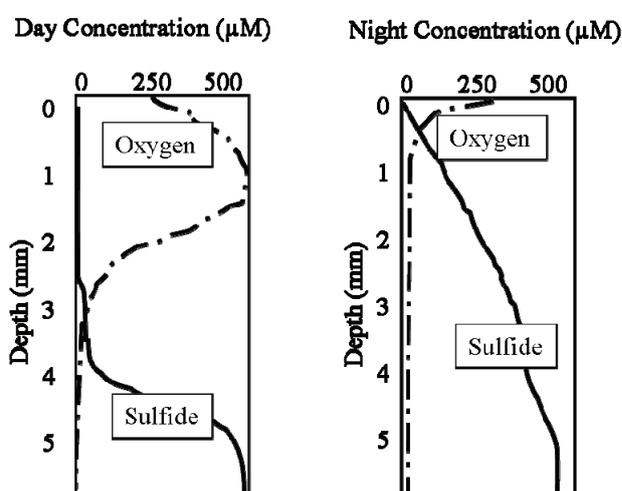


Figure 3. Simplified scheme of diel fluctuations of oxygen and sulfide concentrations in a laminated microbial mat (Van Gernerden, 1993; modified)

Cyanobacterial photosynthesis results in oxygen super-saturation during the day, while at night, anoxic conditions prevail due to continued activity of heterotrophic microorganisms (Jørgensen et al., 1983; Van Gernerden, 1993; Wieland and Kuehl, 2000) (Fig. 3). Cyanobacteria and diatoms mainly contribute to the autotrophic activity in the uppermost parts of mats performing oxygenic photosynthesis (Stal, 1995). However, colorless sulfur bacteria, nitrifiers, methanogens and some sulfate-reducing bacteria perform chemoautotrophy (Madigan et al., 2006). As a result of autotrophy, organic compounds are produced by the carbon-assimilation process and can be released into the environment. Some studies have shown that these compounds can be assimilated and

dependent anoxygenic photosynthesis, and fermentation. Thus, they are successful in a wide range of environments and possess different adaptation strategies to changing environmental conditions (Cohen et al., 1986; de Wit and Van Gernerden 1987; Stal, 1995).

In addition to cyanobacteria, the upper few millimeters in microbial mats are dominated by AHP. This zone represents the biologically most active layer with respect to carbon cycling.

recycled by heterotrophic bacteria (Bauld and Brock, 1974; Bateson and Ward, 1988; Epping et al., 1999; Fig. 4). These excretion products include low molecular weight compounds and EPS. EPS excretion by cyanobacteria was found to be stimulated under conditions of nutrients limitation, high salinity, and desiccation (Myklestad et al., 1989; Staats et al., 2000). EPS is typically composed of polysaccharides, lipids, proteins, and DNA in the form of heteropolymers, such as lipopolysaccharides or glycoproteins (Decho, 1990; Flemming and Wingender, 2001), thus serving as a source of diverse organics for heterotrophs. Bateson and Ward (1988) have shown that cyanobacterial exudates mainly consist of glycolate, the main product of photorespiration. Due to the utilization of soluble photosynthates by aerobic heterotrophs, respiration in the light is

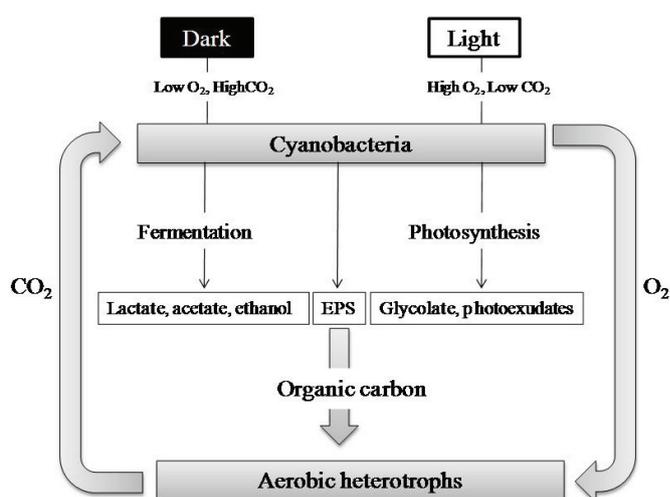


Figure 4. Scheme of proposed carbon cycling in the oxic zone of a cyanobacterial mat. Cyanobacteria perform photosynthesis during daytime and fermentation during night producing organic compounds that can be respired by aerobic heterotrophic microorganisms. The carbon dioxide produced by respiration can be consumed by cyanobacteria for photosynthesis (Bateson and Ward, 1988; from Abed et al., 2010).

higher than in the dark (Bateson and Ward, 1988; Glud et al., 1992; Paerl et al., 1993). Additionally, fermentation products such as acetate, propionate, lactate, or ethanol (Anderson et al., 1987; Jørgensen et al., 1992; Stal, 1995; Nold and Ward, 1996; Stal and Moezelaar, 1997) may provide additional soluble carbon substrates for aerobic heterotrophs and other bacteria. Furthermore, AHP are presumably involved in the utilization of complex, mostly polymeric carbon compounds of

dead cyanobacterial cells (Stal, 1995). However, not only AHP, but also other functional groups, such as anaerobic primary degraders (e.g. polymer degraders), sulfate-reducing bacteria, and phototrophic (anoxygenic) as well as chemotrophic (aerobic) sulfide oxidizers could be nourished by the organics produced by mat autotrophs.

The relevance of AHP for the carbon cycle in microbial mats has been evident for many years. However, most progress in research of mats has been made on cyanobacteria

and bacteria of the sulfur cycle, whereas AHP have been treated merely as a "bulk community". Hence, insights into the identity, diversity, and individual function of AHP in microbial mats are still scarce. Moreover, the complex interactions between AHP and cyanobacteria are not well understood.

3. Intertidal microbial mats of Abu Dhabi

Intertidal flats are the interface between the oceanic and terrestrial environments. As such, they are characterized by frequent fluctuations in temperature, salinity, desiccation, UV-irradiation, and wave action, causing physical and biochemical stress to the resident microorganisms (Decho, 2000).

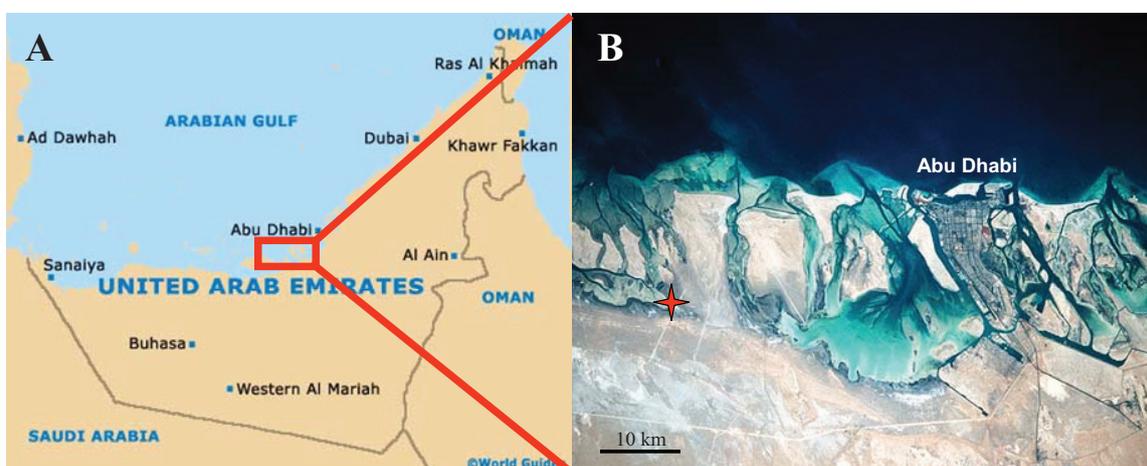


Figure 5. Maps showing the sampling site of the studied microbial mats along the Arabian Gulf coast of Abu Dhabi. (A) Map of the United Arab Emirates (UAE); Image ©World Guides, and (B) Satellite view from the coast of Abu Dhabi; Image ©NASA.

The Arabian Gulf is considered as a unique model area to study marine carbonate sedimentation under extreme arid conditions (Kendall and Skipwidth 1969; Purser 1973; Gischler and Lomando, 2005). The intertidal flats of the Arabian Gulf (Fig. 5) harbor various types of microbial mats that experience different extreme environmental conditions (Kendall and Skipwidth 1968; Golubic, 1991; Abed et al., 2008). Microbial mat zonation was first studied in Khor Al Bazam lagoon west of Abu Dhabi (Kendall and Skipwidth 1968) and later in the more protected lagoons Northeast of the city (Kinsman and Park 1976). These studies investigated the contribution of microbial mats to sedimentary processes. In this context, the dynamics of microbial settlement and mat differentiation were discussed (Golubic, 1991).

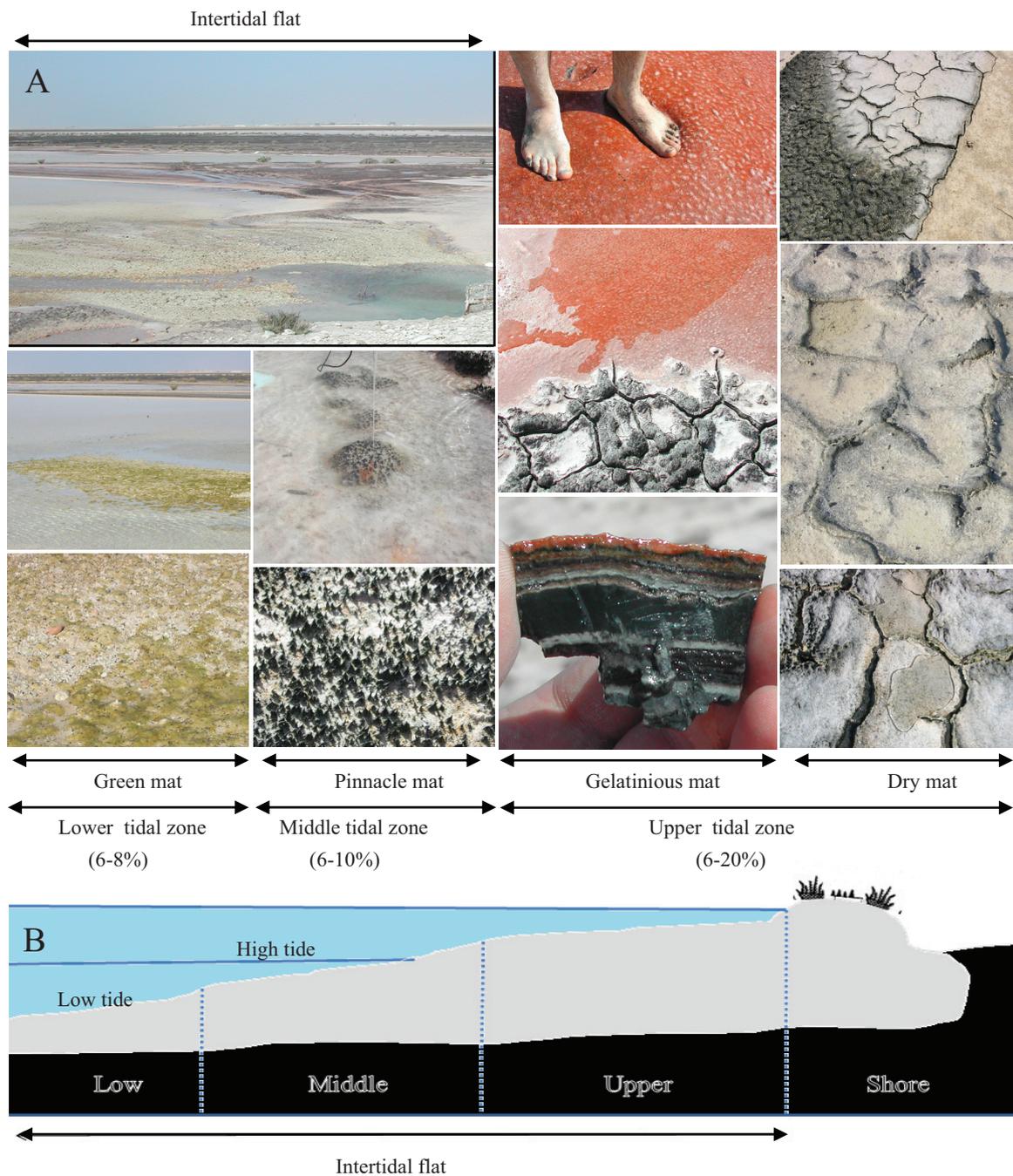


Figure 6. (A) Photographs showing the landscape of microbial mats in the intertidal flat of Abu Dhabi. The mat samples collected from the lower, middle and upper tidal zone vary in texture, color and morphology. (B) Scheme shows the relative position of the mats with respect to tides. Note that the mats are exposed to different salinity fluctuations (in brackets) depending on their tidal position (modified from Abed et al., 2006 and 2008).

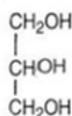
This intertidal zone contains a variety of microbial mats, possessing remarkable morphological differences, due to differences in environmental settings, caused by the tidal regime and the coastal topography (Golubic, 1991; Abed et al. 2008). At the lower intertidal zones, flat and inundated mats with different colors (green, olive, and red) are abundant (Fig. 6; Green mat), whereas pinnacle structures, in the form of upright pointed cones, appear at middle tidal ranges (Fig. 6; Pinnacle mat). At upper tidal zones, parts of the mats are exposed to desiccation, resulting in dry, polygon cracked mat structures (Fig.6; Dry mat). The other parts lie in channels, which are formed throughout the entire mat system, as a result of tidal activities. These mats are permanently covered with seawater, but are set out to extreme salinity fluctuations. They are finely laminated, orange-reddish, and have an extremely gelatinous surface (Fig. 6; Gelatinous mat), which might be caused by an increased productions of EPS. They are flooded with seawater during high tide, whereas some of them are exposed to air and desiccation during low tide. Due to the tidal regime, salinity fluctuates from 6 to 20‰ at a diurnal cycle depending on the mat's tidal position. Hence, the mats are always subjected to hypersaline conditions. Temperature increases up to 55°C in summers and the climate is very dry. The mats are exposed to intense light intensities, leading to high evaporation rates of seawater and desiccation. Air temperature seasonally changes from 15 to 47 °C, temperature of lagoon waters from 15 to 40°C and temperature of the microbial mat surfaces from 15 to 50°C. Additionally, significant temperature variations occur on a daily scale ($\Delta 5^{\circ}\text{C}$ to $\Delta 15^{\circ}\text{C}$). Rainfall is sporadic, about 40 to 60 mm year⁻¹, as compared with evaporation rates of about 1500 mm year⁻¹ (Kinsman and Park, 1976).

All the different types of microbial mats of Abu Dhabi intertidal flats experience combined stress by the simultaneous effect of multiple extreme environmental factors of salinity, salinity fluctuations, temperature, UV and light intensity, and desiccation. In spite of the various and unique environmental conditions of the Arabian Gulf, the diversity and function of mats' microorganisms are poorly investigated and previous studies on this mat system were focused on the geology and landscape (Kendall and Skipwith, 1968, 1969; Purser, 1973; Kinsman and Park, 1976).

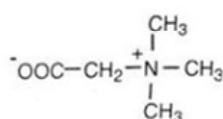
4. Salinity adaptation strategies of microorganisms in hypersaline microbial mats

The microbial mats studied in this thesis experience an array of harsh environmental conditions, most importantly high salinities and strong salinity fluctuations. Consequently, the microorganisms inhabiting these mats need to tolerate highest concentrations of salt, and thus most of them likely belong to halotolerant or even to halophilic microorganisms. Halophiles are defined as organisms that show considerable growth at salt concentrations higher than 10% (Oren, 2002). Salt stress does not only consist of ionic and osmotic stress. Moreover, e.g. oxygen and water activity (water availability) decrease with increasing salinity. Halophilic microorganisms are found in all three domains of life: Archaea, Bacteria, and Eucarya (Oren et al., 1999). However, at salinities exceeding 1.5 M, prokaryotes predominate and eukaryotes become scarce (Grant et al., 1998). It was also shown that bacterial halophiles are abundant up to 2 M sodium chloride,

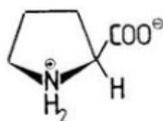
Glycerol



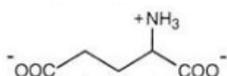
Glycine betaine



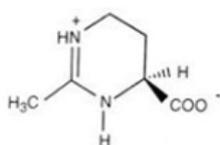
L-Proline



β -Glutamate



Ectoine



Trehalose

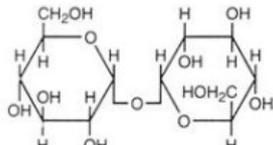


Figure 7. A selection of organic osmotic solutes found in halophilic and halotolerant prokaryotic and eukaryotic microorganisms (modified from Oren, 2008).

belong to polyols, sugars, heterosidas, betaines, thetines, amino acids, glutamine amide derivatives, N-acetylated diamino acids and ectoines (Oren, 2002; Fig. 7). (2) Some

whereas Archaea are dominant at higher salinities (Rodriguez-Valera et al., 1981). However, e.g. many cyanobacteria can dominate hypersaline microbial mats, often exposed to salinities above 2 M.

So far, two mechanisms are known of how halophilic microorganisms avoid water loss by osmosis at high salinities: (1) Some halophiles and halotolerant microorganisms produce and accumulate low-molecular-weight compounds, so called compatible solutes, which have an osmotic potential. These compounds are normally highly polar, highly soluble, uncharged, or zwitterionic (Reed et al., 1986). The compatible solutes possess a great structural diversity and

halophilic microorganisms (mainly Haloarchaea and *Salinibacter ruber*) can accumulate up to 5 M KCl in their cells. To this category belong “extreme halophiles”, which not only tolerate, but require about 10 to 15% NaCl for survival and 20% for growth (Lanyi, 1974). Most of their enzymes work only at high salt concentrations, and can even denature at lower salt concentrations.

5. Methods

The main focus of microbial ecology is to understand the structure and function of microbial communities and their dynamics with regard to biotic and abiotic changes in the environment. In this thesis, we used a polyphasic approach in order to circumvent the limitations of each technique. The information from different techniques, such as microscopy, cultivation, hyperspectral imaging, microsensor measurements, and 16S rRNA based cultivation-independent tools (denaturing gradient gel electrophoresis (DGGE), quantitative PCR (qPCR), cloning, DNA-stable isotope probing (DNA-SIP), fluorescence in situ hybridisation (FISH), catalyzed reporter deposition (CARD)-FISH) were combined to complement and support each other.

5.1 Molecular tools

The 16S rRNA approach has greatly deepened our knowledge about the diversity, abundance, and distribution of microorganisms in a variety of environments. The 16S rRNA genes occur in all microorganisms in high numbers and possess the same function. They have conserved and variable regions, allowing the identification on different phylogenetic levels (Woese and Fox, 1977). The diversity of retrieved 16S rRNA sequences does unlikely represent the real diversity of a sample, due to PCR and cloning biases. However, PCR-based methods are still the most frequently used techniques to study complex microbial communities from environmental samples. Phylogentic analysis of obtained 16S rRNA gene sequences reveals information about potential metabolic functions of these microorganisms, if they are closely related to isolated species. Furthermore, sequence information can be used to select existing probes, or design new specific probes for FISH quantification of microorganisms and estimation of their abundance (Fig. 8).

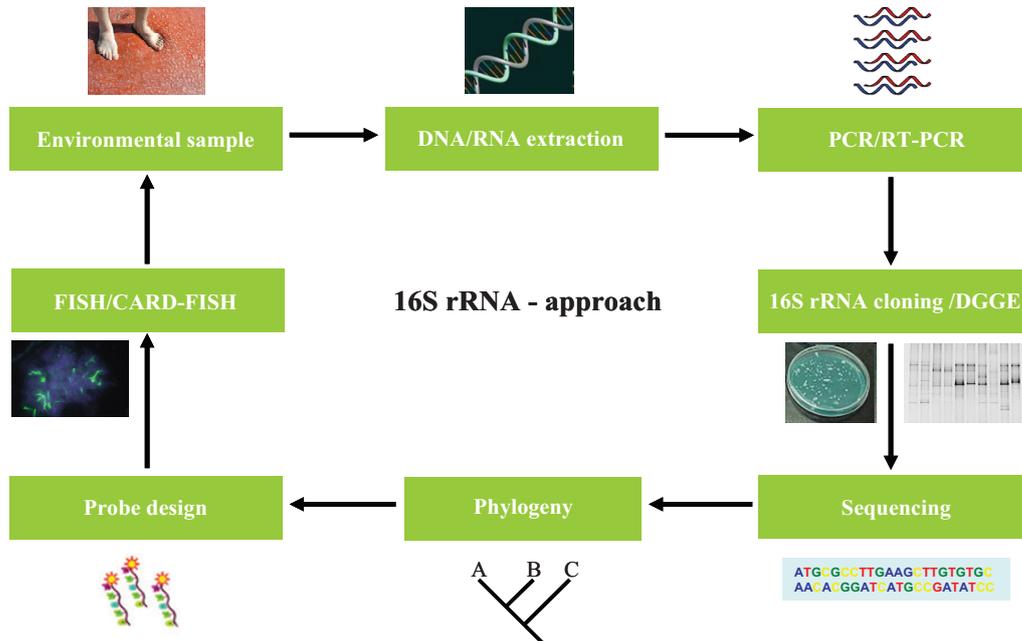


Figure 8. Major steps of the 16S rRNA approach. DNA or RNA from an environmental sample is extracted, amplified and analyzed using DGGE or cloning. Obtained sequences can be used to select or design probes for FISH quantification of specific community members (modified from Amann et al., 1995).

5.2 Microsensors

Microsensors are optimal tools for studies on the microenvironmental level, since they enable fine-scale measurements and quantification of physicochemical gradients, as found in e.g. in microbial mats. They have a tip, which is typically smaller than 20 μm and have a spatial resolution of at least 0.1 mm (Kühl and Revsbech, 1998). They are minimal invasive and the disturbances of the sample and its physical and chemical environment are negligible, because of their small tip size. Three different types of microelectrodes can be distinguished, depending on the measuring principle: amperometric, potentiometric, and voltametric sensors (Taillefert et al., 2000; Kühl and Revsbech, 2001).

In this study, amperometric sensors for measurements of oxygen were used. In these sensors, the analyte is reduced by applying a polarising voltage, and the resulting current is linearly proportional to the concentration of the analyte (Fig. 9).

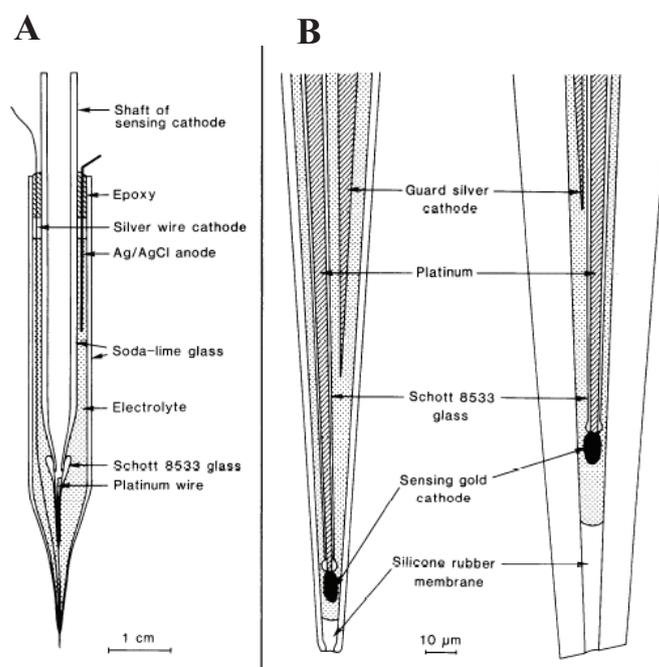


Figure 9. (A) Scheme of an amperometric Clark-type oxygen microsensor, (B) Tip of the microsensor (modified from Revsbech, 1989)

6. Objectives of this thesis

In this thesis, the diversity of cyanobacteria and AHP and the role of their key processes (i.e. oxygenic photosynthesis and respiration, respectively) in carbon cycling in the uppermost layer of different mats were studied using cultivation, molecular and microsensor techniques. This study was initiated to obtain insights into the composition, function, and adaptation strategies of microbial communities of morphologically different microbial mats along an intertidal flat of Abu Dhabi. The study was confined to the upper few millimetres in order to increase our knowledge of AHP, a group that is so far largely overlooked in microbial mats. Furthermore, due to their close association to cyanobacteria, this thesis aimed at investigating the relationship between the two groups.

Following questions were addressed:

- *How is the behavior, function, and diversity of mats' microorganisms affected by extreme and fluctuating environmental conditions, mainly salinity (publications 1, 2 and 3)?*
- *Who are the key players of the carbon cycle in the oxic part of the mats (publications 2, 4 and 5)?*
- *How do cyanobacteria and aerobic heterotrophs interact with respect to carbon cycling (publication 5)?*

B Results and Discussion

In the following section, the results of each study of this thesis are summarized, including those which have been published and those in preparation. At the end of this section, the data are discussed in a broader context, and an outlook for future research is given.

1. Effects of salinity on microbial behavior, metabolism, and community structure

The intertidal mats of Abu Dhabi are subjected to harsh and partly fluctuating environmental conditions, due to tidal regime. One main objective of this thesis was to investigate the effect of mainly hypersaline conditions, and salinity fluctuations on microbial behavior, metabolism and community structure. All studies focused on the upper oxic part of the mats, because of the relevance of the microorganisms in this layer in carbon cycling (see section 2.).

1.1 Salinity-driven migration of cyanobacteria – “Halotaxis”

The gelatinous mat from the upper tidal zone, which is exposed to very strong salinity fluctuations from 6 to 20‰, exhibited a reversible change in its surface color within several hours upon changes in salinity of the overlying water. The mat surface was orange-reddish at salinities above 15‰ and turned dark green at lower salinities. A microbial mat from Baja California showed a similar color change, which was thought to be either due to physiological (photopigment) changes in a stable bacterial community or a change in the community composition (Bebout et al., 2002).

Several different physico-chemical parameters such as ionic composition, pH, specific gravity, surface tension, specific heat, viscosity, conductivity, gas solubility, gas diffusion coefficients, and water activity are affected by changes in salinity (Javor, 1989; Garcia-Pichel et al., 1999). Thus, the main objectives of this study were (i) to find out the reason for the color change in our mats and (ii) to test whether the color change is caused by salinity directly or indirectly.

Filaments of *Microcoleus chthonoplastes*, identified based on morphology and 16S rRNA sequencing, were found to migrate up and down when salinity was decreased

below or increased above 15%, respectively, causing the color change of the mat's uppermost layer (Fig. 1). Further experiments (HPLC, fluorescence microscopy, hyperspectral imaging, absorption spectroscopy, and oxygen microsensor measurements) provided further evidence for the migration of *Microcoleus chthonoplastes* filaments in

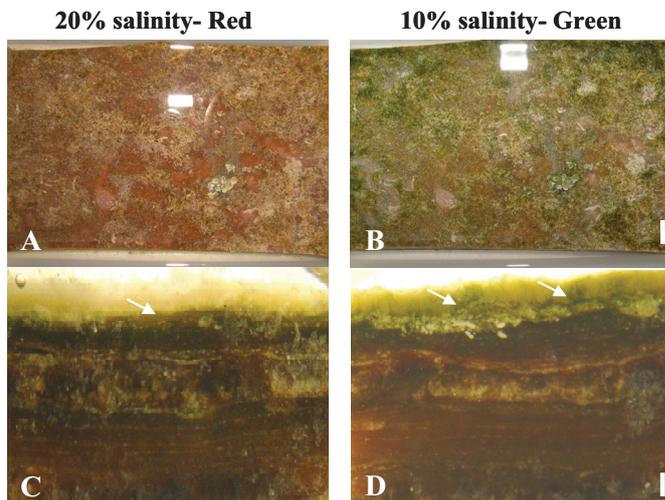


Figure 1. Photographs and photomicrographs of the mats incubated at the overlying water salinities of 20% (red mat, left) and 10% (green mat, right). (A-B) Images of the surface of the same mat sample, photographed from the top; (C-D) Cross-sections of the mats showing green filaments that moved towards the surface (indicated by arrows). The scale bar corresponds to 1 cm and 1 mm for panels (A-B) and (C-D), respectively. (modified from Kohls et al. 2010; publication 1).

response to salinity changes. No indication for a physiological change on a cellular level in the microbial populations that could explain the color change was found. Moreover, a series of migration experiments were carried out and led to the conclusion that the observed migration was most likely due to a direct response to ionic stress. Water activity, oxygen solubility, upward flux of sulfide, surface tension, ionic composition, gravity or light were excluded as triggers. Furthermore, measuring

and modeling of salinity dynamics inside the mat indicated that the migration was a response to salinity gradients.

The clear stress response of *Microcoleus* filaments at salinities higher than 15% was already shown in previous studies on Guerrero Negro mats, which revealed a salinity tolerance of *Microcoleus chthonoplastes* between 6% and 12%, being replaced by other cyanobacteria (*Phormidium*- and *Synechococcus*-related) at salinities higher than 15% (Javor, 1989; Rothschild, 1991; Des Marais, 1995). Further, isolates of *Microcoleus chthonoplastes* were shown to tolerate up to 12% salinity (Karsten, 1996).

Migration of cyanobacteria in microbial mats, desert soils, and mudflats was previously shown to be induced by a variety of factors, including light intensity (Bebout and Garcia-Pichel, 1995; Kruschel and Castenholz, 1998; Nadeau et al., 1999), diel cycle (Richardson and Castenholz, 1987; Garcia-Pichel et al., 1994; Fourçans, 2006), different

oxygen concentrations (Whale and Walsby, 1984), upwards fluxing sulfide (Richardson and Castenholz, 1987; Abed et al., 2006), and wetting (Brock, 1975; Garcia-Pichel and Pringault, 2001; Pringault and Garcia-Pichel, 2003). In this thesis, migration of cyanobacteria in a microbial mat induced by salinity changes is firstly described. We propose to term this salinity-driven cyanobacterial migration as “halotaxis”, a process that might play a vital role in the survival of cyanobacteria in environments exposed to continuous salinity fluctuations, such as intertidal flats.

1.2 Effect of salinity on oxygen consumption and photosynthesis

In this section, the effects of salinity fluctuation on rates of oxygen consumption (in the light and in the dark) and gross photosynthesis were described in three mats along the intertidal flat of Abu Dhabi. From the lower to the upper intertidal zone, the conditions became more extreme, especially with respect to salinity and salinity fluctuations. Mats

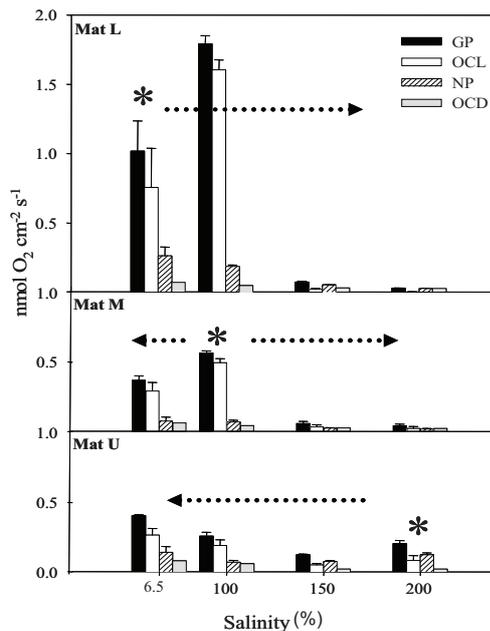


Figure 2. Salinity dependence of areal rates of gross photosynthesis (GP), net photosynthesis (NP), and oxygen consumption in the light (OCL) and in the dark (OCD), in three microbial mats from lower (L), middle (M) and upper (U) tidal positions. The rates marked with asterisks were obtained from measurements at the mats' original salinities during low tide. The arrows indicate the direction of experimental salinity shift. (from Abed et al., 2007; Publication 2)

from the lower, middle, and upper tidal zone were incubated at different salinities (6.5, 10, 15 and 20%) and rates of gross photosynthesis and oxygen consumption were measured with oxygen microsensors (Fig. 2).

At their natural salinities, gross photosynthesis and oxygen consumption of the mats in the light, decreased from the lower to the upper tidal zone, which was probably due to the increasing salinity level. The mats from the lower and middle tidal zone exhibited their optimum rates of photosynthesis and respiration at 10% salinity. However, irrespective of their tidal location, all mats exhibited a decrease in areal gross photosynthesis, as well as light and dark oxygen consumption rates at salinities above 10%. However, the extent of inhibition of these processes at higher salinities was lower in the

mat from the upper tidal zone. These results suggest an increase in salt adaptation of the mat microorganisms from lower to higher tidal mats.

In conclusion, the different salinity regimes of the Abu Dhabi mats seem to influence the rates of photosynthesis and respiration, as well as the halotolerance of microbial communities.

1.3 Effect of salinity on microbial diversity

The effect of different salinity regimes on the microbial diversity in the oxic zone of these mats was examined using denaturing gradient gel electrophoresis (DGGE) and 16S rRNA cloning. Differences in the bacterial composition were correlated to their tidal position (low, middle, high) and *in situ* salinity. The obtained sequence information in this study constitutes the first insights into the microbial diversity of Abu Dhabi mats.

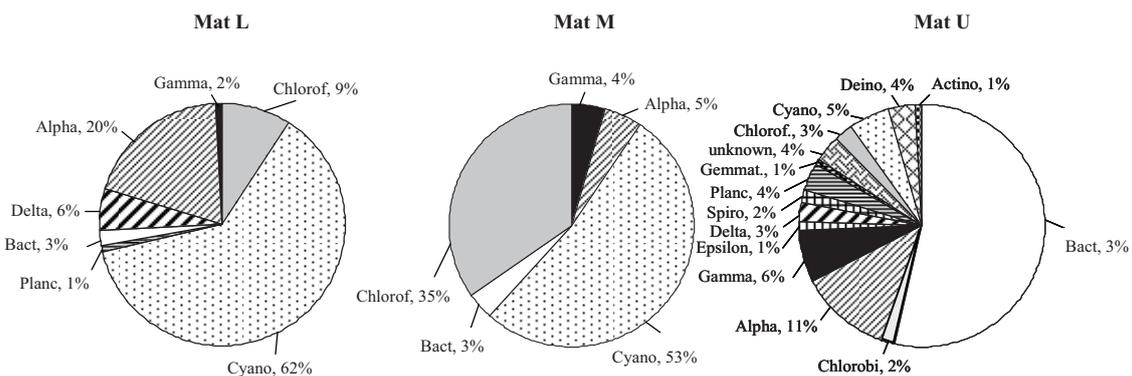


Figure 3. Comparison of the bacterial community composition in three mats collected from lower (L), middle (M) and upper (U) tidal transects as revealed by 16S rRNA cloning. Shown fractions indicate the relative percentage to the total number of clones. Alph: *Alphaproteobacteria*; Gamma: *Gammaproteobacteria*; Beta: *Betaproteobacteria*; Delta: *Deltaproteobacteria*, Epsilon: *Epsilonproteobacteria*, Bact: *Bacteroidetes*; Chlorof; *Chloroflexus*, Planc; *Planctomycetes*, Cyan; *Cyanobacteria*, Actino; *Actinobacteria*, Deino; *Deinococcus*, Gemmat; *Gemmatimonas*, Spiro; *Spirochetaetes* (from Abed et al., 2007; Publication 2).

The DGGE patterns, as well as the obtained sequences and the number of unique operational taxonomic units (OTU), showed clear differences in the mats' bacterial composition. Interestingly, the mat from the upper intertidal zone possessed the highest bacterial diversity among the studied mats although it was set out to the most extreme environmental conditions, e.g. salinity fluctuations from 6 to 20% (Fig. 3). This might partly be explained by the intermediate disturbance hypothesis (Buckling et al., 2000; Johst and Huth, 2005), assuming that disturbances which are neither too rare nor too

frequent (in this case salinity fluctuation), may not affect the resistant fraction of the original community, but may promote the growth of halophilic bacteria, thus leading to an increased biodiversity. Indeed, in the mat from the upper tidal zone, exposed to highest salinities, more halophilic bacteria, such as e.g. *Salinibacter ruber* and *Halothece* sp. were found, explaining the increased salt adaptation regarding photosynthesis and respiration of this mat (see section 1.2). Furthermore, the extreme conditions at the upper tidal zone might provide more ecological niches for other extremophiles, which require these conditions for growth.

Most of the 16S rRNA gene sequences obtained from the mats grouped with ones of *Bacteroidetes*, cyanobacteria, *Chloroflexi*, *Proteobacteria*, *Deinococci*, *Planctomycetes*, *Actinobacteria*, *Chlorobi*, *Spirochaetes* and *Gemmatimonas* groups. The most dominant cyanobacterial sequences belonged to *Microcoleus chthonoplastes* and *Lyngbya aestuarii*. The number of cyanobacterial sequences decreased from the lower to the upper tidal mat, whereas other bacterial groups, such as *Chloroflexi* and *Bacteroidetes*, showed an opposite pattern with the highest dominance in clone libraries of the middle and upper tidal mats, respectively. The decrease in cyanobacterial abundance was further confirmed by chlorophyll *a* concentrations (Chl *a*), which decreased from the lower to the upper tidal mats. Hence, the decrease in photosynthetic activity at increasing salinity correlates well with reduction in cyanobacterial abundance and diversity (see section 1.2). In contrast, the diversity and abundance of aerobic heterotrophic bacteria correlated negatively with the rates of oxygen consumption in the light. Probably, all respiring bacterial populations survived at 20% salinity, but only the halophiles were active. The remaining microorganisms may become active when salinity lowers during high tide. Additionally, under salt stress, accumulation of extracellular polymeric substances (EPS) could attract degradation-specialists of these compounds, such as *Bacteroidetes* (Kirchmann, 2002).

Our results also suggested adaptation strategies of the mat microorganisms to the extreme environmental conditions of temperature, water availability, UV and light intensity, represented by the increased amounts of unsaturated fatty acids and the trans/cis ratio of the cyanobacterial fatty acid n-18:1 ω 9 in the upper tidal mats, the detection of diverse desiccation-tolerant cyanobacteria, such as *Microcoleus*, *Lyngbya*, and

Chroococciopsis, the increase in extracellular EPS from lower to upper tidal mats and also the presence of the sunscreen pigment scytonemin in all mats.

We conclude that the topology of the coast and the extreme environmental conditions across the tidal flat determine the microbial diversity and community composition as well as the adaptation capacities of microorganisms.

2 Interaction between cyanobacteria and aerobic heterotrophs

The second main objective of this thesis was to study the diversity and abundance of aerobic heterotrophic prokaryotes (AHP) in the hypersaline microbial mats of Abu Dhabi and the interaction between the two groups with respect to carbon cycling. AHP are largely overlooked in microbial mats, where most previous research was focused on e.g. cyanobacteria and bacteria involved in sulfur cycling. To obtain cyanobacteria in axenic cultures has been, and still is, a major challenge due to firmly attached AHP. This close association of cyanobacteria and AHP in cultures and in the environment (e.g. microbial mats) signifies a strong interaction. These relationships can be competitive for scarce resources, or mutualistic (Paerl, 1996).

2.1 Diversity and abundance of aerobic heterotrophic prokaryotes in hypersaline microbial mats

The gelatinous mat from the upper intertidal zone (equates to “mat U” in section 1.2 and 1.3) was further investigated with respect to the structure of their AHP communities. This mat hosted a novel and unique diversity of AHP, since many of the detected sequences had no close relatives. Sequences related to ones of cyanobacteria, *Chloroflexi*-related bacteria, *Bacteroidetes*, *Proteobacteria*, *Haloarchaea*, and *Crenarchaeota* dominated the clone libraries. Among these groups, many extremophilic bacterial and archaeal genera, such as e.g. *Deinococcus*, *Salinibacter*, and *Halobacteria* were found. As revealed by quantitative PCR, the AHP in the upper part of this mat consisted of bacteria and archaea in a 10:1 ratio, indicating that archaea are also important in the upper part of this mat. A dominance of reddish *Chloroflexi*-like bacteria in close proximity to bundles of *Microcoleus chthonoplastes* filaments (Fig. 4) was detected using CARD-FISH with *Chloroflexi*-specific probes (GNSB-941 and CFX-1223) on 5µm thin cross-sections of

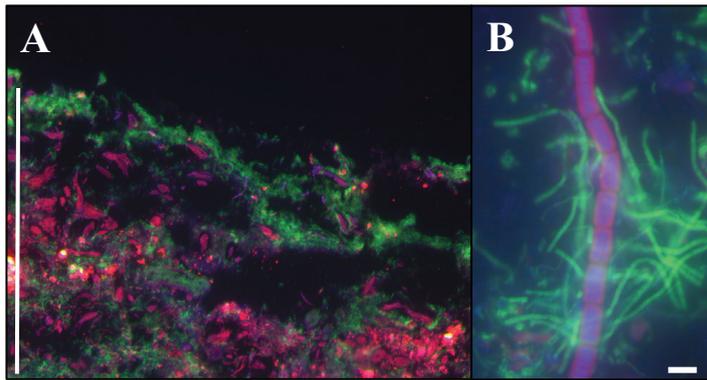


Figure 4. CARD-FISH of 5 µm thick cross-section of the microbial mat using the probes (CFX-1223 and GNSB-941) specific for *Chloroflexi* (A) green color shows probe signal; pink color shows cyanobacterial autofluorescence; blue color shows DAPI signals of the remaining cells; (B) a closer view of the same cross-section. Scale bar corresponds to 500 µm and 5 µm, respectively. (modified from Kohls et al., in preparation; Publication 4)

the oxic part of the mat. This suggests a strong relationship between both groups. Previous studies have shown that such bacterial communities perform a transfer of photoautotrophically fixed carbon from cyanobacteria featuring a symbiotic association (Nold et al., 1996; Ward et al., 1998). Ley and colleagues (2006) suggested a co-metabolism of sulfur, in which *Chloroflexi* may draw down

levels of H₂S stressful for the cyanobacteria, which excrete organics used by *Chloroflexi* bacteria. However, they further found that *Microcoleus* filaments were often disrupted in the presence of *Chloroflexi*, indicating that they may also parasitize cyanobacteria under H₂S stress.

Interestingly, the archaeal clone library presented a high number of sequences affiliated with *Crenarchaeota* belonging to marine benthic group B (MBGB). Although *Crenarchaeota* are widespread in marine habitats (Vetriani et al., 1999), their functions are still unknown (DeLong, 1992; Karner et al., 2001; Schouten et al., 2002; Knittel et al., 2005), but it was speculated that they could be involved directly or indirectly in anaerobic oxidation of methane (AOM) (Knittel et al., 2005; Biddle et al., 2006) or sulfate reduction (Torres et al., 2002), since MBGB were also often found at methane seep sites. However, in the present and in other studies, they were also found in the oxic part of hypersaline microbial mats (Jahnke et al., 2008; Robertson et al., 2009). Thus, also an aerobic heterotrophic metabolism is conceivable.

In spite of the harsh environmental conditions at the intertidal flat of Abu Dhabi, a rich and novel prokaryotic diversity was detected in its microbial mats. The detected potential AHP live in close association with cyanobacteria and hence might play an

important role in carbon cycling, which can be further studied by employing physiological experiments, such as e.g. DNA-stable isotope probing (DNA-SIP).

2.2 DNA-SIP to identify key aerobic heterotrophic prokaryotes involved in carbon cycling using an intact microbial mat

DNA-SIP was applied on an intact microbial mat, incubated under an atmosphere enriched in $^{13}\text{CO}_2$, in order to follow the carbon flow from cyanobacteria to AHP. This study was confined only to bacteria, but not archaea.

Considerable amounts of microbial mat DNA were not ^{13}C -labeled under our incubation conditions. However, bacterial 16S rRNA gene targeted terminal restriction fragment length polymorphism (T-RFLP) fingerprinting revealed clear differences between non-labeled (“light”) and labeled (“heavy”) DNA gradient fractions. Using T-RFLP, a specific ^{13}C -labeling of the populations represented by the 492 bp (*Cyanothece* sp.) and 201 bp terminal restriction fragments (T-RFs) were obtained. The latter could not be identified via cloning, most likely due to an insufficient number of screened clones. From two dominating cyanobacterial population present in the mat, only *Cyanothece* sp., but not *Microcoleus chthonoplastes*, showed strong uptake of $^{13}\text{CO}_2$. The results indicate that unicellular cyanobacteria were much more active in terms of $^{13}\text{CO}_2$ assimilation than filamentous cyanobacteria, such as *Microcoleus chthonoplastes*. Unicellular cyanobacteria are known to be extremely halotolerant and often dominate microbial communities in hypersaline environments (Brock, 1976; Walsby et al., 1983; MacKay et al., 1984; Javor, 1989, Garcia-Pichel et al., 1998; Oren, 2002). Hence, they might also be more active at higher salinities compared to filamentous cyanobacteria.

Clone libraries of selected gradients indicated a preferential allocation of certain groups to the “heavy” fractions (e.g. *Chloroflexi*, *Bacteroidetes*, *Alpha-* and *Gammaproteobacteria*). However, this did not correspond to the abundances of terminal restriction fragments (T-RF) detected with T-RFLP, which could be due to PCR and/or cloning biases.

The results illustrated a low resolution and sensibility of our DNA-SIP experiment. RNA-SIP might reveal a higher resolution (Dumont et al., 2006; Whiteley et al., 2006), but RNA could not be extracted from this mat, although different protocols

were tested. Thus, RNA-extraction protocols need to be optimized in order to obtain a higher resolution of the secondary key players in carbon cycling in this mat.

2.3 Cyanobacterial monocultures as model systems

To gain further insights into cyanobacteria-AHP interactions, cyanobacterial unialgal cultures originating from the Abu Dhabi mats, were chosen as model systems. These cultures were investigated with respect to the identity of associated AHP (only bacteria) and the relationship between the two groups.

Molecular analysis of the AHP in these cultures showed that most of the attached bacteria belonged to the *Bacteroidetes* group but few others were related to *Alpha*- and

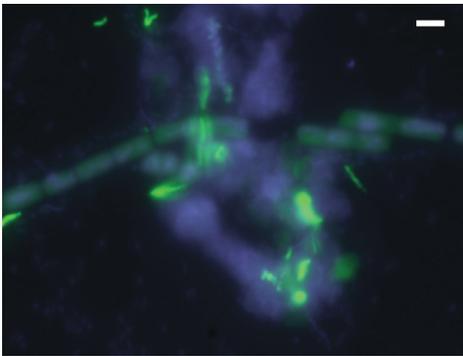


Figure 5. Cyanobacterial culture (*Microcoleus* sp. M7C5) isolated from a mat of Abu Dhabi with associated AHP, visualized by CARD-FISH (probe CF319a; targets *Cytophaga*, *Flexibacter*, *Bacteroidetes*). Scale bar: 2 μ m. Green color shows the probe signal, blue color shows the DAPI signal. Representative photograph of cyanobacterial filament which seems to be cleaved by AHP, belonging to the *Bacteroidetes* group is shown (modified from Kohls et al., in preparation; Publication 5).

Gammaproteobacteria, as well as *Chloroflexi*. *Bacteroidetes* are believed to grow on EPS produced by cyanobacteria. The association between cyanobacteria and *Chloroflexi* seemed to be very important in the studied mats, and requires further investigation. Closely related cyanobacteria isolated from the same mat possessed nearly identical community structures of associated AHP, whereas distantly related cyanobacteria had different accompanied bacteria. Further, closely related cyanobacteria isolated from different mats, varied in their communities of AHP, but also had some bacteria in common. Hence, the community structure of AHP associated with cyanobacteria seems to be species-specific and additionally

depends on the specific environment, from which the cyanobacterium was isolated.

Cultivation of a selected axenic cyanobacterial culture with and without AHP showed that the growth of cyanobacteria was stimulated after addition of AHP. This point out to a significant role of AHP in the growth of cyanobacteria, probably by using photosynthetically produced oxygen and organics and thereby protecting the cyanobacteria from photo-oxidative stress (Abeliovich et al., 1972; Eloff et al., 1976;

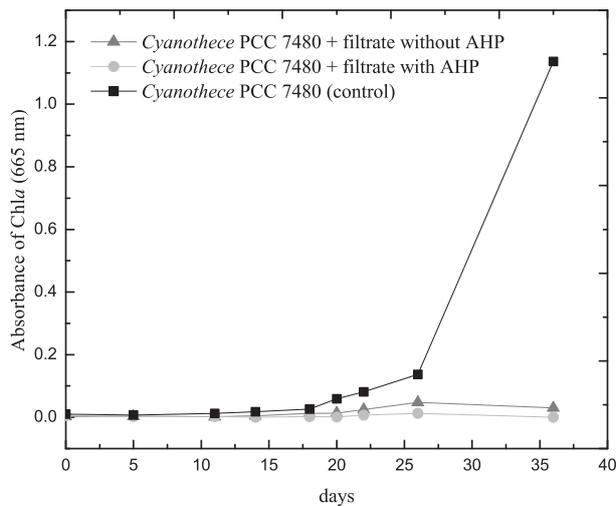


Figure 6. Growth experiments of axenic cyanobacterial culture *Cyanothoece* PCC 7480 with addition of filtrates (with and without AHP) obtained from a non-axenic culture *Cyanothoece* sp. M7CRI (modified from Kohls et al., in preparation; Publication 5).

bacteria were shown, using CARD-FISH (Fig. 5) and a growth experiment, to lyse cyanobacterial filaments and grow on their fragments, similar to previous results from Sallal and colleagues (1994). Interestingly, *Cyanothoece* PCC 7418, as well as three further tested cyanobacterial cultures (*Leptolynbya* PCC 8103, *Xenococcus* PCC 7304, and *Microcoleus* PCC 7420) were inhibited after inoculation with filtrates (with and without associated bacteria) obtained from culture *Cyanothoece* sp. M7CRI (Fig. 6). Additionally, our experiments indicate that the growth inhibiting substance originates from the associated bacteria and not from the cyanobacteria (see publication 5). We thus hypothesize that an inhibiting substance, e.g. an antibiotic, was released by the cyanobacteria-associated AHP into the medium. This might be a protecting strategy for *Cyanothoece* M7CRI, helping to compete against others, whereas the associated bacteria might benefit from cyanobacterial exudates. Hence, our results suggest that the competitiveness of cyanobacteria can be enhanced due to interactions with AHP. Furthermore, the production of bioactive substances, as effective competition and defense strategies by microorganisms represents an important resource for the discovery and development of e.g. new antibiotics.

In conclusion, the interactions between cyanobacteria and AHP are manifold, very complex, highly specific, and cannot be generalized.

Paerl and Kellar, 1978). AHP and cyanobacteria might also provide each other with necessary vitamins and/or other growth factors (Burkholder, 1963; Paerl, 1996). In this thesis, known denitrifying and nitrite reducing bacteria (i.e., *Nisaea denitrificans* and *Nisaea nitritireducens*) and the *nifH* gene (data not shown) were detected in the *Cyanothoece* culture (M7CRI), hinting to a nitrogen cycling between AHP and cyanobacteria. In contrast to these beneficial relationships, *Flexibacter*-like

C Final discussion

Extreme environments such as those with high/low temperatures, high salinity, low water availability, high radiation, high pressure, high/low oxygen concentrations, and high/low pH are considered inhospitable for most organisms. Although it was expected that these environments possess low species diversity, research revealed a high diversity of prokaryotes living under conditions that are generally considered extreme. Typically, as conditions become more extreme, environments become exclusively populated by prokaryotes. While some microorganisms are able to tolerate these harsh conditions (i.e. extremotolerants), others require them for growth (i.e. extremophiles). For example, some organisms are unable to grow below 90°C or in less than 3 M NaCl (Madigan and Oren, 1999). For these organisms, such environments are “normal” and moderate environmental conditions would be rather “extreme”. The high prokaryotic diversity in environments, which are inhospitable for many eukaryotes, demonstrates that the term “extreme” is not easy to delimit, since it strongly depends on the point of view. The seemingly inhospitable environment studied in this thesis with multiple extremes of high salinity, temperature, desiccation, and UV radiation levels supports this statement.

This work aimed at investigating the effect of extreme and strongly fluctuating environmental conditions (mainly salinity) on the microbial community composition, function and adaptation of intertidal microbial mat microorganisms. The focus was on cyanobacteria and aerobic heterotrophic bacteria, because of their key role in carbon cycling in microbial mats.

1. Adaptation strategies of microorganisms to extreme conditions

Microbial cells possess different survival strategies or adaptation mechanisms for different stress factors. In the following, extreme environmental factors and mechanisms how microorganisms cope with these factors are discussed with respect to the environmental conditions present at the intertidal flat of Abu Dhabi, i.e. temperature, UV and light intensities, desiccation, and salinity.

The microorganisms with the ability to live at high temperatures require heat stable nucleic acids, proteins, and lipids to keep the whole cell machinery alive.

Generally, there is an increasing trend in glycolipid content from psychrophiles to moderate and extreme thermophiles. A high content of sugar-containing lipids increases the hydrogen bonding capacity of the lipid bilayer surface, and thus could stabilize the membrane at high temperatures, perhaps through additional interactions with cell wall components (Russell and Funkunga, 1990). Abed and colleagues (2006) showed that the photosynthesizing community in the Abu Dhabi mats are adapted to high temperatures. Maximum rates of photosynthesis were detected at 45°C. Only above 50°C, photosynthesis was completely inhibited, however most likely due to high sulfide concentrations. Additionally, fatty acids with high trans/cis ratios, which are relevant at increasing temperatures and salinities, were detected in these mats (publication 3).

Mechanisms against strong UV light intensities include e.g. the production of the sunscreen pigment scytonemin. Scytonemin is produced by some cyanobacteria, such as *Lyngbya aestuarii*, *Entophysalis* spp., and *Chroococcus* sp. (Fleming and Castenholz, 2007) and was also detected in high concentrations in the mats of Abu Dhabi (publication 3). Some cyanobacteria migrate to deeper layers of microbial mats to escape from high light intensities (Bebout and Garcia-Pichel, 1995; Kruschel and Castenholz, 1998; Nadeau, 1999), a process known as phototaxis. An example of bacteria which are resistant to very high doses of ionizing radiation (e.g. caused by high UV light intensities) are some members of the *Deinococci* group (Moseley, 1983; Minton, 1994), which were also found in the studied mats (publications 2 and 4). Due to their potent DNA-repair capabilities, they are also able to survive prolonged periods of desiccation (Mattimore and Battista, 1998).

The production of scytonemin seems to be also relevant for desiccation-tolerant bacteria (Singh et al., 2002). To survive longer periods of desiccation, mechanisms which maintain the structural integrity of cell membranes, appear to be of importance (Singh et al., 2002). Some desiccation-tolerant bacterial cells accumulate large amounts of one or both of the disaccharides trehalose and sucrose (Crowe and Crowe, 1992) which might be effective at protecting enzymes during drying. Another protection mechanism against desiccation is the production of extracellular polymeric substances (EPS), which was also shown in our study to increase from lower to upper tidal mats (publication 3). EPS is thought to prevent cells from water loss and maintain the structure and functions of

biological membranes during desiccation (Tamaru et al., 2005).

High salinity possesses, besides an osmotic, also an ionic stress component and two main mechanisms are known to cope with this. In the first mechanism, cells maintain high intracellular salt concentrations (mainly KCl), osmotically at least equivalent to the external concentrations (“salt-in” strategy). Therefore, the whole cellular system needs to be adapted to high salt concentrations. In the second mechanism, the osmotic pressure is balanced by the production of compatible solutes, which does not require special adaptation of the intracellular system (Oren, 2002; Pikuta et al., 2007).

We observed an expected increase in salt adaptation of the mats’ microorganisms of Abu Dhabi, with respect to photosynthesis and oxygen consumption, from lower to upper intertidal mats, corresponding to increasing ambient salinities (publication 2). Additionally, we discovered a novel process, termed as “halotaxis”, which might be crucial for the survival of cyanobacteria in environments with regular salinity fluctuations, such as intertidal flats. Cyanobacteria were found to migrate to lower salinities (publication 1). This finding further broadened our knowledge of bacterial adaptation mechanisms to salinity, which was restricted so far to the knowledge of cellular uptake of compatible solutes or salt (see above). It could also explain how *Microcoleus chthonoplastes* can survive up to 25% salinity, although its isolates could not grow at salinities above 12% (Karsten et al., 1996). This phenomenon is important for the fundamental understanding of how life exists in nature and how critical it is to extrapolate culture-based observations to *in situ* conditions.

2. Aerobic heterotrophic prokaryotes in the Abu Dhabi mats

The processes studied in this thesis (i.e. photosynthesis, oxygen consumption, and halotaxis) were restricted to the upper, fully oxygenated part of microbial mats. The high oxygen concentration within this zone of the mat (up to 1 mM; Publication 1) represents another extreme factor, since molecular oxygen is a potent inhibitor of key microbial processes, such as e.g. photosynthesis, N₂ fixation, and sulfate reduction (Paerl and Pinckney, 1996). This zone is dominated by cyanobacteria and aerobic heterotrophic prokaryotes (AHP), which could diminish high metabolic constraints of O₂ concentrations (Paerl and Pinckney, 1996) by aerobic respiration. In this zone, carbon flux driven by

photosynthesis is highest (Ley et al., 2006). Gröttschel and colleagues (2002) showed that the respiratory activity of AHP was always coupled to cyanobacterial photosynthesis, most likely, because of their growth on cyanobacterial exudates. This indicates their potential importance for the turnover of organic carbon, mainly released by cyanobacteria. Another study showed that *Chloroflexi*-like bacteria were responsible for most of the respiration in the top 1 mm of a hypersaline microbial mat, while the contribution of other aerobic heterotrophs was negligible (Polerecky et al., 2007). Isolated *Chloroflexus* spp. were indeed shown to be nutritionally versatile, able to grow aerobically as a light-independent heterotroph, and anaerobically as a photoautotroph or photoheterotroph (Madigan et al., 1974; van der Meer et al., 2003). Furthermore, some active sulfate-reducing bacteria were found in the oxic part of hypersaline microbial mats and could use organic carbon as electron donor for reducing sulfate to hydrogen sulfide (Canfield and Des Marais, 1993; Visscher et al., 1998; Minz et al., 1999; Ley et al., 2006; Fourçans et al., 2008). Thus, some of them can also be considered in the broader sense as aerobic heterotrophs, being able to perform aerobic respiration (Dilling and Cypionka, 1990; Cypionka, 2000; Jonkers et al., 2005) and aerobic sulfate reduction (Canfield and Des Marais, 1991), although they were previously thought to live strict anaerobically.

The use of the term “aerobic” in this thesis is not necessarily be valid for all heterotrophs in the oxic layer of the mats, since not all microbes respire oxygen. But although sulfate is the most abundant electron acceptor in the oxic part of the mat, according to the canonical progression of electron acceptors, $O_2 \rightarrow NO_3^- \rightarrow Mn^{+4} \rightarrow Fe^{+3} \rightarrow SO_4^{-2} \rightarrow CO_2$, as predicted by thermodynamic considerations (Franks and Stolz, 2009), oxygen will be used first for respiration.

3. Microbial consortia in extreme environments: Interaction between aerobic heterotrophic prokaryotes and cyanobacteria

Since AHP live in close proximity to cyanobacteria, we further investigated the interaction between the two groups (publication 5).

The basic hypothesis was that cyanobacterial photosynthesis in the light, fermentation in the dark, and nitrogen fixation may provide the respiring aerobic heterotrophs with oxygen, organic compounds, and fixed nitrogen required for their

metabolism and growth. In return, aerobic heterotrophs create suitable conditions for the cyanobacteria, e.g. by protecting them from photooxidative stress (Abeliovich and Shilo, 1972; Eloff et al., 1976) and N₂-fixation inhibition due to high oxygen concentrations (Paerl and Keller, 1978), and by providing the cyanobacteria with vitamins and growth factors (Paerl, 1996). Partially, these kinds of interactions can also be regarded as survival strategies in a smaller habitat scale. So heterotrophs help e.g. to circumvent the harmful increase in oxygen concentrations around cyanobacteria by consuming it. However, these relationships are not that simple and can be manifold, highly specific, and even harmful in some cases.

The associations between cyanobacteria and AHP are of a very strong nature, since it is still challenging to separate cyanobacterial cultures from AHP. These bacteria form functional consortia with strong interdependencies. For example, Steppe and colleagues (1996) proposed a mutualistic N₂-fixing consortium, in which non-N₂-fixing *Microcoleus* spp. provide a habitat for epiphytic diazotrophic bacteria, supplying the cyanobacteria with fixed nitrogen. This raises the question why this cyanobacterium is evolutionary not provided with the capability to fix nitrogen, or in other words, what is the advantage of these consortia, or functional units compared to omnipotent free living cells, especially with regard to life in extreme environments?

Microbial consortia or multiple interacting microbial populations, can perform more complicated functions than individual populations and thus consortia can be more robust to environmental fluctuations (Brenner et al., 2008), which is especially relevant in dynamic environments, such as intertidal flats. Compared to suspended cells, adhered communities might be more resistant to antimicrobial agents and to invasion by other species, most likely due to synergistic interactions within their community (Burmolle et al., 2006). Cowan and colleagues (2000) showed that the organization and morphology of a dual-species biofilm changed in response to selective pressure of increasing toxicant concentration. The contact between two different bacterial strains was increased, whereby one of them became dispersed throughout the biofilm. This arrangement appears to have enhanced the survival of this strain, possibly because the local concentration of the toxicant in its vicinity was reduced. Hence, the arrangement of cells in the biofilm facilitated the survival of the strain, which otherwise would have been eliminated from

the biofilm. Furthermore, communities might be able to endure periods of nutrient limitation better than free living cells, because of the variety of coexisting metabolic modes and the ability to share metabolites among each other (Fay, 1992). Consequently, a community is thought to promote stability through time for its members (Brenner et al., 2008). Eitemann and colleagues (2008) engineered two strains of *Escherichia coli* in the way that one metabolizes only glucose and the other only xylose, and which can be regulated so that they consume their substrates at similar rates. Interestingly, by combining these two strains in a single process, xylose and glucose are consumed faster than by a single organism performing both functions. This shows that the division of labor in microbial communities results in a higher efficiency and thus in a better utilization of energy. But why is that so? In a similar study, using maltose and lactose, it was proposed that the interference of generalists compared to specialist is caused, because the number of sites for sugar permease molecules in the membrane of *E. coli* is limited, such that when both maltose and lactose operons are induced, there is not enough space for the full number of both types of permeases (Dykhuizen and Davis, 1980).

Our study (publication 5) showed that some bacterial communities associated with cyanobacteria might give competitive advantage to their host by killing other potentially competitive cyanobacteria. But are there also disadvantages in living in a community, like e.g. competition for limiting nutrients? The answer of this question strongly depends on the specific environment and its inhabitants. For microbial mats, the question can be rather negated, since these community structures are very stable. Every member of the community has its own traits and thus fills a certain niche. This is what ecologists call "niche differentiation". Thus, all the members of the community fulfill a certain function to sustain the whole system. Still, the question remains, what happens with functional similar species? How can they coexist without outcompeting each other? One possibility is that the niches of all of these seemingly similar species really differ in aspects that are not easily detectable (Scheffer and van Nes, 2006). However, a rather new ecological theory (Hubbell, 2001) assumes that the differences between members of an ecological community of trophically similar species are "neutral," or irrelevant to their success. It is assumed that some organisms are simply too similar to outcompete each other, thus explaining their coexistence. However, there are many controversial opinions

about these ecological theories.

In conclusion, despite of some harmful interactions, we probably can consider microbial communities within microbial mats, more as positively than as competitively interacting functional communities. In these complex structures, all microbial cells of different species are distributed according to their functions and to physicochemical gradients that allow more effective system support, self-protection and energy distribution (Pikuta et al., 2007), which is especially favorable for the survival in extreme habitats, as described above.

4. Relevance of studying microbial communities in extreme habitats

Mats of today e.g. are representatives of the earliest microbial communities on Earth (i.e. stromatolites) and are therefore regarded as a fundamental, evolutionary step from single cell stage into complex ecosystems. Studying extremophiles will enrich our understanding of the physical and chemical constraints on life and may accelerate new theories on how life originated and developed on earth.

The discovery of organisms that have the capability to cope with environmental conditions formerly considered inhospitable for life, redefined the previous constraints for possibilities for life, showing that it is not bound to the “normal” conditions of temperature, salinity, water content, pressure, ionic strength, pH, etc. normally required by the more complex eukaryotes. Consequently a new concept is developing: life is robust and can adapt to many different extreme conditions (Amils et al., 2007). Thus, from the characteristics of extremophilic microorganisms found on the present day earth, some insights on the question of habitability of other planets and about possible bioindicators that may be suitable when searching for extraterrestrial life can be derived (Seckbach and Chela-Flores, 2007). Hence, extreme environments were started to be regarded as analogues to other planets from our solar system and astrobiologists try to use the knowledge about microbial life in extreme habitats for studying potential extraterrestrial life.

Furthermore, microorganisms from extreme habitats provide a huge biotechnological potential (Herber, 1992; Pennisi, 1997; Abed et al., 2009). Often, industrial, medical and environmental applications involve exposure of e.g. enzymes to

extremes of temperature, pressure ionic strength, pH, and organic solvents, and hence there is a continuing need to study and isolate microorganisms from extreme environments (Herber, 1992). One example is the enzyme Taq polymerase, isolated from the Yellowstone hot spring bacterium *Thermus aquaticus*, which is able to perform at high temperatures in the polymerase chain reaction (PCR). Even if isolated enzymes turn out not to be useful for industry, chemists hope to learn how to redesign conventional enzymes to perform in harsh conditions (Pennisi, 1997). Another example is the investigation of ways to use bacteria from the genus *Deinococci*, which were also found in this study (publications 2 and 4), for bioremediation of nuclear waste sites (Lange et al., 1998; Brim et al., 2000; Daly, 2000). From our studies, the cyanobacterial culture M7CRI can be potentially important for biotechnology (publication 5), because its heterotrophic community was able to kill potential cyanobacterial competitors, most likely by producing bioactive substances or antibiotic compounds.

5. Conclusion and outlook

In conclusion, the present work provided an insight into the unique and novel diversity of extremotolerant and extremophilic microorganisms and their behavior regarding carbon cycling with respect to changes in salinity in hypersaline microbial mats from an intertidal flat of Abu Dhabi. Most importantly, a novel strategy of cyanobacteria to circumvent high salinities was found (halotaxis) and unexpected physiological features of cyanobacterial-bacterial co-cultures were observed, which could have a high potential for biotechnology, due to its bactericidal activity. In the future, more studies are needed to investigate how widespread halotaxis is among different species of cyanobacteria and whether it is a general mechanism in microbial mats from intertidal flats. Further studies on adaptation strategies of the mats' microorganisms to coexistent extreme conditions of salinity, UV, desiccation, and temperature, and their response to changes in these parameters due to tidal regime should be carried out. Furthermore, bioactive compounds from cyanobacterial-bacterial co-cultures should be identified and their possible biotechnological or industrial potential should be evaluated. Finally, more physiological studies are needed to identify the key players of carbon cycling and their interactions with each other, using functional studies.

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Part II: Publications

A List of publications

I. Publications presented in this thesis

- 1 **Kohls, K., Abed, R.M.M., Polerecky, L., Weber, M., and de Beer, D.** (2010) Halotaxis of cyanobacteria in an intertidal hypersaline microbial mat. *Environ Microbiol* 12: 567-575.

Concept by K. Kohls, practical work by K. Kohls with contribution of L. Polerecky and M. Weber, writing by K. Kohls with editorial help from R. Abed, L. Polerecky, and D. de Beer

- 2 **Abed, R.M.M., Kohls, K., and de Beer, D.** (2007) Effect of salinity changes on the bacterial diversity, photosynthesis and oxygen consumption of cyanobacterial mats from an intertidal flat of the Arabian Gulf. *Environ Microbiol* 9:1384-1392.

Concept by R. Abed, practical work by R. Abed and K. Kohls, writing by R. Abed with editorial help from K. Kohls and D. de Beer

- 3 **Abed, R.M.M., Kohls, K., Schoon, R., Scherf, A. K., Schacht, M., Palinska, A.K., Al-Hassani, H., Hamza, W., Rullkötter, J., and Golubic, S.** (2008) Lipid biomarkers, pigments and cyanobacterial diversity of microbial mats across intertidal flats of the arid coast of the Arabian Gulf (AbuDhabi, UAE). *FEMS Microbiol Ecol* 65:449-462.

Concept by R. Abed, practical work by R. Abed, K. Kohls and other coauthors, writing by R. Abed with help from K. Palinska, K. Kohls and S. Golubic

- 4 **Kohls, K., Leloup, J., Arnds, J., de Beer, D., Abed, R.M.M.** (in preparation) Abundance and community composition of Bacteria and Archaea in the oxic layer of a hypersaline intertidal cyanobacterial mat

Concept by R. Abed and K. Kohls, practical work by K. Kohls with help from J. Leloup and J. Arnds, writing by K. Kohls with editorial help from R. Abed, J. Leloup, J. Arnds and D. de Beer

- 5 **Kohls, K., Abed, R.M.M., Lueders, T., Palinska, K., Tulpule, K. and De Beer, D.** (in preparation) Molecular identification of aerobic heterotrophic bacteria in hypersaline microbial mats and their interaction with associated cyanobacteria

Concept by R. Abed and K. Kohls, practical work by K. Kohls with contribution of T. Lueders, R. Abed, K. Tulpule, and K. Palinska, writing by K. Kohls with editorial help by R. Abed, T. Lueders and D. de Beer

II. Publications not presented in this thesis

Sulfate-reducing bacteria in marine sediment (Aarhus Bay, Denmark): abundance and diversity related to geochemical zonation.

Leloup, J., Fossing, H., Kohls, K., Holmkvist, L., Borowski, C., and Jørgensen, B.B. (2009) *Environmental Microbiology* 11: 1278-1291.
Help with DGGE

Abstract

In order to better understand the main factors that influence the distribution of sulfate-reducing bacteria (SRB), their population size and their metabolic activity in high- and low-sulfate zones, we studied the SRB diversity in 3- to 5-m-deep sediment cores, which comprised the entire sulfate reduction zone and the upper methanogenic zone. By combining EMA (ethidium monoazide that can only enter damaged/dead cells and may also bind to free DNA) treatment with real-time PCR, we determined the distributions of total intact bacteria (16S rDNA genes) and intact SRB (*dsrAB* gene), their relative population sizes, and the proportion of dead cells or free DNA with depth. The abundance of SRB corresponded in average to 13% of the total bacterial community in the sulfate zone, 22% in the sulfate-methane transition zone and 8% in the methane zone. Compared with the total bacterial community, there were relatively less dead/damaged cells and free DNA present than among the SRB and this fraction did not change systematically with depth. By DGGE analysis, based on the amplification of the *dsrA* gene (400 bp), we found that the richness of SRB did not change with depth through the geochemical zones; but the clustering was related to the chemical zonation. A full-length clone library of the *dsrAB* gene (1900 bp) was constructed from four different depths (20, 110, 280 and 500 cm), and showed that the *dsrAB* genes in the near-surface sediment (20 cm) was mainly composed of sequences close to the Desulfobacteraceae, including marine complete and incomplete oxidizers such as Desulfosarcina, Desulfobacterium and Desulfococcus. The three other libraries were predominantly composed of Gram-positive SRB.

Inorganic carbon fixation by sulfate-reducing bacteria in the Black Sea water column.

Neretin, L.N., Abed, R.M.M., Schippers, A., Schubert, C.J., Kohls, K., and Kuypers, M.M.M. (2007) *Environmental Microbiology* 9: 3019–3024.
Cone libraries and phylogeny

Abstract

The Black Sea is the largest anoxic water basin on Earth and its stratified water column comprises an upper oxic, middle suboxic and a lower permanently anoxic, sulfidic zone. The abundance of sulfate-reducing bacteria (SRB) in water samples was determined by quantifying the copy number of the *dsrA* gene coding for the alpha subunit of the dissimilatory (bi)sulfite reductase using real-time polymerase chain reaction. The *dsrA*

gene was detected throughout the whole suboxic and anoxic zones. The maximum *dsrA* copy numbers were 5×10^2 and 6.3×10^2 copies ml^{-1} at 95 m in the suboxic and at 150 m in the upper anoxic zone, respectively. The proportion of SRB to total Bacteria was 0.1% in the oxic, 0.8–1.9% in the suboxic and 1.2–4.7% in the anoxic zone. A phylogenetic analysis of 16S rDNA clones showed that most clones from the anoxic zone formed a coherent cluster within the *Desulfonema*–*Desulfosarcina* group. A similar depth profile as for *dsrA* copy numbers was obtained for the concentration of non-isoprenoidal dialkyl glycerol diethers (DGDs), which are most likely SRB-specific lipid biomarkers. Three different DGDs were found to be major components of the total lipid fractions from the anoxic zone. The DGDs were depleted in ^{13}C relative to the $\delta^{13}\text{C}$ values of dissolved CO_2 ($\delta^{13}\text{C}_{\text{CO}_2}$) by 14–19%. Their $\delta^{13}\text{C}$ values [$\delta^{13}\text{C}_{\text{DGD(II-III)}}$] co-varied with depth showing the least ^{13}C -depleted values in the top of the sulfidic, anoxic zone and the most ^{13}C -depleted values in the deep anoxic waters at 1500 m. This co-variation provides evidence for CO_2 incorporation by the DGD(II–III)-producing SRB, while the 1:2 relationship between $\delta^{13}\text{C}_{\text{CO}_2}$ and $\delta^{13}\text{C}_{\text{DGD(II-III)}}$ indicates the use of an additional organic carbon source.

A series of microbial processes kills corals exposed to organic-rich sediments

Weber, M., Lott, C., Kohls, K., Polerecky, L., Abed, R. M. M., Ferdelman, T., Fabricius, K. E., and de Beer, D.

In preparation for Public Library of Science (PLOS)

Help with molecular analysis

Abstract

Land runoff of nutrients and nutrient-rich sediments from cultivated land commonly leads to the organic enrichment of coastal sediments. Here we investigated the mechanisms leading to physiological stress and rapid death of corals when exposed to sediments rich in organic matter, postulating that they are microbially mediated. Microsensor measurements were conducted both in mesocosm experiments and in naturally accumulated sediment on inshore and offshore reefs. Microsensor profiles showed that in the organic-rich but not in organic-poor sediment, pH and oxygen started to decrease immediately after onset of exposure. Organic-rich sediments started inflicting tissue necrosis within one day, whereas organic-poor sediments had no effect after six days. In the former, sulfide concentrations were low after one day, but progressively increased, predominantly due to the degradation of coral mucus and dead tissue rather than sulfate reduction in the sediment. Dark incubations of corals showed that separate exposure to darkness, anoxia, low pH or sulfide did not cause mortality within four days, while the combination of anoxia and low pH lead to death within 24 h, and within 15 h when also adding sulfide after 12 h of anoxia and low pH exposure. We conclude that sedimentation kills corals through a series of microbial processes triggered by the organic matter in the sediments. The increased microbial activity results in reduced O_2 and pH, which initiates tissue necrosis, rising sulfide concentrations that diffuses to and accelerates death of the neighboring tissues. Our data suggest that the organic enrichment of coastal sediments may be a key process in the commonly observed degradation of runoff-exposed inshore coral reefs.

Microbial response to substrate addition during anaerobic degradation of organic carbon in Namibian shelf sediment

Julies, E., Leloup, J., Kohls, K., and Brüchert, V.

In preparation

*Help with DGGE***Abstract**

We investigated the carbon degradation rates, response time, and temporal evolution of anaerobic bacteria in anoxic organic-rich sediment from the Namibian shelf when supplemented with high molecular weight (HMW) carbohydrates and low molecular weight (LMW) carboxylic acids. A continuous flow-through setup was used to amend sediment with laminarin as model polysaccharide. Denaturing gradient gel electrophoresis (DGGE) allowed us to monitor the temporal evolution of the microbial community composition. In a different experiment, different concentrations of the carboxylic acids acetate and lactate (25 μ M, 50 μ M, 100 μ M, 500 μ M, 1 mM, 5 mM, and 10 mM) were injected into undisturbed sediment cores and metabolic turnover was studied by measuring 35 S-sulfate reduction rates. Addition of laminarin resulted in a significant (up to 6 fold) stimulation of the metabolic activity of microorganisms. The fermenting bacteria that responded the fastest to laminarin addition belonged to the *Bacteroidetes* phylum. Both the addition of LMW and HMW organic substrates stimulated sulfate reduction rates, but the response was weaker with increasing depth. A DNA clone library based on the DSR sequence bands suggested the dominance of comparably fast-growing incomplete oxidizing SRB in the top 2 cm, while slower growing SRB become dominant in the deeper parts of the sediment. The combined information from these experiments suggests a differential response of the microbial community to substrate addition. Whereas the surface sediment is dominated by bacteria capable of accelerating hydrolysis and carbon oxidation rates in response to enhanced organic matter flux, bacteria present in sediment buried to only 14 cm depth exhibit a slower response.

B Publications

Publication 1

Halotaxis of cyanobacteria in an intertidal hypersaline microbial mat

Halotaxis of cyanobacteria in an intertidal hypersaline microbial mat

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Summary

An intertidal hypersaline cyanobacterial mat from Abu Dhabi (United Arab Emirates) exhibited a reversible change in its surface colour within several hours upon changes in salinity of the overlying water. The mat surface was orange-reddish at salinities above 15% and turned dark green at lower salinities. We investigated this phenomenon using a polyphasic approach that included denaturing gradient gel electrophoresis, microscopy, high-performance liquid chromatography, hyperspectral imaging, absorption spectroscopy, oxygen microsensor measurements and modelling of salinity dynamics. Filaments of *Microcoleus chthonoplastes*, identified based on 16S rRNA sequencing and morphology, were found to migrate up and down when salinity was decreased below or increased above 15%, respectively, causing the colour change of the mat uppermost layer. Migration occurred in light and in the dark, and could be induced by different salts, not only NaCl. The influence of salinity-dependent and independent physico-chemical parameters, such as water activity, oxygen solubility, H₂S, gravity and light, was excluded, indicating that the observed migration was due to a direct response to salt stress. We propose to term this salinity-driven cyanobacterial migration as 'halotaxis', a process that might play a vital role in the survival of cyanobacteria in environments exposed to continuous salinity fluctuations such as intertidal flats.

Introduction

Intertidal flats of the Arabian Gulf coast near Abu Dhabi (United Arab Emirates) harbour extensive areas covered with cyanobacterial mats. These mats are exposed to strong daily fluctuations in environmental parameters such as temperature, light intensity, desiccation and salinity, which are induced by the tidal regime and depend on the intertidal position. Salinity typically varies between 6% and 22% during high and low tides respectively. Upon transfer of these mats to the laboratory and simulation of the tidal salinity changes, a clear reversible change in the colour of the mat surface from red at 20% salinity to green at 10% salinity was observed within several hours. A similar colour change was previously reported in a microbial mat from Baja California and was thought to be either due to physiological (photopigment) changes in a stable bacterial community or due to a change in the community composition (Bebout *et al.*, 2002).

Salinity was shown to influence the diversity and function of microbial communities in microbial mats. Very high salinities inhibit gross and net photosynthesis and oxygen consumption in the light and in the dark (Garcia-Pichel *et al.*, 1999; Wieland and Kühl, 2006; Abed *et al.*, 2007). Hypersaline conditions in Bahamian lagoons resulted in decreasing rates of CO₂ and N₂ fixation and thus in a lower productivity of microbial mat communities (Pinckney *et al.*, 1995). It was also shown that sulfate reduction rates decreased with increasing salinities (Brandt *et al.*, 2001; Porter *et al.*, 2007). Long-term changes in salinity caused a community shift in microbial mats (Bebout *et al.*, 2002) and microbial diversity was shown to decrease with increasing salt concentrations (Rodriguez-Valera, 1988; Benlloch *et al.*, 2002; Casamayor *et al.*, 2002; Jungblut *et al.*, 2005; Rothrock and Garcia-Pichel, 2005). As pointed out by Javor (1989) and Garcia-Pichel and colleagues (1999) many parameters such as ionic composition, pH, specific gravity, surface tension, specific heat, viscosity, conductivity, gas solubility, gas diffusion coefficients and water activity are salinity-dependent. Thus, the observed colour change in our mats could be either due to a direct effect of salinity, or due to an indirect effect by other parameters that change with salinity.

The main goals of this study were (i) to find out the reason for the salinity-associated colour change in our mats and (ii) to test whether the colour change is caused

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2 K. Kohls et al.

by salinity directly or indirectly, or whether it is additionally induced by other physico-chemical parameters. We employed direct light and epifluorescence microscopy, pigment analyses by high-performance liquid chromatography (HPLC), absorption spectroscopy and hyperspectral imaging, denaturing gradient gel electrophoresis (DGGE) and oxygen microsensor measurements to elucidate the reason for the colour change. Furthermore, to exclude possible indirect causes, we investigated the phenomenon under constant light intensity and in the dark, in the presence of different dissolved salts, in upright and inverted mat pieces, at constant oxygen concentrations in the overlying water, with and without the sulfidic part of the mat, and at constant water activities. Finally, to better understand how salt ions diffuse through the mat, the dynamics of salinity inside the mat were modelled assuming diffusive transport, and measured directly in the pore water of mat sections.

Results

The cause of the mats colour change with changing salinity

At salinities above 15%, the mat surface appeared orange-reddish (Fig. 1A). Upon an abrupt reduction of the overlying water salinity below 15%, the colour changed to green within 3–8 h (Fig. 1B). When the salinity was increased back to 20% the mat turned red again. The phenomenon was always reversible.

Microscopy of mat cross-sections showed that *Microcoleus*-like bundles of green filaments migrated from a depth of 1–3 mm to the surface layer (0–1 mm) when the salinity of the overlying water was lower than 15% (Fig. 1B, D and F), causing the green colour of the surface.

Using DGGE with 16S rRNA specific cyanobacterial primers, the banding patterns of the surface layer of the red and green mats were similar except for a dramatically increasing intensity of one band (Fig. 2A, band 2). The sequence of this band was closely related to the cyanobacterium *Microcoleus chthonoplastes* (Fig. 2B, band 2), confirming the microscopic identification. Other dominant, not migrating cyanobacteria in the uppermost layer were closely related to *Euhalothece* and *Leptolyngbya* sp. (Fig. 2B).

The migration of *Microcoleus* filaments was further confirmed using pigment and spectral analyses. HPLC analysis revealed that, from the pigments extracted by the applied protocol, only chlorophyll *a* (Chl *a*) and β -carotene exhibited a significant change in their concentrations in the uppermost layer after the transition from red to green. Concentrations of both pigments increased about 2.5-fold; however, their ratio remained constant (Fig. 3A). Spectral reflectance measurements of the mat surface

showed that, in addition to these pigments, phycocyanin (absorption maximum 630 nm), a cyanobacteria-specific pigment, was also significantly enhanced in the green mat (Fig. 3B). Furthermore, autofluorescence microscopy of 5 μ m thick mat cross-sections (Fig. 1I–J), as well as hyperspectral imaging of 1 mm thick cross-sections (Fig. 1G–H), showed that, for the red mat, cyanobacteria-specific signals were present in a close-packed, 0.5–1 mm thick layer located about 1–3 mm below the surface, whereas this signal spread and moved towards the surface in the green mat.

The migration of *M. chthonoplastes* was also supported by the oxygen microprofiles measured in illuminated mat samples (Fig. 4A). Volumetric rates of net photosynthesis (Fig. 4B), derived from the microprofiles, exhibited two discernable oxygen production zones in the red mat, reflecting two different photosynthetically active communities before migration. After salinity was decreased to 10%, the lower zone shifted gradually upwards and after ~8 h merged with the upper one.

In summary, these data showed that *M. chthonoplastes* filaments migrate in response to salinity changes in this mat, and that there is no indication for a physiological change on a cellular level in the microbial populations that could explain the colour change. Furthermore, experiments conducted in a wider range of salinities (5–25%) showed consistently that the migration occurred in the direction towards and away from the surface when the salinities were below and above 15% respectively.

Migration of Microcoleus chthonoplastes at different ambient conditions

Visual observations of the mat surface colour (Fig. 1A and B) revealed the following patterns in the migration of *M. chthonoplastes*. Migration of *M. chthonoplastes* upon salinity changes occurred at a constant light intensity as well as in the dark. It was also induced by a variety of salts, including NaCl, Na₂SO₄ and MgCl₂, indicating that the migration is not dependent on the ionic composition. Also, it did not depend on the orientation to gravity, as it occurred also in mat samples that were incubated upside down at 10% salinity. In dead or fixed mats no colour change was observed upon salinity changes, showing that the colour change was not of a purely physical character (i.e. change in surface tension). Furthermore, the migration was clearly independent of salinity-associated changes in the oxygen concentrations in the overlying water, as changes of the mat colour were still detectable after changing the salinity from 20% to 10% while keeping the oxygen levels constant. The removal of the anoxic/sulfidic layer of the mat did not affect the migration behaviour of *M. chthonoplastes*, and was thus independent of H₂S gradients in the mat. Even decreasing the water

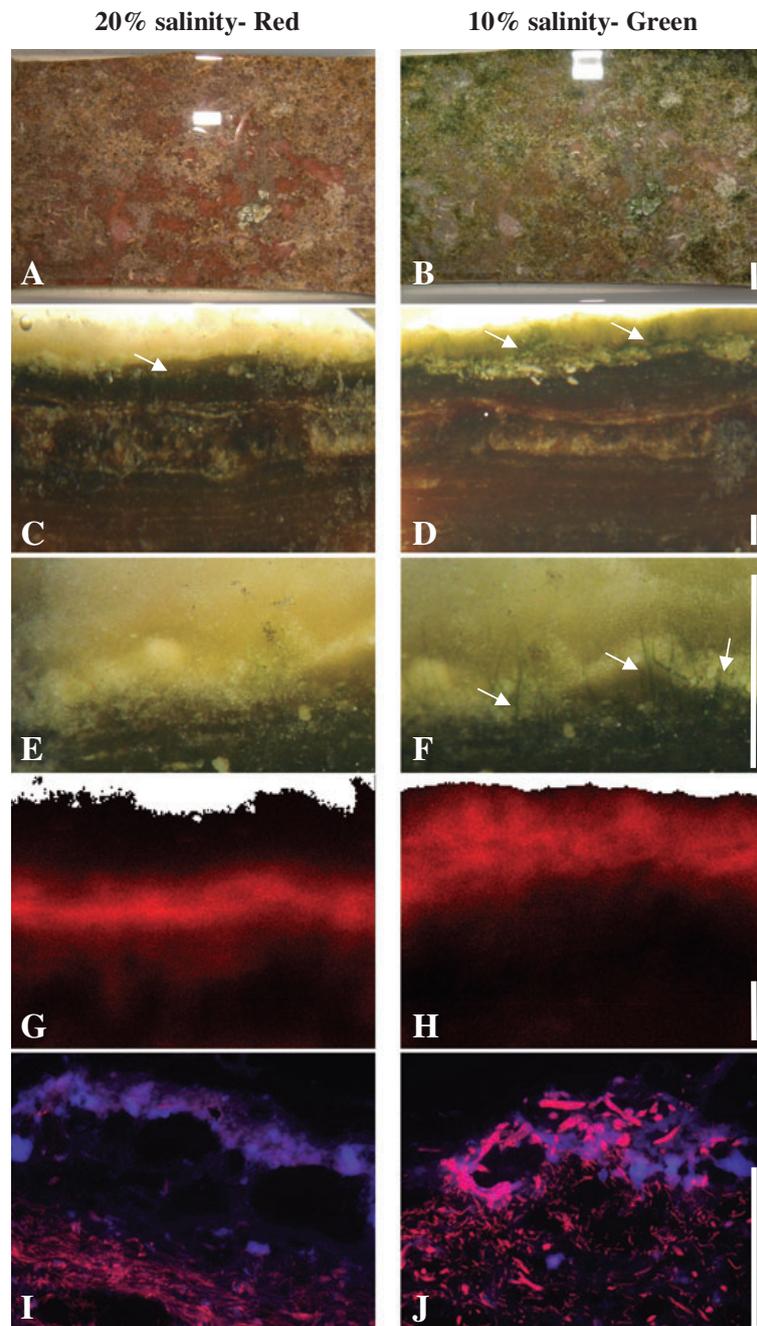


Fig. 1. Photographs and photomicrographs of the mats incubated at the overlying water salinities of 20% (red mat, left) and 10% (green mat, right).
 A and B. Images of the surface of the same mat sample, photographed from the top.
 C and D. Cross-sections of the mats, showing green filaments that moved towards the surface (indicated by arrows).
 E and F. Closer view of the cross-sections in (C–D).
 G and H. Hyper-spectral images of the mat cross-sections, showing the relative distribution of phycocyanin, a cyanobacteria-specific accessory pigment (brighter red colour corresponds to a higher pigment concentration).
 I and J. Epifluorescence microscope images, showing autofluorescence of cyanobacteria (pink) and the signal from the mat matrix (blue). The scale bar corresponds to 1 cm and 1 mm for panels (A–B) and (C–J) respectively.

4 K. Kohls et al.

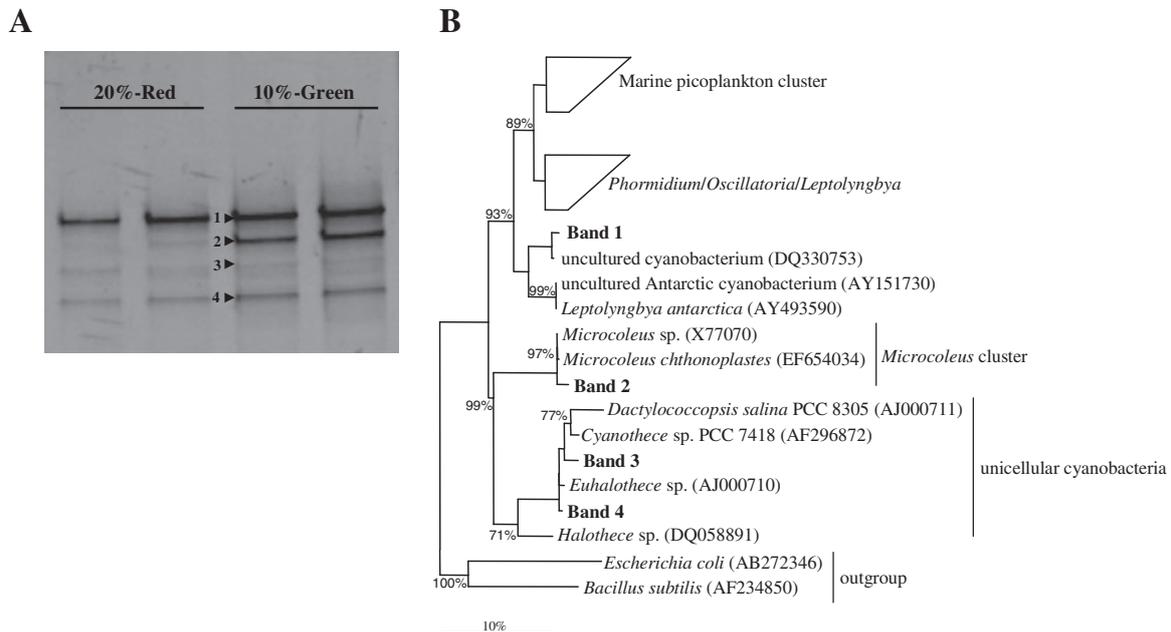


Fig. 2. A. DGGE banding patterns of cyanobacterial 16S rRNA from the uppermost layer of the microbial mats incubated at 20% and 10% salinity, shown in duplicates. The intensity of band 2 increased strongly at salinity 10%, whereas intensities of bands 1, 3 and 4 were similar at both salinities. Bands 1–4 were excised, reamplified and sequenced. B. 16S rRNA gene-based phylogenetic reconstruction based on maximum likelihood methods, showing the affiliation of cyanobacterial sequences retrieved from DGGE bands obtained from the uppermost layer of the studied microbial mat. The partial sequences were inserted into the reconstructed tree by applying parsimony criteria without allowing changes in the overall tree topology. Accession numbers are indicated in parentheses. Bootstrap values (1000 replicates) are shown next to the relevant nodes. The scale bar indicates 10% sequence divergence. Sequences obtained in this study are shown in bold.

activity by the addition of sucrose did not inhibit the migration of the cyanobacteria to the surface, suggesting that they did not follow the water. Collectively, these results showed that the migration of *M. chthonoplastes* was most likely directly induced by salinity changes.

Modelling and measurements of salinity dynamics in the mat

The modelled vertical profiles of salinity inside the mat varied dynamically upon the salinity change (Fig. 5A and B). Even after prolonged periods of constant salinity in the overlying water, salinity in the mat did not reach a steady state. Depending on the diffusion coefficient, the salinity in the depth interval where the migrating cyanobacteria are present (~1.5 mm) increased from 10% to 18% within 4–24 h after an abrupt increase from 10% to 20% in the overlying water. Inversely, about 4–24 h was required until the salinity at this depth reached 12% after it was changed in the overlying water from 20% to 10%. Salinity in this depth interval exhibited pronounced gradients in the range of 0.5–3% mm⁻¹ during 24 h after the change from 20% to 10%. These gradients were opposite but equal in

magnitude for the inverse change in salinity (Fig. 5C and D). The measured salinity profiles were comparable to the modelled data and confirmed the presence of pronounced salinity gradients in the mat after an abrupt salinity change in the overlying water (Fig. 5E).

Discussion

The change of the mat surface colour from red to green upon decreasing salinity was found to be due to the migration of *M. chthonoplastes* to the mat surface. Cyanobacteria were previously shown to migrate in microbial mats, desert soils and mudflats in response to a variety of factors, including light intensity (Bebout and Garcia-Pichel, 1995; Kruschel and Castenholz, 1998; Nadeau, 1999), diel cycle (Richardson and Castenholz, 1987; Garcia-Pichel *et al.*, 1994; Fourçans, 2006), different oxygen concentrations (Whale and Walsby, 1984), upwards fluxing sulfide (Richardson and Castenholz, 1987; Abed *et al.*, 2006) and wetting (Brock, 1975; Garcia-Pichel and Pringault, 2001; Pringault and Garcia-Pichel, 2003). This is the first time that migration of cyanobacteria in a microbial mat was shown to be induced by salinity changes.

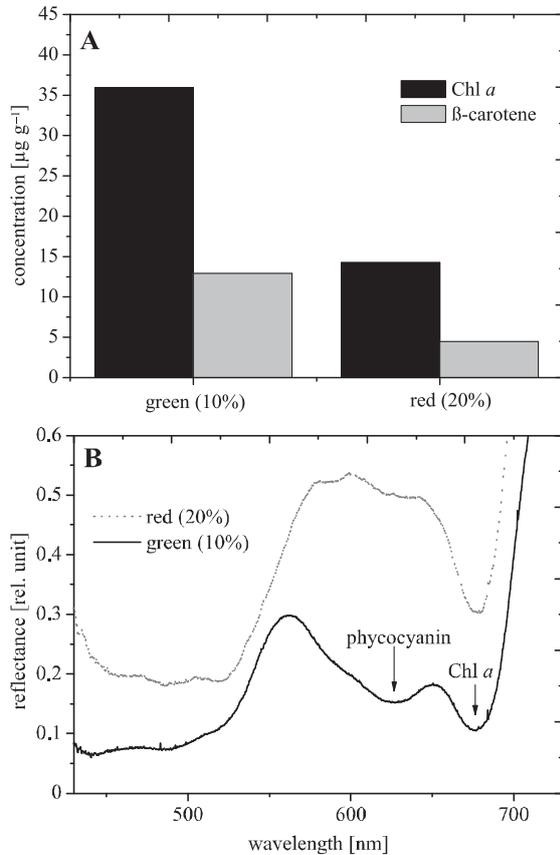


Fig. 3. A. Pigment concentrations in the top 1 mm of a sample from the green and red mat, as measured by HPLC. Mean values of two measurements are shown (percentage errors ranged between 0.032% and 0.128% and are thus not displayed). B. Spectral reflectance of the surface of the same mat sample incubated at 20% ('red' mat) and 10% ('green' mat) salinity. The dominant reflectance around 550 nm explains the green appearance of the mat incubated at 10% salinity. Pronounced valleys (marked by arrows) are due to enhanced absorption of light by pigments in the surface layer.

Since salinity influences a variety of other physico-chemical parameters, one aim of this study was to find out whether salinity is indeed the direct elicitor for the migration. For example, salt stress and other osmotic stresses like desiccation and dissolved organics were generally considered to be similar and were often put in the same category of stressors (Smith and Brock, 1973a,b; Csonka, 1989), because all these cases are associated with decreased water availability (i.e. water activity, a_w). However, salinity stress, when compared with other osmotic stresses has, besides the osmotic component, also an ionic component, and cyanobacteria were indeed shown to respond differently to low water activities caused by high salinity and by organic solutes

(Fernandes *et al.*, 1993). This raises a question whether the migration observed in this study is due to the ionic component or due to the change in water activity, and must thus be considered as the previously described hydrotactic migration (Brock, 1975; Garcia-Pichel and Pringault, 2001; Pringault and Garcia-Pichel, 2003). Our results indicated that the observed migration was triggered by ionic stress and not by hydrotaxis. Other potential triggers for the migration, like changes in oxygen solubility, gravity, surface tension, ionic composition and upwards fluxing sulfide, were also excluded experimentally. Furthermore, the migration was not light-dependent, and since pH strongly depends on light due to CO_2 fixation, the influence of pH on the migration was theoretically excluded.

Although it could be presumed that the cyanobacteria migrate deeper into the mat to avoid higher salinity that is

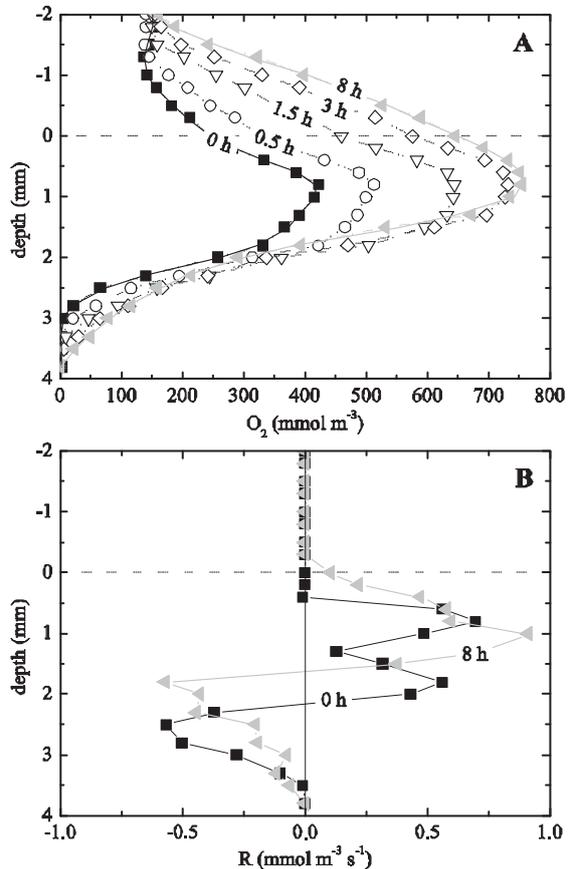


Fig. 4. A. Vertical oxygen microprofiles in the mat measured after the overlying water salinity was abruptly changed from 20% to 10%. Times after the change are indicated. B. Volumetric rates of net photosynthesis, calculated from the initial and final oxygen profiles shown in (A).

6 K. Kohls et al.

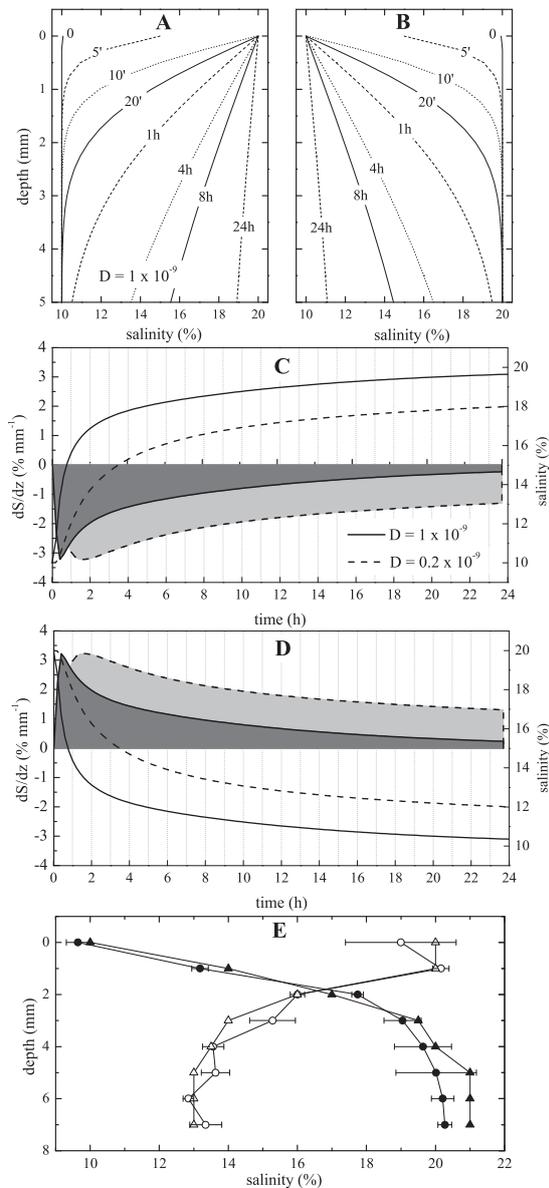


Fig. 5. Modelled vertical profiles of salinity in the mat as a function of time after abrupt changes of the overlying water salinity from 10% to 20% (A) and from 20% to 10% (B). Times after the change are indicated over the profiles. C and D. Salinity (lines, right axis) and salinity gradient (gray areas, left axis) at depth 1.5 mm, modelled as a function of time after an abrupt change of the overlying water salinity from 10% to 20% (C) and 20% to 10% (D). Results are shown for diffusion coefficients $1 \times 10^{-9} \text{ m}^2 \text{ s}^{-1}$ (solid lines, darker gray area) and $0.2 \times 10^{-9} \text{ m}^2 \text{ s}^{-1}$ (dashed lines, lighter gray area). E. Measured vertical profiles of salinity in the mat pore water, determined by a refractometer (circles) and a resistivity sensor (triangles) 1 h after an abrupt change of the overlying water salinity from 10% to 20% (open symbols) and from 20% to 10% (closed symbols). Mean values of three measurements with error bars are shown for the resistivity sensor.

diffusing from above, the modelling results suggest that they do not succeed. Even if they could migrate sufficiently fast, the unfavourable salinity levels ($> 15\%$) would reach them after several hours, provided that there was no diffusion barrier against the ions that cause the stress. This raises a question as to why they migrate at all. When considering that chemotaxis in general is a response to the gradient of a given chemical parameter, we have to look at the migration behaviour with respect to salinity gradients, and not with respect to the salinity levels themselves. Indeed, the modelling as well as salinity measurements showed that the cyanobacteria experience negative salinity gradients during many hours after increasing the overlying water salinity, and respond to it by migrating downwards, i.e. away from the higher salinity levels. Inversely, after decreasing the overlying water salinity to 10%, the salinity gradient at the position where the cyanobacteria dwell rapidly changes direction, to which the cyanobacteria respond by migrating upwards, again away from the higher salinity levels. Additionally, we observed that the filaments formed a dense compact layer embedded in their polysaccharide sheaths at depths of 1–3 mm at salinities above 15%, as opposed to a much more spread distribution at lower salinities. Such dense compaction of filaments could reduce water loss and mass transfer (Javor and Castenholz, 1981; Holtkamp, 1985), possibly offering an additional protection against ionic stress. However, other factors such as increased sulfide concentrations or decreased light availability could also be a reason why the filaments do not migrate deeper than ~3 mm but form a compact layer at higher salinities. High spatial resolution measurements of salinity inside the mat and around the individual filaments, which were not possible in the present study, are needed to resolve this issue.

Although *M. chthonoplastes* has been characterized as euryhaline, i.e. able to survive and tolerate broad range of salinities (Golubic, 1980), the upper salinity limit that is tolerated by isolates was reported to be 12% (Karsten, 1996). Additionally, studies on Guerrero Negro mats showed a salinity tolerance of *M. chthonoplastes* between 6% and 12%, being replaced by other cyanobacteria (*Phormidium*- and *Synechococcus*-related) at salinities higher than 15% (Javor, 1989; Rothschild, 1991; Des Marais, 1995). Similar to these studies, our results showed a clear stress response of *Microcoleus* filaments at salinities higher than 15%.

In conclusion, the present study showed for the first time, that *M. chthonoplastes* migrates in a microbial mat in the direction towards lower salinities. We showed that the migration was very likely due to a direct effect of salinity and not due to changes in other salinity-dependent and independent factors. This phenomenon was not shown before and we hereby call it ‘halotaxis’, a process that might play an important role in the survival of cyanobac-

teria in environments exposed to continuous salinity fluctuations, such as intertidal flats.

Experimental procedures

Origin of mat samples

Mat samples were collected in December 2006, from an intertidal flat in Abu Dhabi (Arabian Gulf coast, United Arab Emirates). Air temperature in this region typically reaches over 50°C during hot summers and between 15°C and 35°C in winter. The water temperature in summer was up to 32°C at high tide, whereas it reached 50°C in pools that remained separated during low tide. The mats used for this experiment were collected during low tide from the upper intertidal zone where salinity fluctuated from 6% to 22% due to tidal regime. During sampling the salinity was 16% and the mat surface appeared reddish. The collected mat samples were shipped to the Max Planck Institute in Bremen and were kept in 10% salinity seawater in glass aquaria directly upon arrival. The seawater originated from the North Sea and the salinity was adjusted using NaCl. The salinity fluctuations due to tidal regime were simulated by leaving the seawater to evaporate until the salinity reached 20–25%, after which the aquarium was refilled again with distilled water.

Direct light and epifluorescence microscopy

For direct light microscopic observations, mat samples incubated at 20% salinity (red surface) and 10% (green surface) were cut in 1 mm thick cross-sections and photographed with a digital camera through a dissection microscope (Axiolab, Carl Zeiss). Furthermore, a freshly sliced piece of the red mat was pressed against the window of an aquarium, the salinity was reduced to 10% and direct images of the cross-section were recorded every 15 min through a dissection microscope.

For epifluorescence microscopy, a green and a red mat sample were first fixed over night in 4% formaldehyde and then incubated in O.C.T. compound (Plano) at 4°C for another 24 h. Afterwards the mat pieces were transferred into cryotrays and left at –20°C for 5 h. Thin cross-sections (5 µm) were prepared using a cryomicrotome (HM 505E, Microm) at –31°C and placed onto polysine coated slides. A Zeiss Axio-phot2 microscope equipped with a high-pressure mercury bulb (100 W) and an AxioCam MRm camera was used to visualize the cyanobacterial autofluorescence of the slices through the Zeiss filter-sets 02 and 43.

Molecular analysis and phylogeny

The uppermost layers (1 mm thick, ~100 mg wet weight) of the red and of the green mat samples were subjected to nucleic acid extraction after Lueders and colleagues (2004). Amplification of cyanobacterial 16S rRNA genes was carried out by polymerase chain reaction (PCR) using the primers CYA359F (with 40 nucleotide GC clamp at the 5' end) and CYA781R (Nübel *et al.*, 1997). DGGE was carried out using a Bio-Rad D-Code system and run at 60°C and a constant voltage of 200 V for 3.5 h. DGGE bands were excised and

sequenced as described before (Abed *et al.*, 2008). The obtained sequences were aligned and analysed using the ARB software (Ludwig *et al.*, 1998) version 071207 and the official database (<http://www.arb-silva.de>) of October 2008 for small subunit RNA sequences (ssuref_96_silva_04-10-08_opt.arb) (Pruesse *et al.*, 2007). The phylogenetic tree was calculated by maximum likelihood, based on long 16S rRNA gene sequences (> 1300 bp). The partial sequences were inserted into the pre-established tree using the parsimony ARB tool, while maintaining the overall tree topology without changes. Bootstrapping was performed for 1000 replicates.

Pigment analyses

Pigment analysis by HPLC was carried out in dim light. For about 50 mg of the uppermost layer of the red and green mat, 2 ml of ice-cold methanol (99.8% for preparative chromatography) were added. After sonication, samples were stored for 24 h at 20°C. The further procedure was carried out as described before (Abed *et al.*, 2008). Each sample was measured twice. The pigments that were found to differ significantly in the red and green mat samples (i.e. Chl *a* and β-carotene) were identified and quantified by comparing the retention time, spectrum and chromatogram area with commercially available plant pigment standards (DHI, Denmark).

Autofluorescence from freshly cut mat cross-sections (1 mm thick) was imaged with a modular spectral imaging (MOSI) system (Polerecky *et al.*, 2009). Sections were placed in hypersaline seawater between two glass slides, which were sealed with silicone grease to prevent leaking. The samples were illuminated by an amber light emitting diode (LXHL-LL3C, Lumileds). The distribution of phycocyanin in the mat, a characteristic pigment of cyanobacteria, was derived by localizing the spectral signature of this pigment in the hyper-spectral data set using HS_ImAn software (Polerecky *et al.*, 2009; <http://www.microsen-wiki.net>).

Spectral reflectance of the mat surface was measured with a fibre-optic irradiance microsensor (Lassen *et al.*, 1992) connected to a spectrometer (USB-2000, Ocean Optics). Illumination was provided by a halogen lamp with a broad spectral range (400–900 nm). The light spectrum back-scattered from the sample was normalized to that reflected from a white diffuser (Spectralon, Labsphere).

Oxygen microsensor measurements and photosynthetic rate calculations

Oxygen microprofiles in the mat during the transition of the surface colour from red to green were measured with a microsensor system described previously (Polerecky *et al.*, 2007; <http://www.microsen-wiki.net>). The mat was illuminated with photosynthetically active radiation (PAR) of constant intensity (~400 µmol photons m⁻² s⁻¹). A two-point linear calibration was performed at both salinities using the readings in the anoxic zone of the mat and in the overlying aerated seawater. The oxygen solubility at the experimental temperature and salinity was obtained from the table available at <http://www.unisense.dk>. Volumetric rates of net photosynthesis were calculated by dividing the difference in diffusive oxygen fluxes at the boundaries of a specific zone with the

8 K. Kohls et al.

thickness of the zone ($R = \Delta J / \Delta z$). This was done in steps of 200 μm throughout the entire oxic layer of the mat. The diffusive fluxes were calculated from the measured oxygen concentrations, using Fick's law of diffusion ($J = -D_{\text{O}_2} \Delta C / \Delta z$). The oxygen diffusion coefficient in the mat, D_{O_2} , was measured with a diffusivity microsensor (Revsbech *et al.*, 1998), and amounted to $1.2 \times 10^{-9} \text{ m}^2 \text{ s}^{-1}$.

Migration experiments

The previous experiments showed that the colour change was due to the migration of *M. chthonoplastes*. Subsequently, in order to find out whether this effect was directly or indirectly induced by salinity or whether there were additional stimulating factors, mat pieces were incubated as follows:

(i) at different salinities under constant light conditions as well as in the dark, to test whether migration is light-dependent;

(ii) in the presence of different salts such as Na_2SO_4 and MgCl_2 , to find out whether salts other than NaCl induce migration;

(iii) upside down at 10% salinity to test whether gravity has an influence on migration;

(iv) in the presence of inactive controls treated with 10% ZnCl_2 and with 4% formaldehyde (over night at room temperature), to test whether purely physical effects, such as salinity-induced changes of the surface tension, and not cell motility, drive the migration;

(v) in the presence of constant oxygen concentrations in the overlying water. The salinity was changed from 20% to 10%, but the oxygen concentration in the overlying water was kept constant, using a mixture of nitrogen gas and air. The oxygen concentration was followed using oxygen microsensors. This experiment was designed to test whether salinity-associated changes in oxygen solubility drive the migration of cyanobacteria;

(vi) in the absence of the anoxic mat layer. Only the upper 3 mm section was incubated at 20% and 10% seawater to induce migration, to test whether H_2S gradients affect migration;

(vii) at a reduced water activity, to find out whether the migration is a hydrotactic response due to an increased water activity (water availability) at lower salinities. The salinity was decreased from 20% to 10%, but the water activity was reduced with the addition of 60 g sucrose to 100 ml of water.

Modelling and measurements of salinity dynamics

Salinity dynamics inside the mat was modelled in Comsol Multiphysics (v3.3), assuming that transport was governed by diffusion. The salinity transitions in the overlying water approximated the experimental conditions. Specifically, salinity was assumed to be initially 20% throughout the mat and was then changed abruptly in the overlying water to 10%, or vice versa, the initial salinity of 10% was changed abruptly to 20%. The diffusion coefficients of the different ions inside the mat that constitute 'salinity' were assumed to be in the range of $0.2\text{--}1 \times 10^{-9} \text{ m}^2 \text{ s}^{-1}$.

Direct salinity measurements were conducted in two mat samples. First, the mat samples were incubated for 2 days in

seawater of constant salinity (one at 20% and the other one at 10%) to allow for the establishment of constant salinity throughout the mat. Then, the incubation seawater was exchanged for 1 h, after which the mat samples were horizontally cut in 1 mm intervals. Salinity of the pore water extracted from these sections by centrifugation (at 10 000 r.p.m. for 1 h) was then determined using a portable refractometer and a resistivity sensor (tip diameter $\sim 1 \text{ mm}$) calibrated in seawater with salinities ranging between 5% and 25%.

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Publication 2

Effect of salinity changes on the bacterial diversity, photosynthesis and oxygen consumption of cyanobacterial mats from an intertidal flat of the Arabian Gulf

Effect of salinity changes on the bacterial diversity, photosynthesis and oxygen consumption of cyanobacterial mats from an intertidal flat of the Arabian Gulf

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Summary

The effects of salinity fluctuation on bacterial diversity, rates of gross photosynthesis (GP) and oxygen consumption in the light (OCL) and in the dark (OCD) were investigated in three submerged cyanobacterial mats from a transect on an intertidal flat. The transect ran 1 km inland from the low water mark along an increasingly extreme habitat with respect to salinity. The response of GP, OCL and OCD in each sample to various salinities (65‰, 100‰, 150‰ and 200‰) were compared. The obtained sequences and the number of unique operational taxonomic units showed clear differences in the mats' bacterial composition. While cyanobacteria decreased from the lower to the upper tidal mat, other bacterial groups such as *Chloroflexus* and *Cytophaga/Flavobacterium/Bacteroidetes* showed an opposite pattern with the highest dominance in the middle and upper tidal mats respectively. Gross photosynthesis and OCL at the ambient salinities of the mats decreased from the lower to the upper tidal zone. All mats, regardless of their tidal location, exhibited a decrease in areal GP, OCL and OCD rates at salinities > 100‰. The extent of inhibition of these processes at higher salinities suggests an increase in salt adaptation of the mats microorganisms with distance from the low water line. We conclude that the resilience of microbial mats towards different salinity regimes on intertidal flats is accompanied by adjustment of the diversity and function of their microbial communities.

Introduction

Salinity controls the diversity and functions of microbial communities. Several studies along salinity gradients showed a decreasing trend in the diversity of bacterial, archaeal and eukaryotic communities when the salinity increased (Gerdes *et al.*, 1985; Rodrigues-Valera, 1988; Guixa-Boixareu *et al.*, 1996; Benloch *et al.*, 2002; Casamayor *et al.*, 2002; Jungblut *et al.*, 2005; Rothrock and Garcia-Pichel, 2005). These studies were confined to closed inland basins (e.g. solar salterns and evaporation ponds) and lakes, where salinity within individual samples along the gradient was constant. The effect of salinity changes on submerged intertidal microbial communities on arid coasts, where salinity fluctuates drastically on a daily basis, due to tides and high evaporation rates, was less frequently studied. The intertidal cyanobacterial mats of the Arabian Gulf represent an ideal model system for studying the salinity fluctuation effects on the structure and function of bacterial communities. Mats that are located higher up on the tidal flat are exposed to a wider range of salinity (i.e. 60–200‰) during a diurnal cycle compared with those present at the lower tidal zones (i.e. 60–80‰).

Unlike studies on the effect of salinity on bacterial diversity, few studies were performed on the role of salinity in the regulation of bacterial metabolic processes at the community level (Garcia-Pichel *et al.*, 1999; Sørensen *et al.*, 2004). Studies on cyanobacterial cultures (e.g. *Microcoleus*, *Anabaena* and *Spirulina* spp.) showed a significant reduction in rates of processes like photosynthesis, respiration and nitrogen fixation by osmotic stress (Vonshak *et al.*, 1988; Fernandes *et al.*, 1993; Karsten, 1996). However, extrapolation of physiological adaptations of individual microorganisms to complex natural bacterial communities, where various metabolic processes closely interact, may not always be valid. Microsensors are suitable tools to study the regulation and interaction of key processes in complex phototrophic microbial communities under *in situ* conditions and in response to environmental stimuli (Revsbech and Jørgensen, 1986; Kühl and Revsbech, 2000). Few studies have used this technique to monitor functional response of mat microorganisms to

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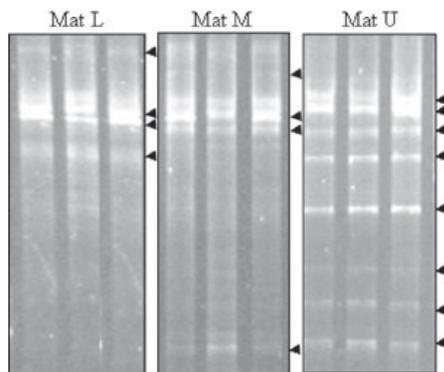


Fig. 1. Denaturing gradient gel electrophoresis fingerprints of PCR-amplified 16S rRNA gene fragments obtained from microbial mats at lower (L), middle (M) and upper (U) tidal zones, using the bacterial primers GM5 and 907RC. Arrows show the visible dominant bands.

salinity changes (Garcia-Pichel *et al.*, 1999; Wieland and Kühl, 2006).

We compared bacterial diversity and rates of gross photosynthesis (GP), oxygen consumption in the light (OCL) and in the dark (OCD) in three submerged microbial mats along a tidal salinity gradient [i.e. lower (L), middle (M) and upper (U) tidal zones] of the coastal flats of Abu Dhabi (United Arab Emirates). The upper tidal mat was selected from channels that retained seawater at lower tide. The effect of changes in salinity on GP, OCL and OCD were also monitored using oxygen microsensors. Our goals were (i) to obtain insights into the bacterial diversity in these poorly investigated mats; (ii) to assess whether the extent of salinity changes, dictated by the position on the tidal transect, affects the structure and activity of microbial communities in mats and (iii) to assess the physiological response of the different communities to salinity.

Results

Bacterial composition of the studied mats

The structure of bacterial communities within the three mats along the tidal salinity gradient was studied using denaturing gradient gel electrophoresis (DGGE) and 16S rDNA cloning. Denaturing gradient gel electrophoresis was performed in order to assess the variability of bacterial communities within each mat and to compare these communities among the three mats. The community structure within each mat was homogenous because the triplicate cores displayed a similar pattern (Fig. 1). Denaturing gradient gel electrophoresis fingerprinting showed distinct banding pattern for every mat sample (Fig. 1).

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Table 1. Diversity–richness measures of three mats collected along a tidal salinity gradient.

	Mat L	Mat M	Mat U
Number of clones	105	116	112
Number to total OTUs	51	39	58
Number of unique OTUs	40	32	52
Number of phyla	7	5	14
Coverage (%)	52	66	48
Species richness	24.7	18.4	27.8
Species evenness	1.5	0.5	1.9
Shannon–Weaver index	2.6	0.7	3.3

Cloning also revealed clear differences in the community composition of the mats (Fig. 3 and Table 1). The rarefaction curves showed that all clone libraries were far from saturation (Fig. 2) with a homologous coverage between 48% and 66% (Table 1). A total of 333 sequences were obtained and were distributed among 14 different major bacterial groups and 148 operational taxonomic units (OTUs) (Table 1). The clone libraries of the three mats showed clear differences in the phylogeny and percentage distribution (Fig. 3) of the obtained sequences as follows.

Cyanobacteria

Cyanobacterial diversity was higher in mats L and M than in mat U. In mat L, 50% of the cyanobacterial clones fell next to sequences of *Phormidium*, *Leptolyngbya* and *Halomicronema* whereas 20% were related to *Scytonema hofmanni* and *Oscillatoria princeps*. The remaining sequences were distributed among *Microcoleus*, *Spirulina* and *Lyngbya*. In mat M, most sequences were affiliated to *Leptolyngbya* and *Microcoleus* species. One sequence was closely related to the species *Lyngbya hieronymussi* while another was related to a *Symploca* species. In mat U, only six cyanobacterial clones related to *Leptolyngbya*, *Oscillatoria* and *Synechococcus* were detected.

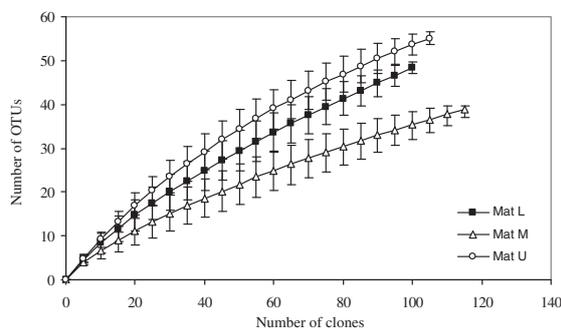


Fig. 2. Calculated rarefaction curves of observed OTUs richness among the clone libraries from lower (L), middle (M) and upper (U) tidal mats.

1386 R. M. M. Abed, K. Kohls and D. de Beer

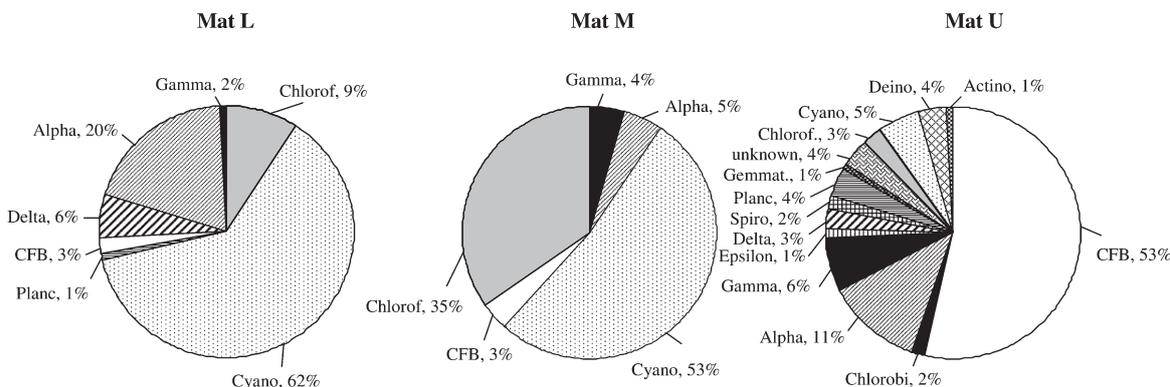


Fig. 3. Comparison of the bacterial community composition in three mats collected from lower (L), middle (M) and upper (U) tidal transects as revealed by 16S rRNA cloning. Shown fractions indicate the relative percentage to the total number of clones. Alpha, *Alphaproteobacteria*; Gamma, *Gammaproteobacteria*; Beta, *Betaproteobacteria*; Delta, *Deltaproteobacteria*; Epsilon, *Epsilonproteobacteria*; CFB, *Cytophaga/Flavobacteria/Bacteriodes*; chlorof., *Chloroflexus*; Planc., *Planctomycetes*; Cyan, *Cyanobacteria*; Actino, *Actinobacteria*; Deino, *Deinococcus*; Gemmat, *Gemmatimonas*; Spiro, *Spirochaete*.

Cytophaga/Flavobacteria/Bacteriodes (CFB) and Chloroflexus groups

Cytophaga/Flavobacteria/Bacteriodes constituted 53% of the total clones in mat U, 45% of which formed two clusters related to the species *Salinibacter ruber*. *Cytophaga/Flavobacteria/Bacteriodes* were much less dominant (3%) in mats L and M. *Chloroflexus*-related clones were detected in all mats but were most dominant in mat M (35% of the clones). Most sequences of this group were distinct from known *Chloroflexus* species. In mat M, the *Chloroflexus*-related sequences formed a cluster that had more than 6% sequence divergence to the closest relative *Candidatus chlorothrix*.

Proteobacteria

The three mats exhibited different distribution of the groups of *Proteobacteria*. While *Alpha*- and *Gammaproteobacteria* were detected in all mats, *Betaproteobacteria* were not detected in any. *Epsilonproteobacteria* were only detected in mat U but *Deltaproteobacteria* were detected in mats L and U and not in mat M. The alphaproteobacterial clones from mat L were related to *Salipiger mucescens*, *Rhodomicrobium vannielii* and *Hyphomicrobium* sp. but they were related mainly to *Hyphomicrobium* sp. in mat U. The gammaproteobacterial clones were related to *Chromatium* species in mats L and M whereas they were closely affiliated to *Acinetobacter*, *Serratia* and *Pseudomonas* species in mat U.

Other groups

The remaining clones were all relatively rare, and were related to *Deinococcus*, *Planctomycetes*, *Actinobacteria*,

Chlorobi, *Spirochaete* and *Gemmatimonas* groups. *Planctomycetes* were detected only in mats L and U whereas the remaining groups were present only in mat U. The clones related to *Deinococcus* species in mat U formed a cluster with the known UV and solar radiation-resistant species, *Deinococcus radiophilus* and *Deinococcus proteolyticus* (Rainey *et al.*, 1997).

Microsensor measurements at in situ salinities

Microsensor measurements were performed in the mats at salinities measured at low tide directly after sampling (see Table 2). The three mats at these salinities showed clear differences in net oxygen production with the highest oxygen peak and interfacial flux for mat L at the lowest salinity (Fig. 4, boxes marked with asterisk on the top right corner). The oxygen penetration depth increased from mat L to mat U. The calculated areal rates of GP and OCL showed a decreasing trend from lower tide to upper tide with maximum rates detected in mat L (Fig. 5, bars marked with asterisk). Gross photosynthesis and OCL areal rates in mat L were five- and 10-fold higher than in mat U respectively.

Salinity-shift experiments

Upon salinity shifts, either upward or downward, all mats, irrespective of their tidal location, showed a decrease in net photosynthesis (NP) with increasing salinity above 100‰. The degree of reduction was most dramatic in mat L (from 1.38 mol m⁻³ at 65‰ to 0.17 mol m⁻³ at 200‰), the mat that exhibited the highest oxygen concentration of all mats at 65‰ salinity. Mat U showed a flatter decrease in net oxygen production with increasing salinity compared with a sharper decrease in mats L and M. Oxygen

Table 2. Characteristics of the studied microbial mats and their tidal positions.

Sample	Field characteristics			Mat characteristics	
	Salinity (‰) ^a	Salinity range (‰) ^b	Tidal location	Description	Dominant cyanobacteria ^c
Mat L	65	60–80	Lower tide	Light red, rough, flat	<i>Microcoleus chthonoplastes</i> , <i>Leptolyngbya</i>
Mat M	100	60–100	Middle tide	Bright red, soft, flat	<i>Leptolyngbya</i> , <i>Halotheca</i>
Mat U	200	60–200	Upper tide	Dark red, very gelatinous, soft	<i>Leptolyngbya</i> , <i>Oscillatoria</i>

- a. Salinity at the time of sampling (low tide).
- b. Measured in a daily cycle.
- c. By direct microscopy.

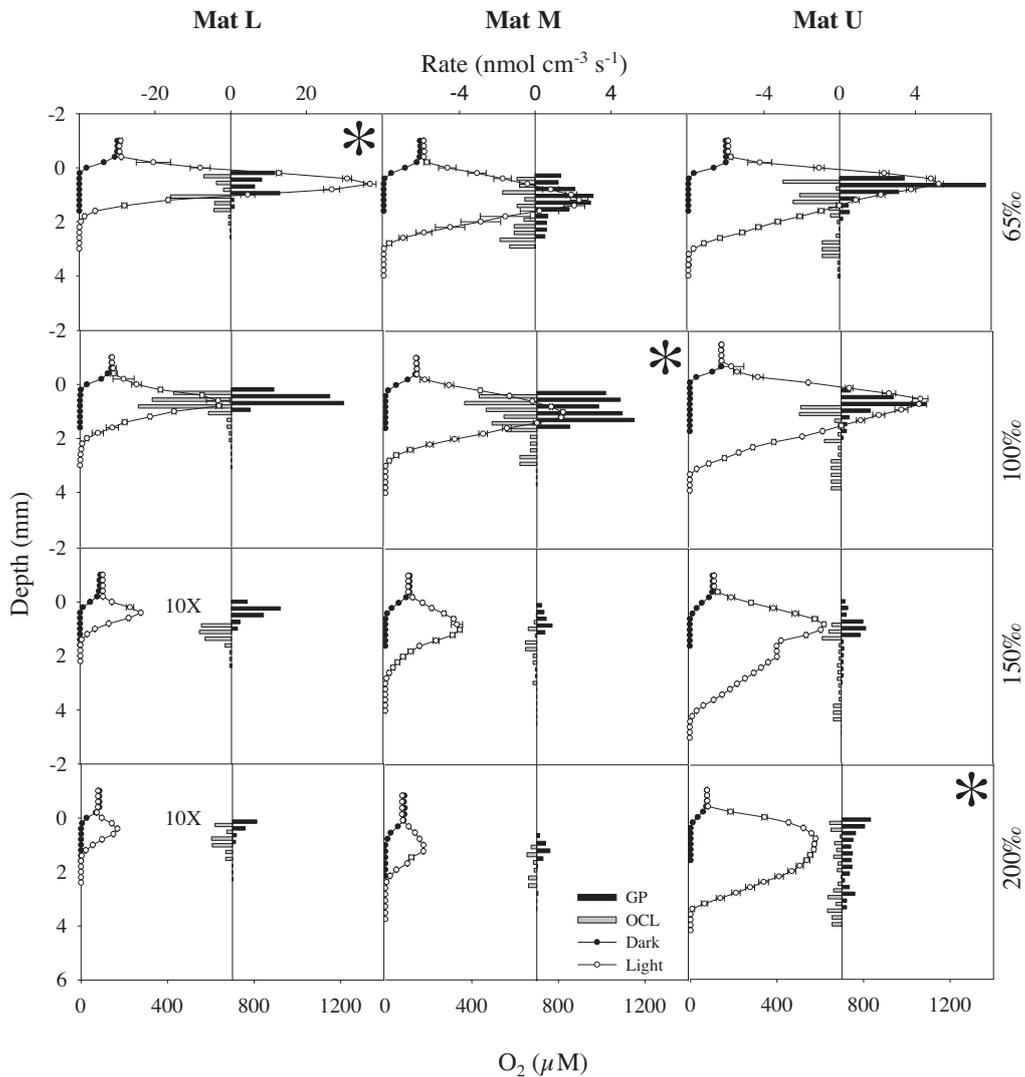


Fig. 4. Mean depth profiles of oxygen in the light and in the dark measured at different salinities. Black bars represent average rates ($n = 3$) of volumetric GP whereas grey bars indicate average rates ($n = 3$) of volumetric light oxygen consumption (OCL). Volumetric GP and OCL rates for mat L at salinities of 150‰ and 200‰ were multiplied by 10 for clarity. Note that the upper scale for mat L is different from those for mats M and U. Boxes with asterisk in the top-right corner are measurements performed at the *in situ* salinities of the mats (i.e. during the lower tide). Standard deviations from three measurements are indicated by error bars.

1388 R. M. M. Abed, K. Kohls and D. de Beer

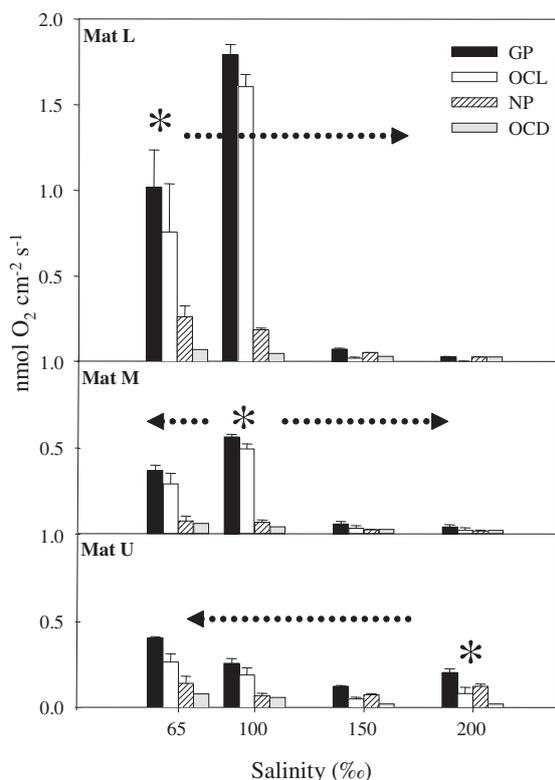


Fig. 5. Salinity dependence of areal rates of GP, NP and OCL and OCD in three microbial mats from lower (L), middle (M) and upper (U) tidal positions. The rates marked with asterisk were obtained from measurements at the mats' original salinities during the lower tide. The arrows indicate the direction of experimental salinity shift.

penetration decreased with increasing salinity in mat L (from 2 mm to 1.2 mm) and mat M (from 3 mm to 2 mm) while it did not change significantly in mat U (3.4 mm).

Gross photosynthesis and OCL showed similar response to different salinities as inferred from their calculated areal rates (Fig. 4). All mats, regardless of their tidal position, showed lower rates of the two processes at higher salinities (150‰ and 200‰) compared with lower salinities (65‰ and 100‰). Areal rates of GP in mat L and mat M were highest at 100‰ salinity, although net oxygen production at this salinity was lower than at 65‰ salinity. Above 100‰ salinity, areal rates of GP and OCL strongly decreased. In mat U, the two processes decreased with increasing salinity and maximum rates were detected at 65‰. At 200‰ salinity, mat U maintained 5–10-fold higher areal rates of GP and OCL than mat L and mat M. Consequently, the extent of inhibition of the two processes was dependent on the tidal position of the mat (i.e. the lower tidal mat was more sensitive than the upper tidal mat). Dark O₂ profiles did not change measurably and O₂ in the dark was fully consumed in the uppermost 0.5 mm of each mat.

Discussion

Microbial mats on intertidal flats experience fluctuations in several environmental parameters, such as salinity, temperature, desiccation, UV and light intensity. We consider salinity fluctuations in the studied site to be the most significant because variations in the other factors were minimal. Temperature differences among the mats were below 3°C and desiccated mats were excluded from this study. The salinity regimes of the studied mats appeared to determine the diversity and halotolerance of bacterial communities as well as the rates of GP and OCL.

Comparing the mats at their ambient salinities

The degree of salinity effect on the studied mats was clearly dependent on the mat's tidal position and the salinity range they experience. Each of the studied mats apparently developed its own microbial communities in correlation with distinctly different salinity regimes. Only 12 OTUs (out of a total of 148) occurred in all mats. The abundance and diversity of cyanobacteria was highest in the lower and middle tidal mats, but dropped significantly in the uppermost tidal mat, probably in favour of species that are better adapted to high salinities and salinity fluctuations (Golubic, 1980). Studies on Guerrero Negro mats also showed that *Microcoleus chthonoplastes*, which dominated the mats at salinities between 60‰ and 120‰, was replaced by other *Phormidium*- and *Synechococcus*-related cyanobacteria at salinities > 150‰ (Javor, 1989; Rothschild, 1991; Des Marais, 1995). A reversed pattern of distribution was shown in the CFB group, which was more abundant and diverse in the upper tidal mat than in the middle and lower tidal mats. This could be attributed to the clearly visible high amounts of extrapolymeric substances (EPS) in the upper tidal mat (Kirchman, 2002), which supported the growth of CFB. Extrapolymeric substance production by phototrophs was shown to be stimulated under high salt stress (Liu and Buskey, 2000; Abdullahi *et al.*, 2006). The clone library of mat U showed many sequences related to the extremely halophilic *Salinibacter ruber* (Anton *et al.*, 2002; Benlloch *et al.*, 2002), which uses a similar mode of haloadaptation to the Archaea of the family *Halobacteriaceae* and does not accumulate organic osmotic solutes as in halophilic aerobic bacteria (Oren *et al.*, 2002; Corcelli *et al.*, 2004). The detection of *Salinibacter*-related species in mat U and not in mats L and M is consistent with the high salinities at the upper tidal zone.

Comparing rates of GP and OCL at the ambient salinities of the mats (Fig. 5, bars marked with asterisk), both processes were reduced with increasing salinity, although disproportionately. Moving from the low tidal mat to the middle tidal mat, GP was inhibited more than OCL (44%

and 34% respectively), resulting in a lower rate of NP. In contrast, OCL was inhibited more strongly relative to GP when moving to high salinity in the upper tidal mat (84% and 65% respectively), resulting in a relatively high NP. Over time, this distinction may be reflected in the accumulated mat biomass (i.e. thickness).

The reduction of photosynthetic activity along the increasing salinity gradient correlated well with reduction in cyanobacterial abundance and diversity. In contrast, the diversity and abundance of aerobic heterotrophs showed an opposite trend to the rates of OCL. It is conceivable that all respiring bacterial populations survived at 200‰ salinity, but only the halophilic part of them were active. The rest of the community may become active when salinity lowers during the tidal cycle. This assumption is supported by the known intermediate disturbance hypothesis (Buckling *et al.*, 2000; Johst and Huth, 2005) according to which disturbances that are neither too rare nor too frequent (in this case salinity fluctuation), may not affect the resistant fraction of the original community but may promote the growth of halophilic bacteria, thus leading to increased biodiversity. It is also possible that under stressed conditions degradation of organic matter becomes selective, which results in accumulation of recalcitrant compounds like EPS, attracting specialists such as CFB.

Regulation of photosynthesis and respiration by salinity

The inhibition of OCL and GP at high salinities was consistent with previous reports on other mat systems (Garcia-Pichel *et al.*, 1999; Wieland and K uhl, 2006). The three mats exhibited inhibition of OCL and GP at salinities > 100‰, irrespective of their tidal position. However, the extent of inhibition in the upper tidal mat was less than in the lower and middle tidal mats, indicating an increase in the mats' halotolerance by moving away from the waterline. The adaptation of mats microorganisms to salinity changes is apparently determined by the exposure frequency to tidal seawater and the extent of evaporation.

The photosynthesis inhibition at higher salinities could be explained by direct and indirect salinity effects. Salinity exerts an osmotic stress on microorganisms, which might directly limit their metabolic activities. Cyanobacteria were shown to tolerate high salinities by accumulating inorganic (K⁺ ions) and organic (sugars, polyols, and quaternary amines, etc.) osmoregulators intracellularly in order to reach an osmotic equilibrium with the surrounding environment (Blumwald and Tel-Or, 1983; Mackay *et al.*, 1983; Reed *et al.*, 1984; Csonka, 1989; Fernandes *et al.*, 1993; Galinsky, 1995). The production of these compounds requires a reallocation of a significant fraction of cell resources, consequently affecting other metabolic processes (Pinckney *et al.*, 1995). Inhibition of photosynthesis by high salinity could also be due to the limited

availability of CO₂, given that the solubility and diffusion coefficient of gases decrease with increasing salinity. Garcia-Pichel and colleagues (1999) proposed a different mechanism to explain inhibition of photosynthesis by salinity in hypersaline microbial mats from Mexico. They postulated that the elevated oxygen tensions within mats, due to increasing diffusion barriers to oxygen escape in the light at high salinities, may have a negative effect on photosynthesis. High oxygen tensions result in photooxidation in mats and oxygen acts as a competitive inhibitor of RubisCO carboxylase activity. If GP rate should remain constant with increasing salinity, the oxygen partial pressure should increase to reach pressures that could result in structural damage to the community (2 atm at 150‰ and 4 atm at 230‰ salinity). As these partial pressures are unlikely to be reached, the inhibition of GP is an obvious consequence.

The response of OCL to increasing salinity was in all cases similar to the response of GP. This is because these two processes depend on each other (Canfield and Des Marais, 1993; K uhl *et al.*, 1996) and inhibition of either will consequently result in the inhibition of the other. OCL inhibition might also be due to the decrease in oxygen solubility and diffusion coefficient at elevated salinities (the brine at 200‰ salinity has almost half the capacity to hold oxygen than the brine at 65‰ salinity). The influence of salinity on OCD was more conservative than on OCL because OCD was solely dependent on diffusion limitation of oxygen from the overlying water.

In conclusion, large fluctuations in salinity resulted in lower rates of GP and OCL as well as shifts in the community composition in the favour of aerobic heterotrophic bacteria. The rates of GP and OCL decreased at high salinities, irrespective of the salinity regime of the mats. The halotolerance of microbial mats along intertidal flats increases from lower to upper tidal zones, due to the exposure to a wider range of salinity. Microbial mats that are located at different tidal positions adjust the structure and function of their bacterial communities in response to the salinity range they experience.

Experimental procedures

Origin of mat samples

Mat samples were collected in December 2004, from the Sabkha of Abu Dhabi (Arabian Gulf coast, United Arab Emirates) during lower tide. Triplicate mat pieces (8 × 15 cm) were collected from different tidal positions (0.5 km apart) along a transect perpendicular to the low water line, i.e. lower, middle and upper. The mats were different in salinity, texture and appearance (Table 2). The salinity of the overlying water was measured during the lower tide using a portable refractometer and was found to be 60‰, 100‰ and 200‰ for mats L, M and U respectively, but during inundation all mats had 60‰ salinity. During lower tide, the mats were subjected to

1390 R. M. M. Abed, K. Kohls and D. de Beer

continuous evaporation but they were always covered with seawater. Seawater from the same sampling sites was collected for use in incubation experiments. The mat samples were transported immediately to the laboratories of United Arab Emirates University (UAEU, Al-Ain), where incubation experiments and microsensor measurements were completed within 3 weeks. Frozen samples were shipped to the Max-Planck Institute for Marine Microbiology in Bremen, Germany, where the molecular work was performed.

Molecular analysis

The microbial communities within the three mats were investigated using DGGE and 16S rRNA clone library construction. The upper 2–3 mm of triplicate cores of each mat (c. 300–500 mg each) were subjected to nucleic acid extraction, polymerase chain reaction (PCR) and DGGE as previously described (Abed and Garcia-Pichel, 2001). Polymerase chain reaction was carried out using the primers GM5F (with GC-clamp) and 907R (Muyzer *et al.*, 1995). Denaturing gradient gel electrophoresis was carried out using a Bio-Rad D-Code system and was run at 60°C and at a constant voltage of 200 V for 3.5 h.

For cloning, the 16S rRNA genes were PCR amplified using the primers GM3F and GM4R (Muyzer *et al.*, 1995) at an annealing temperature of 42°C. The PCR products were purified using the QIA quick PCR purification kit (Qiagen, Hilden, Germany) and were cloned using the TOPO TA Cloning Kit (Invitrogen, Karlsruhe, Germany) according to the manufacturer's instructions. The obtained clones were screened for the presence of inserts and the positive clones were then sequenced.

Phylogenetic analysis and biodiversity indices

The obtained sequences of the clones were analysed using the ARB program (Ludwig *et al.*, 1998). Phylogenetic trees were calculated by maximum parsimony based on long 16S rRNA sequences (more than 1300 bp). The obtained sequences were then inserted into the reconstructed tree by applying parsimony criteria, without allowing changes in the overall topology.

For determining the number of OTUs, similarity matrices among the sequences were calculated with the ARB program. One OTU was defined for sequences which have more than 97% similarity. Rarefaction curves were calculated using the freeware program aRarefactWin (available at <http://www.uga.edu/~strata/software/Software.html>). The coverage of the clone libraries, species richness, species evenness and Shannon–Weaver index of diversity were calculated as previously described in Good (1953), Atlas and Bartha (1998) and Singleton and colleagues (2001).

Salinity shift experiment

This experiment was designed to study the effect of salinity on OCL and GP in the mats L, M and U. The mat samples were incubated in small aquaria filled with seawater that had the original salinity of their respective sites (during lower tide) and left for at least one day to acclimate. The water in the

aquaria was continuously aerated and the mats were kept under natural light conditions until analysis. Salinity was maintained constant throughout the experiment by continuously replenishing the evaporated water. Seawater with the salinities 60‰, 100‰, 150‰ and 200‰ was either obtained directly from the field sites or prepared by mixing the original seawater of different salinities till the desired salinity was reached. These brines were filtered prior to use. Mat L was first incubated at 60‰ (i.e. the site salinity) and then subjected to upward salinity shifts whereas mat U was incubated first at 200‰ salinity and then subjected to downward salinity shifts. In case of mat M, the salinity shifts followed the order 100‰ (field salinity), 60‰, 150‰ and then 200‰. Each mat was incubated for 2–3 days at each salinity before microsensor measurements were carried out.

Microsensor set-up

Oxygen profiles were measured at steady state in the dark (overnight) and in the light (after 3–4 h illumination), for each incubation salinity. The measurements were performed in 200 µm steps inside the mat till a maximum depth of 5 mm. Microprofiles were measured using an automated set-up controlled by a computer. Microsensor positioning with 1 µm precision was done using a VT-80 linear positioner (Micos, Germany) equipped with a DC motor (Faulhaber, Germany). The oxygen microsensors were connected to fast-responding picoamperimeters. The sensors were positioned at the mat surface with the help of a dissection microscope (Zeiss, SV6, Germany). Signals were acquired through data acquisition card (DAQCard-AI-16XE-50, National Instruments, Austin, TX, USA) on a computer.

Microsensor measurements

Oxygen concentrations in the mats were determined with fast-responding Clark-type oxygen microsensors with guard cathodes (Revsbech, 1989). The oxygen microsensors had tip diameters of 20–30 µm, a stirring sensitivity of < 2%, and a response time of $t_{90} < 0.5$ s. A two-point linear calibration of oxygen electrodes at each salinity was performed (i.e. reading in the anoxic layer equals 0% oxygen and reading in the overlying constantly aerated seawater equals 100% air saturation) (Revsbech, 1989). The oxygen solubility at the experimental temperature and salinity were obtained from the table available at <http://www.unisense.dk>.

Gross photosynthesis rates were measured by the light-dark shift method (Revsbech and Jørgensen, 1986). Gross photosynthesis was recorded in 250 µm steps inside the mat from linear regression of each local oxygen depletion rate during the first 1–3 s period after darkening. From oxygen concentration and GP rate profiles, OCL rates were determined based on the mass balance.

$$\text{OCL} = \text{GP} - \text{NP} \quad (1)$$

Net photosynthesis was determined by fitting the steady-state oxygen profile with a diffusion model (Berg *et al.*, 1998). The diffusion coefficient in the mat was taken as 60% of that in the overlying seawater (Gröttschel *et al.*, 2002).

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Calculation of areal rates

Areal rates (in mol O₂ m⁻² s⁻¹) of GP were calculated by integrating the volumetric GP rates over the depth of the photosynthetic zone. Areal NP rate was obtained by calculating the O₂ flux in the diffusive boundary layer (DBL) above the mat surface using Fick's first law of diffusion (Jørgensen and Revsbech, 1985; Jensen and Revsbech, 1989) and the oxygen diffusion coefficient in seawater (D_o) corrected for temperature and salinity. Areal rates of OCL were calculated by subtracting the areal NP from the areal GP rate. Areal rates of OCD were obtained by calculating the flux at the DBL from the profiles measured in the dark.

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1392 R. M. M. Abed, K. Kohls and D. de Beer

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Supplementary material

The following supplementary material is available for this article online:

Fig. S1. 16S rRNA-based phylogenetic reconstruction based on maximum-parsimony methods, showing the affiliation of the 16S rRNA sequences obtained by cloning from the lower tidal mat (L). The sequences of the clones were inserted into the reconstructed consensus tree by applying parsimony criteria without allowing changes in the overall tree topology. The tree was simplified for clarity by omitting all sequences between clusters. The bar indicates 10% sequence divergence.

Fig. S2. 16S rRNA-based phylogenetic reconstruction based on maximum-parsimony methods, showing the affiliation of the 16S rRNA sequences obtained by cloning from the middle tidal mat (M). The sequences of the clones were inserted into the reconstructed consensus tree by applying parsimony criteria without allowing changes in the overall tree topology. The tree was simplified for clarity by omitting all sequences between clusters. The bar indicates 10% sequence divergence.

Fig. S3. 16S rRNA-based phylogenetic reconstruction based on maximum-parsimony methods, showing the affiliation of the 16S rRNA sequences obtained by cloning from the upper tidal mat (U). The sequences of the clones were inserted into the reconstructed consensus tree by applying parsimony criteria without allowing changes in the overall tree topology. The tree was simplified for clarity by omitting all sequences between clusters. The bar indicates 10% sequence divergence.

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Publication 3

Lipid biomarkers, pigments and cyanobacterial diversity of microbial mats across intertidal flats of the arid coast of the Arabian Gulf (AbuDhabi,UAE)



RESEARCH ARTICLE

Lipid biomarkers, pigments and cyanobacterial diversity of microbial mats across intertidal flats of the arid coast of the Arabian Gulf (Abu Dhabi, UAE)

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cyanobacterial mats; arid climate; extreme environment; scytonemin; denaturing gradient gel electrophoresis (DGGE); Arabian Gulf.

Abstract

Variations in morphology, fatty acids, pigments and cyanobacterial community composition were studied in microbial mats across intertidal flats of the arid Arabian Gulf coast. These mats experience combined extreme conditions of salinity, temperature, UV radiation and desiccation depending on their tidal position. Different mat forms were observed depending on the topology of the coast and location. The mats contained 63 fatty acids in different proportions. The increased amounts of unsaturated fatty acids (12–39%) and the *trans/cis* ratio (0.6–1.6%) of the cyanobacterial fatty acid *n*-18:1 ω 9 in the higher tidal mats suggested an adaptation of the mat microorganisms to environmental stress. Chlorophyll *a* concentrations suggested lower cyanobacterial abundance in the higher than in the lower intertidal mats. Scytonemin concentrations were dependent on the increase in solar irradiation, salinity and desiccation. The mats showed richness in cyanobacterial species, with *Microcoleus chthonoplastes* and *Lyngbya aestuarii* morphotypes as the dominant cyanobacteria. Denaturing gradient gel electrophoresis patterns suggested shifts in the cyanobacterial community dependent on drainage efficiency and salinity from lower to higher tidal zones. We conclude that the topology of the coast and the variable extreme environmental conditions across the tidal flat determine the distribution of microbial mats as well as the presence or absence of different microorganisms.

Introduction

Microbial mats are among the oldest and most compact ecosystems on earth, sharing very close similarities with the Precambrian fossil stromatolites (Walter *et al.*, 1992; van Gemerden, 1993). They are distributed worldwide in a diverse range of extreme environments, typically where the abundance and activity of grazing organisms is restricted (Javor & Castenholz, 1984; Farmer, 1992). Cyanobacteria, the dominant oxygenic phototrophs in microbial mats, are conjectured to have been the predominant forms of life on early earth for more than 2 billion years, and were likely

responsible for the creation of earth's atmospheric oxygen, through their oxygenic photosynthetic metabolism. In modern ecosystems, cyanobacteria prevail whenever conditions become more extreme (Golubic, 1991). Special attention has, therefore, been paid to extreme environments and the organisms therein with the aim being to understand life on early earth and to enable the search of life on other planets. The diversity of cyanobacteria in microbial mats has been studied under extreme conditions of salinity (e.g. hypersaline lakes and marine intertidal flats), temperature (hot springs) and light intensity and UV radiation (hot deserts) (Ferris *et al.*, 1996; Ward *et al.*, 1997; Nübel *et al.*,

1999, 2000; Ward & Castenholz, 2000; Abed & Garcia-Pichel, 2001; Garcia-Pichel & Pringault, 2001).

Coastal intertidal flats of the Arabian Gulf harbor various types of mats that experience the following extreme environmental parameters combined: salinity that may reach over 22‰ (depending on the mat's tidal position), solar irradiation and temperature increased up to 55 °C in hot summers. Rainfall is sporadic, about 40–60 mm year⁻¹, as compared with evaporation rates of about 1500 mm year⁻¹ (Kinsman & Park, 1976). The mats are exposed to intense sunlight, leading to high evaporation rates of seawater and desiccation. These conditions are expected to attract microbial extremophiles. In spite of the unique environmental settings of the Arabian Gulf mats, little is known about their structure and bacterial diversity. Most previous studies on this mat system focused on the geological record of the area and the description of the landscape (Kendall & Skipwith, 1968, 1969; Purser, 1973; Kinsman & Park, 1976).

The present study applied molecular methods to characterize microbial mats on intertidal flats on the south-eastern coast of the Arabian Gulf at Abu Dhabi, United Arab Emirates (Fig. 1). The structure, distribution and composition of cyanobacterial communities were studied using a polyphasic approach that included direct light microscopy, enrichment cultivation, denaturing gradient gel electrophoresis (DGGE), 16S rRNA gene sequencing and distribution of lipid biomarkers and pigments. A combination of these techniques was applied to circumvent the limitations of each approach. The aim of the study was (1) to investigate the effect of multiple extreme conditions of salinity, temperature, desiccation and irradiation on the development and distribution of mat types as well as on the distribution of their fatty acids and pigments and (2) to explore the

diversity of extremophilic cyanobacteria that tolerate the harsh conditions of the arid climate of the Gulf.

Materials and methods

Mat samples and their environmental settings

Mat samples were collected in December 2004, during low tide (Fig. 2). Triplicate mat pieces were collected from different tidal positions along a transect perpendicular to the low waterline, at low, middle and high intertidal ranges. The mat samples were frozen on site and shipped to the Max-Planck Institute in Bremen. The collected mats were different in appearance, texture and ambient salinity (Fig. 2). All mats were exposed to 6‰ salinity during high tide as measured using a portable refractometer. The salinity of the overlying water during the lower tide was found to be 6‰, 8–10‰ and 20‰ (low, middle and upper intertidal). Air temperature in this region typically reaches above 50 °C during hot summers and between 15 and 35 °C in winter. Thus, the mats experience seasonal temperature difference ranges of 25–35 °C. The water temperature in summer was 32 °C at high tide, whereas in pools remaining separated during low tide, it reached 50 °C.

Fatty acid analysis

Fatty acids were extracted from the uppermost centimeter of the studied mats using a method modified after Bligh & Dyer (1959), White *et al.* (1979), and Fang & Findlay (1996). A single-phase solvent mixture of methanol/dichloromethane/ammonium acetate buffer [pH 7.6, 2:1:0.8 (v/v/v)] was added to the homogenized mat samples. Dichloromethane and distilled water were added to the pooled extracts until a ratio of methanol/dichloromethane/

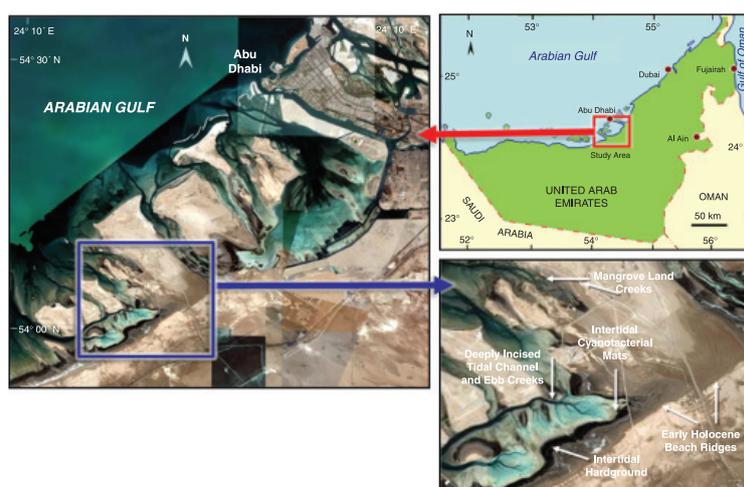


Fig. 1. Map showing the location of the studied cyanobacterial mats on the Arabian Gulf coast near Abu Dhabi and satellite images of the sampling site modified from Google Earth. Image © 2007 DigitalGlobe.

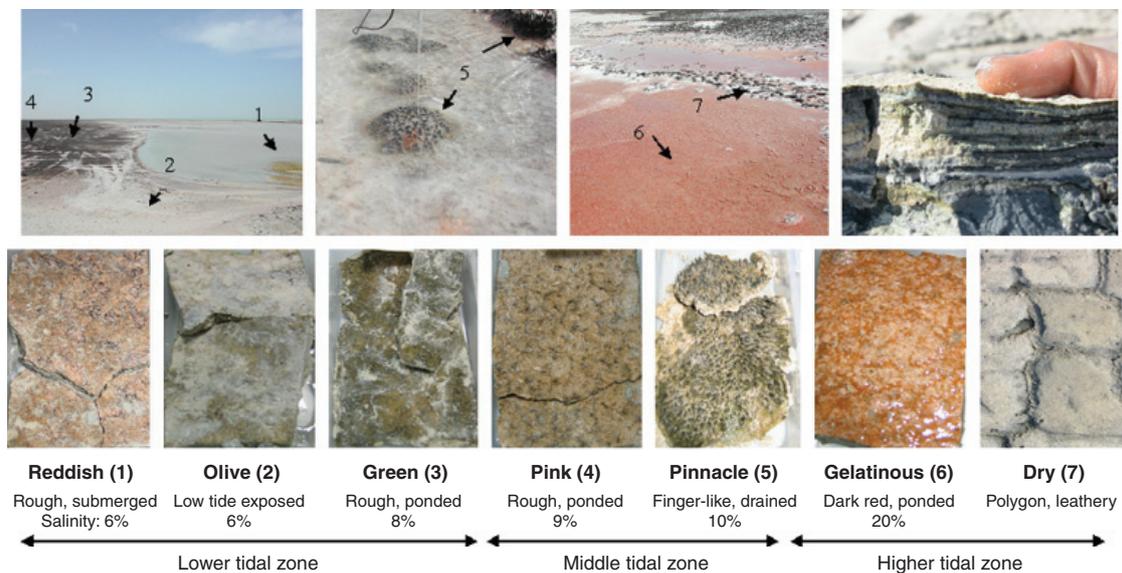


Fig. 2. Photographs showing the landscape of microbial mats in the intertidal flat of Abu Dhabi (top line). The numbers in the top photographs show the tidal positions of the collected mats (bottom line). The mat samples collected from the lower, middle and higher tidal zones show variations in texture, color and morphology.

ammonium acetate buffer of 1 : 1 : 0.9 (v/v/v) was obtained. The organic phase with the extracted lipids was separated, evaporated to dryness and stored at -20°C . Subsequently, lipid extracts were dissolved in dichloromethane/methanol 9 : 1 (v/v) and separated using liquid chromatography according to Zink & Mangelsdorf (2004). Four fractions were eluted from the column: (1) neutral lipids, (2) free fatty acids, (3) glycolipids and (4) phospholipids. Aliquots of the phospholipid fractions were transesterified with trimethylsulfonium hydroxide as described by Müller *et al.* (1998). Identification and quantification of the resulting methyl esters were performed by GC-MS analysis using a Finnigan MAT SSQ 710B mass spectrometer (Finnigan-Thermoquest, San Jose, CA). Individual components were identified by comparing their mass spectra with published spectra or with bacterial fatty acid methyl ester standards (Sigma Aldrich, Taufkirchen, Germany).

Pigment analysis

Triplicate subsamples (top 2–3 mm) of the studied mats were used for pigment extraction. The samples were washed in ice-cooled sodium chloride solution (8% NaCl), vortexed and then centrifuged for 10 min at 2200 g. Extraction of pigments was performed with ice-cooled 100% methanol after sonication and incubation at -20°C for 24 h. The supernatants were filtered through a 0.45- μm Acrodisc[®] CR 4-mm syringe filter (Pall Gelman Laboratory). The whole

procedure was carried out on ice under dim light. The pigments were analyzed using reverse-phase HPLC that consisted of a Waters 996 photo diode array detector (PDA) and a Waters 2690 separation module (Waters, MA). A 125 mm \times 4.6 mm vertex column packed with a Eurospher-100 C18 of 5- μm particle size was used (Knauer GmbH, Berlin, Germany). The pigments were identified by comparing the retention time and the spectrum with commercially available pigment standards: chlorophyll *a* (Chl *a*) and β -carotene from DHI Water and Environment, Denmark; bacteriochlorophyll *a* (Bchl *a*) from *Rhodospseudomonas sphaeroides* from Sigma-Aldrich; and scytonemin from Merck, Germany.

Light microscopy and enrichment cultivation

Morphological observations were made using Axiovert and Axioplan Zeiss photomicroscopes equipped with phase-contrast and Nomarski (DIC) optical systems, applying sample preparation as described previously (Palinska *et al.*, 1998). Morphological identification was carried out in accordance with traditional phycological (Geitler, 1932; Komárek & Anagnostidis, 1999, 2005) and bacteriological (Castenholz, 2001) systems, while awaiting further confirmation by molecular sequencing.

Single filaments and cell colonies from the field samples were separated using a binocular microscope and then placed on three different types of media: 'Castenholz'

(Castenholz, 1981), 'RC' (van Rijn & Cohen, 1983) and ASNIII (Rippka *et al.*, 1979) solidified with 0.9% agar. Unicyanobacterial cultures were obtained after several transfers of single filaments to fresh media. All cyanobacterial cultures were maintained in ASNIII medium at 35 °C. The cultures were illuminated using Osram tungsten light tubes providing a photosynthetic photon flux density (PPFD) of 30.5 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ (measured using a LICOR LI-185B quantum radiometer/photometer equipped with a LI-190SB quantum sensor) and with a light/dark cycle of 12 h/12 h. The 16S rRNA gene sequences of the obtained cultures were phylogenetically analyzed.

Molecular analysis

The photic zones (2–3 mm) of mat cores (*c.* 300–500 mg each) were subjected to nucleic acid extraction, PCR and DGGE as follows: mat cores or pellets, 10 mL of each culture (centrifuged at 4602 g, 5 min, RT), were used for the DNA extraction, resuspended in 1 mL buffer (100 mM Tris, 100 mM EDTA, 1.5 M NaCl, 1% cetyltrimethylammonium bromide, pH 8.0). After addition of lysozyme (1% final concentration), the samples were incubated at 37 °C for 1 h. After five cycles of freeze (in liquid nitrogen) and thaw (at 65 °C), 5 μL proteinase K (100 $\mu\text{g mL}^{-1}$) and 90 μL 10% sodium dodecyl sulfate was added and the samples were incubated at 52 °C for 150 min. The samples were centrifuged in a microcentrifuge at 12 000 g for 5 min and the supernatants were extracted twice with phenol, phenol/chloroform and chloroform. The DNA was precipitated from the aqueous phase with 0.6 vol. of 2-propanol, washed with 70% ethanol, vacuum dried and stored in 100 μL TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0).

PCR for the amplification of 16S rRNA genes was carried out using cyanobacteria-specific primers CYA106F (for cultures) or CYA359F (with a 40-nucleotide GC clamp at the 5' end; for DGGE) and CYA781R (Nübel *et al.*, 1997). Thermocycling was performed using a Mastercycler gradient cycler (Eppendorf, Hamburg, Germany). After an initial denaturation step (5 min at 95 °C), followed by 80 °C for 1 min, Super Taq DNA polymerase (HT Biotechnology, Cambridge, UK) was added. Thirty-five cycles were performed at 94 °C for 1 min, 60 °C for 1 min (annealing temperature) and 72 °C for 1 min. The presence of PCR products was detected by standard agarose gel electrophoresis and ethidium bromide staining.

DGGE was carried out using a Bio-Rad D-Code system and run at 60 °C and a constant voltage of 200 V for 3.5 h. The DGGE bands were excised manually under the UV light, the DNA allowed to diffuse out in 50 μL of PCR water at 4 °C overnight and PCR reamplified using the same primers (CYA359F and 781R) as described above. The amplification products of the DGGE bands and cultures were purified

using the QIAquick PCR Purification Kit (Qiagen, Hilden, Germany) and sequenced in both directions. The primers were the same as for amplification.

Phylogenetic analysis

Sequence alignment and phylogeny of 16S rRNA gene fragments obtained from DGGE bands as well as from the cultures were carried out using the ARB software (Ludwig *et al.*, 1998) version 07.12.17prv and the official database (www.arb-home.de) of February 2005 for small subunit RNA sequences (ssu_jan04_corr_opt.arb). Additionally, cyanobacterial gene sequences available from the GenBank were imported and aligned in the database of the ARB software. These sequences were then aligned with the sequences in the ARB database using the alignment ARB tool. The alignment was corrected manually. The phylogenetic tree was calculated by maximum likelihood, based on long 16S rRNA gene sequences (>1300 bp). The sequences were inserted into the pre-established tree using the parsimony ARB tool, while maintaining the overall tree topology without changes. The final tree was minimized for simplicity in presentation.

Nucleotide sequence accession numbers

The sequences determined in this study have been deposited in the GenBank database under accession numbers EU024357–EU024387. Sequences were checked for chimera formation using the CHIMERA CHECK program of the Ribosomal Database Project II database (Cole *et al.*, 2003). The assumption was that true chimeras consist of fragments that each have closer database relatives than the full-length sequence has.

Results

Intertidal mat landscape

Seven different mat types were identified across the intertidal zone of the coast of Abu Dhabi (Fig. 1) by moving from the waterline landward. These mats can be tentatively classified by their position across the range of intertidal flooding and water retention, and recognized by their color and texture (Fig. 2).

Inundated flat mats (Fig. 2, Reddish, Olive and Green mats) dominate the lower intertidal zones. These mats are smooth, leathery and typically consist of a thin (*c.* 3 mm) layer that is firmly adhering to the sediment beneath. The reddish mats are always submerged whereas the olive mats are exposed briefly during the lowest tides. The dark green mats are in ponds in a slightly elevated position.

The middle tidal ranges are dominated by two strikingly different mat types. A flat mat (Fig. 2, Pink mat) is

periodically air-exposed but water logged, thus leaving a laminated sediment beneath. The mat surface is pink in color, smooth and often covered by gypsum crystals. In contrast, the pinnacle mat (Fig. 2, Pinnacle) covers well-drained elevated hills between pools, channel levees and their slopes. The surfaces of this mat grow in the form of upright pointed cones (i.e. pinnacles) that are 1–2 cm high. Below the surface layer of cyanobacteria, there is a thin, often incomplete layer of purple sulfur bacteria and an equally faint black layer indicating a transient anoxic zone.

Upper tidal ranges are characterized by shallow pools and slightly higher margins covered by a polygonally cracked mostly air-exposed dry mat. The pools are covered by finely laminated, slimy mats (Fig. 2, Gelatinous), with each layer representing a distinct functional group of microorganisms, with oxygenic phototrophs on top, underlain by purple and green anoxygenic phototrophs and then by sulfate-reducing bacteria. These mats are, unlike others, exceptionally gelatinous on the surface. They occupy the ponded channels and depressions exclusively in the upper intertidal zone, and hence are always inundated. Polygonally cracked dry mats (Fig. 2, Dry) dominate large areas of the higher intertidal zone, where the water is trapped between tides, but the mat surface is exposed to evaporation. These mats are leathery and often have a convoluted surface. Upon extensive desiccation, these mats crack, forming polygons.

Polyphasic characterization of the cyanobacterial communities

Fatty acid fingerprints

The studied mats yielded different lipid patterns based on a total of 63 fatty acids: saturated, branched, cyclopropyl, mono- and diunsaturated, in the range of C₉–C₂₈ (Fig. 3). Although the saturated straight-chain fatty acids 16:0 and 18:0 and partially the monounsaturated fatty acids 16:1 and 18:1 dominated all samples (Fig. 3a), important quantitative differences were found in the fatty acid distributions in different mat types (Fig. 3b). The 16:0 fatty acids accounted for relative amounts between 18.3% (Dry mat) and 25.6% (Pinnacle mat) of the total fatty acids. Branched saturated fatty acids were found in amounts of 8.9% (Pinnacle mat) to 18.5% (Pink mat). They consisted mainly of *iso* (*i*) and *anteiso* (*ai*) fatty acids. Several other methyl-branched saturated fatty acids with 13–18 carbon atoms were found. Most of them were of insignificant relative abundance, except for the *me* – 16:0 fatty acids, which accounted for 5.1% of the detected fatty acids in the reddish mat. The amount of cyclopropyl fatty acids ranges between 2.9% (Pink mat) and 21% (Reddish mat). The amounts of monounsaturated fatty acids ranged from 11.5% in the Gelatinous mat to 34.4% in the Pinnacle mat. Nearly equal concentrations of the *cis* and *trans* configurations of the fatty

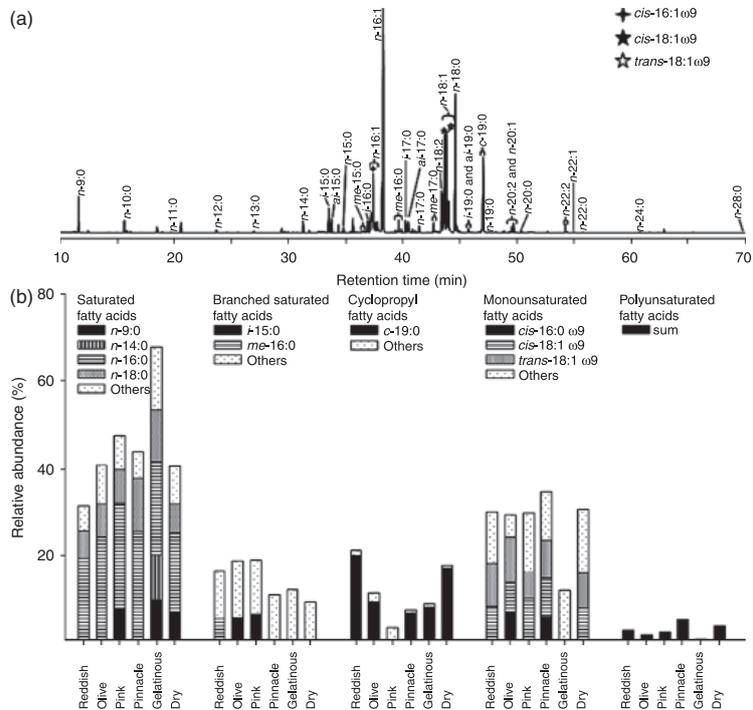


Fig. 3. Distribution of fatty acids in the intertidal microbial mats of Abu Dhabi. (a) Total ion current chromatograms of the studied microbial mats. (b) Relative abundances and distribution of different fatty acid groups in the mats.

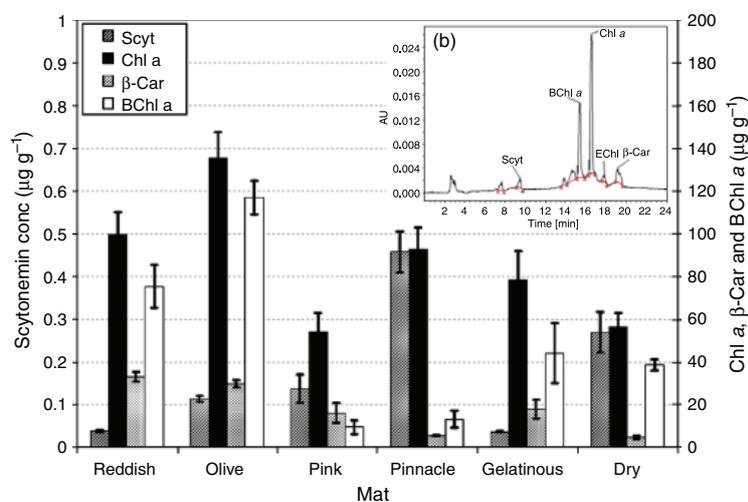


Fig. 4. Distribution of intracellular and extracellular pigments among the studied microbial mats. Note that the concentration of Chl *a* is higher in the lower compared with the higher tidal mats while the concentration of scytonemin increases from the lower to the middle tidal mats and then decreases in the higher tidal mats. The insert shows a representative HPLC chromatogram. Note that the scale for scytonemin is different than for other pigments.

acid *n*-18:1 ω 9 were detected in all mats. Their sum concentration ranged between 7.7% (Gelatinous mat) and 17.9% (Pinnacle mat) of the total fatty acids. The only polyunsaturated fatty acids found in the studied mats had two double bonds and were represented by an 18:2 fatty acid, two 20:2 and two 22:2 fatty acids. The highest amounts of polyunsaturated fatty acids were estimated in the pinnacle mat (4.7%). The sum of saturated long-chain fatty acids reached 3.9% in the dry mat, which was more than in any other analyzed mat.

Pigment analysis

The pigments Chl *a*, scytonemin, β -carotene and Bchl *a* were detected in each mat (Fig. 4), consistent with the presence of cyanobacteria (Chl *a*, scytonemin and β -carotene) and anoxygenic phototrophic bacteria (Bchl *a*). An additional peak that had a retention time of 17.7 min in the HPLC chromatograms was also detected in all mats; its spectrum resembled that of echinenone. Chl *a* and Bchl *a* concentrations (P values 0.004, $df=10$ and 0.002, $df=10$, respectively) were significantly higher in the lower tidal mats compared with the higher tidal mats. However, the ratio of Bchl *a* to Chl *a* concentration in the middle tidal mat (pink mat and pinnacle mat) was much lower than in other mats (P value 0.001; $df=16$). There is a positive correlation between mat exposure to excessive solar irradiation and scytonemin production. Scytonemin exhibited a 10-fold increase in concentration between the submersed (Reddish and Gelatinous) mats and the frequently drained or air-exposed (Pinnacle and Dry) mats.

Light microscopy

Using light microscopy, a total of 15 different cyanobacterial morphotypes were identified (Fig. 5, Table 1). Each mat

harbored a distinct cyanobacterial community with at least five morphotypes. An interesting observation was the detection of a large number of morphotypes in the gelatinous mat and the dry mat (11 and 10 morphotypes, respectively), where salinity and desiccation were most extreme. In all mats, the mat-forming *Microcoleus chthonoplastes* Thuret ex Gomont (Fig. 5a) and *Lyngbya aestuarii* Liebman ex Gomont (Fig. 5b) were often observed as the most dominant cyanobacteria. *Lyngbya aestuarii* was characterized by the presence of a dark scytonemin-colored sheath and was always present at the surface of the mat above *M. chthonoplastes* filaments. The sunscreen pigment scytonemin was also present in the envelopes of the coccoid cyanobacteria *Entophysalis major* Ercegovic (Fig. 5g and h) and *Chroococcus* sp. (Fig. 5i). Morphotype *Schizothrix splendida* Golubic (Fig. 5c) largely replaced *Microcoleus* in Pinnacle mat. Other morphotypes such as *Spirulina subtilissima* Kützing ex Gomont (Fig. 5d) occurred commonly interspersed in deeper layers of the mat, often colonizing the sheaths of *Microcoleus*. The number of very thin chlorophyll-pigmented filaments corresponding to *Leptolyngbya* and *Geitlerinema* increased in the lower layers of the mat (Fig. 5e and l). *Aphanothece* sp. (Fig. 5j) characterized the Gelatinous mats in the upper intertidal ranges. *Rhabdoderma* sp. (Fig. 5f), *Chroococcidiopsis* sp. (Fig. 5k) *Gomphosphaeria salina* Komárek et Hindak (Fig. 5m), *Aphanocapsa* sp. (Fig. 5n) and *Oscillatoria* spp. (Fig. 5o) were occasionally observed in a single mat but not in others. All mats contained at least two cyanobacteria of those known to produce scytonemin (*L. aestuarii*, *Entophysalis* spp. and *Chroococcus* sp.).

Using enrichment cultivation, a total of 10 strains of cyanobacteria were isolated, most of them showing a simple morphotype of *Leptolyngbya* or *Phormidium* (Fig. 5, M1C2, M5C6, M1C10, M4C4, M6C11 and M5C7). Only three cultures show-

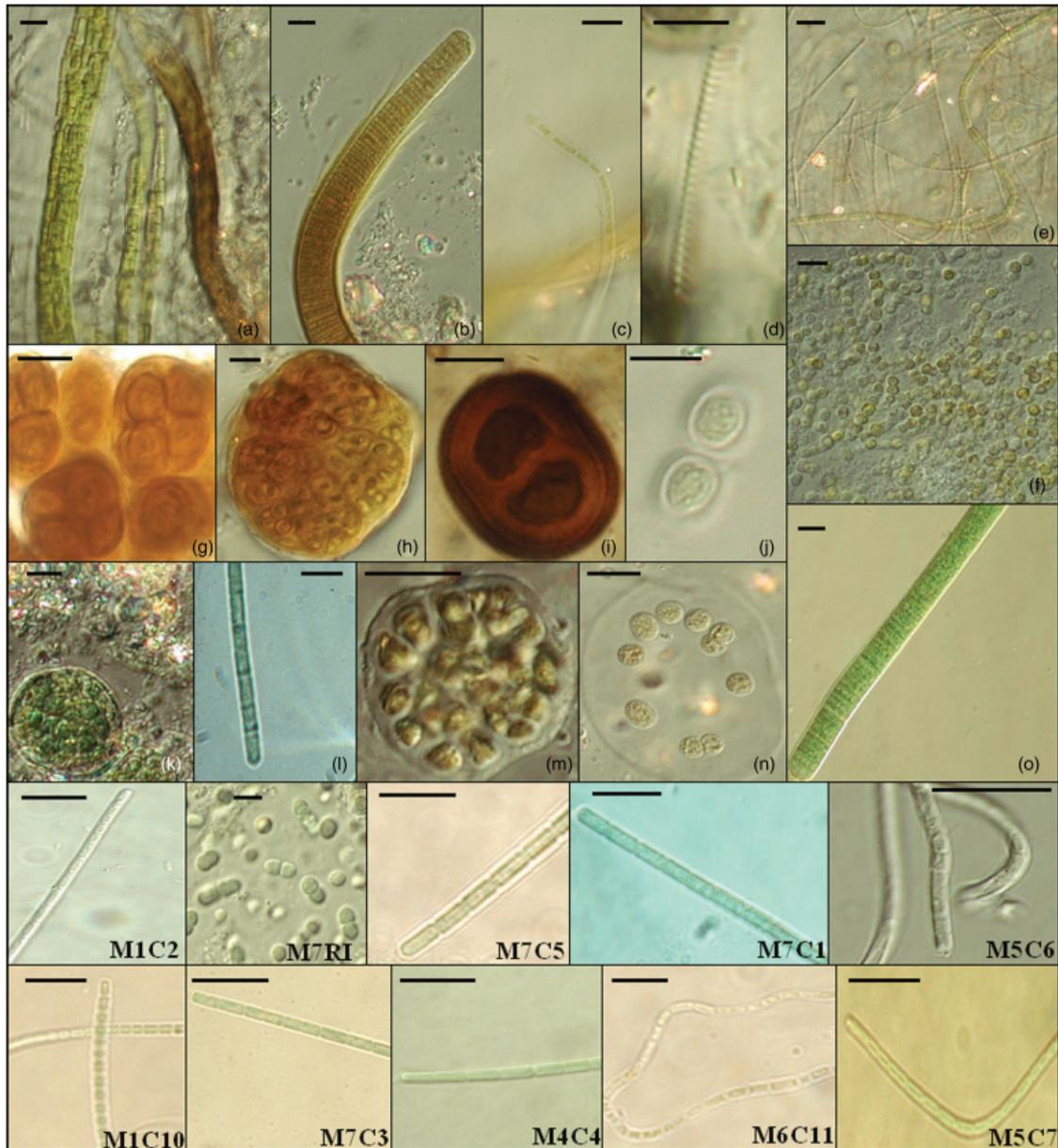


Fig. 5. Photomicrographs of identified filamentous and unicellular morphotypes of cyanobacteria in the studied mat samples. Scale bar is 10 μm for all pictures. See text and Table 1 for the identification and distribution of these morphotypes among the mats.

ed morphotypic similarity to *M. chthonoplastes* (Fig. 5, M7C5, M7C1 and M7C3) and one to *Rhabdoderma* sp. (M7RI).

DGGE patterns and molecular diversity

The DGGE gel showed a distinct banding pattern for each mat (Fig. 6). The phylogenetic tree revealed that the

sequences retrieved by DGGE showed variations in the cyanobacterial community composition among different mats (Fig. 7). Because DGGE displays dominant members of the bacterial community, the diversity of cyanobacteria detected by DGGE within each mat was, as expected, lower than by direct microscopy. The Pinnacle mat displayed the highest number of DGGE bands, whereas the Dry mat

Table 1. Comparison of the cyanobacterial community among the studied microbial mats as determined by light epifluorescence microscopy and morphological traits of identified morphotypes (see photographs in Fig. 5)

Morphotype	Probable taxon*	Cell width (µm)	Cell shape	Colony colour	Mat sample					
					Reddish	Olive	Pink	Pinnacle	Gelatinous	Dry
a	<i>Microcoleus chthonoplastes</i>	1.9–5.0	Isodiametric	Dark green	++	++	++	+	++	++
b	<i>Lyngbya aestuarii</i>	9.0–16.0	Short discoid	Brown green	++	++	++	++	++	++
c	<i>Schizothrix splendida</i>	1.5–3.5	Isodiametric	Pale green	–	+	+	++	+	–
d	<i>Spirulina subtilissima</i>	0.8–0.9	Isodiametric	Pale green	–	+	–	–	–	–
e	<i>Leptolyngbya</i> sp. 1	1.0–2.1	Isodiametric	Colourless	++	–	+	+	+	+
f	<i>Rhabdoderma</i> sp.	3.0–4.0	Cylindrical	Green	–	–	–	–	–	+
g	<i>Entophysalis major</i>	6.0–8.0	Spherical	Reddish brown	++	++	+	+	++	–
h	<i>Entophysalis</i> sp.	2.5–4.5	Spherical	Reddish brown	+	+	+	–	+	–
i	<i>Chroococcus</i> sp.	5.5–8.5	Spherical oval	Dark green	–	+	+	–	+	–
j	<i>Aphanothece</i> sp.	4.0–6.5	Oval isodiametric	Yellowish green	–	–	+	–	++	–
k	<i>Chroococcidiopsis</i> sp.	3.0	Spherical	Colourless	–	–	–	–	–	+
l	<i>Leptolyngbya</i> sp. 2	2.0–3.1	Isodiametric	Colourless	–	–	+	–	–	+
m	<i>Gomphosphaeria</i> sp.	3.0–6.0	Club-shaped	Green	–	+	+	–	–	–
n	<i>Aphanocapsa</i> sp.	5.5–7.0	Spherical	Colourless	–	+	+	–	+	–
o	<i>Oscillatoria</i> sp.	7.5–8.9	Short discoid	Dark green	–	–	–	–	+	–
M1C2	<i>Leptolyngbya</i> sp.	1.0–1.5	Isodiametric	Yellowish green	+	–	–	–	–	–
M7RI	<i>Rhabdoderma</i> sp.	4.6–5.1	Oval spherical	Yellowish green						+
M7C5	<i>Microcoleus</i> sp.	2.1–2.3	Isodiametric	Dark green						+
M7C1	<i>Microcoleus</i> sp.	2.5–5.0	Isodiametric	Dark green						+
M5C6	<i>Halomicronema</i> sp.	0.8–1.1	Oval	Green				+		
M1C10	<i>Leptolyngbya</i> sp.	1.0–1.9	Isodiametric	Dark green	+					
M7C3	<i>Microcoleus</i> sp.	2.2–2.5	Oval	Dark green						+
M4C4	<i>Phormidium</i> sp.	2.4–5.0	Oval	Green			+			
M6C11	<i>Leptolyngbya</i> sp.	1.2–2.0	Isodiametric	Dark green					+	
M5C7	<i>Halomicronema</i> sp.	0.8–1.1	Isodiametric	Green				+		

*Taxonomic names have been assigned according to Komárek & Anagnostidis (1999, 2005) nomenclature systems.

++, dominant; +, secondary; –, absent.

showed a single strong band with few much less prominent ones. The designations based on names submitted with the sequences to GeneBank do not have taxonomic validity unless independently confirmed. Sequences related to *M. chthonoplastes* were detected only in the lower tidal mats (Figs 6 and 7, Reddish-DGGE band 1, Green-DGGE band 5 and Pink-DGGE band 8) but not in the pinnacle, gelatinous and dry mats, although *M. chthonoplastes* was microscopically observed within these mats. Sequences related to *Leptolyngbya* and *Phormidium* were found in all tidal zones, whereas sequences phylogenetically affiliated with *Plectonema* only in the lower tidal mats (Green-DGGE band 6, Reddish-DGGE band 2 and Olive-DGGE band 3). Sequences related to the extremely halotolerant *Halotheca* group were detected only in the Pink and Gelatinous mats (Pink-DGGE band 9 and Gelatinous-DGGE band 15). A single sequence related to *Spirulina subsalsa* was detected in the Pinnacle mat (Pinnacle-DGGE band 10).

The phylogenetic affiliations of the cultured cyanobacteria showed closeness to sequences of the marine and hypersaline *Phormidium*, *Halomicronema*, *Oscillatoria*, *Microcoleus* and the unicellular *Cyanotheca* GeneBank designations.

Cultures related to *Microcoleus* sp. were obtained from only one particular mat (Dry mat, higher tidal zone). The remaining isolates (M1C2, M5C6, M5C7 and M7RI) were obtained from different mats in higher middle and lower intertidal zones. The sequences of only four cultures (M1C2, M7C3, M7C5 and M7C1) were related to sequences obtained from DGGE bands.

Discussion

Effect of multiple extreme environmental conditions on macro- and microstructure of microbial mats

The extent of extreme conditions of salinity, temperature, desiccation and solar irradiation, and their interplay during the tidal cycles across the coastal flats of Abu Dhabi determined the shape of microbial mats as well as the composition of microbial communities, fatty acids and pigments. The distinctive zonal distribution of different mat types reflects the harsh environmental constraints selecting for highly adapted and tolerant genotypes among

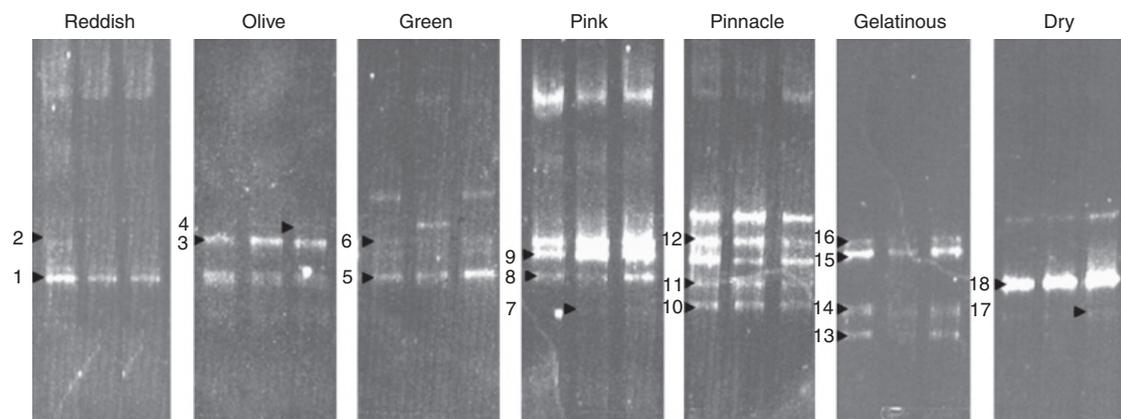


Fig. 6. DGGE fingerprints of PCR-amplified 16S rRNA gene fragments obtained from the studied microbial mats using cyanobacteria-specific primers. The shown pictures all belong to the one gel, which was cut and ordered for display. The indicated bands were excised, reamplified and sequenced.

cyanobacteria, which are the dominant primary producers and architects of these mats (Golubic, 1991). The studied mats are stratified microbial communities, vertically differentiated at mm scale responding to steep physical and chemical gradients, which are especially sharp across oxic–anoxic interfaces. These mats are also horizontally differentiated at different scales. They are significantly drier in the higher compared with the lower tidal zones due to extended exposure to intense solar irradiation and the accompanying high rates of evaporation. The flat mats prevailed in the low tidal zones and in depressions of the middle and high tidal areas where they have adequate water supply. The frequent alteration of air exposure and inundation promoted the growth of contiguous pinnacle mats on well-drained elevations in the middle tidal zones, whereas severe dryness in the higher tidal zones resulted in cracking and polygon formation. Cracking of the mats in channels begins transverse to the flow, and the size of desiccation polygons depends on their tidal position; larger polygons form in the moist center of channels but become gradually smaller towards the edges, where drying is faster (Golubic, 1991). The number of extracellular polymeric substances (EPS), as assessed by direct visualization, increased from lower to higher tidal mats, which correlated with increased salinities. Previous reports showed that EPS production by phototrophs was stimulated under high salt stress (Liu & Buskey, 2000; Abdullahi *et al.*, 2006).

The extracted Chl *a* quantities from the studied mats suggested that cyanobacteria were most abundant in lower intertidal zones where environmental conditions are optimal. Measured photosynthesis rates using oxygen micro-sensors decreased from lower to higher tidal mats (Abed *et al.*, 2007). Because the salinity and temperature can reach 20% and 55 °C, respectively, in the higher tidal mats, it is

likely that the cyanobacteria within these mats are both halotolerant and thermotolerant. The optimum temperature for photosynthesis in these mats was 45 °C (Abed *et al.*, 2006). Photosynthesis was still possible up to 60 °C and photosynthesis was detectable up to 20% salinity (Abed *et al.*, 2006, 2007). The observed strains of *Aphanothece*, *Halomiconema* and *Microcoleus* are known halotolerant and thermotolerant cyanobacteria (Karsten, 1996; Nübel *et al.*, 2000; Abed *et al.*, 2002a,b). The detection of *M. chthonoplastes* in mats where salinity reaches 20% is interesting because previous studies on various *Microcoleus* isolates showed a maximum salt tolerance up to 12% salinity (Karsten, 1996). The exclusive occurrence of the desiccation-tolerant *Chroococidiopsis* in the dry mat is consistent with the conditions prevalent in this mat. Our DGGE patterns support previous reports that changes in environmental extremes are accompanied by selection and shifts in cyanobacterial community composition (Benlloch *et al.*, 2002; Rothrock & Garcia-Pichel, 2005).

Fatty acids and environmental adaptation

The fatty acids obtained could be assigned to known major bacterial groups in microbial mats. Monounsaturated fatty acids are typical for aerobic microorganisms whereas *n*-18:1 ω 9, and *n*-16:1 ω 9 together with the polyunsaturated fatty acids 18:2, 20:2 and 22:2, indicate the presence of cyanobacteria (Grimalt *et al.*, 1992). The presence of sulfate-reducing bacteria and other anaerobic bacteria is indicated by the detection of cyclopropyl and the terminally branched (*i*- and *ai*-17:0) fatty acids (Findlay & Dobbs, 1993). The *i*, *ai*-15:0 and *i*-16:0 fatty acids are characteristic of Gram-positive bacteria whereas the *c*-19:0 acid was reported to be

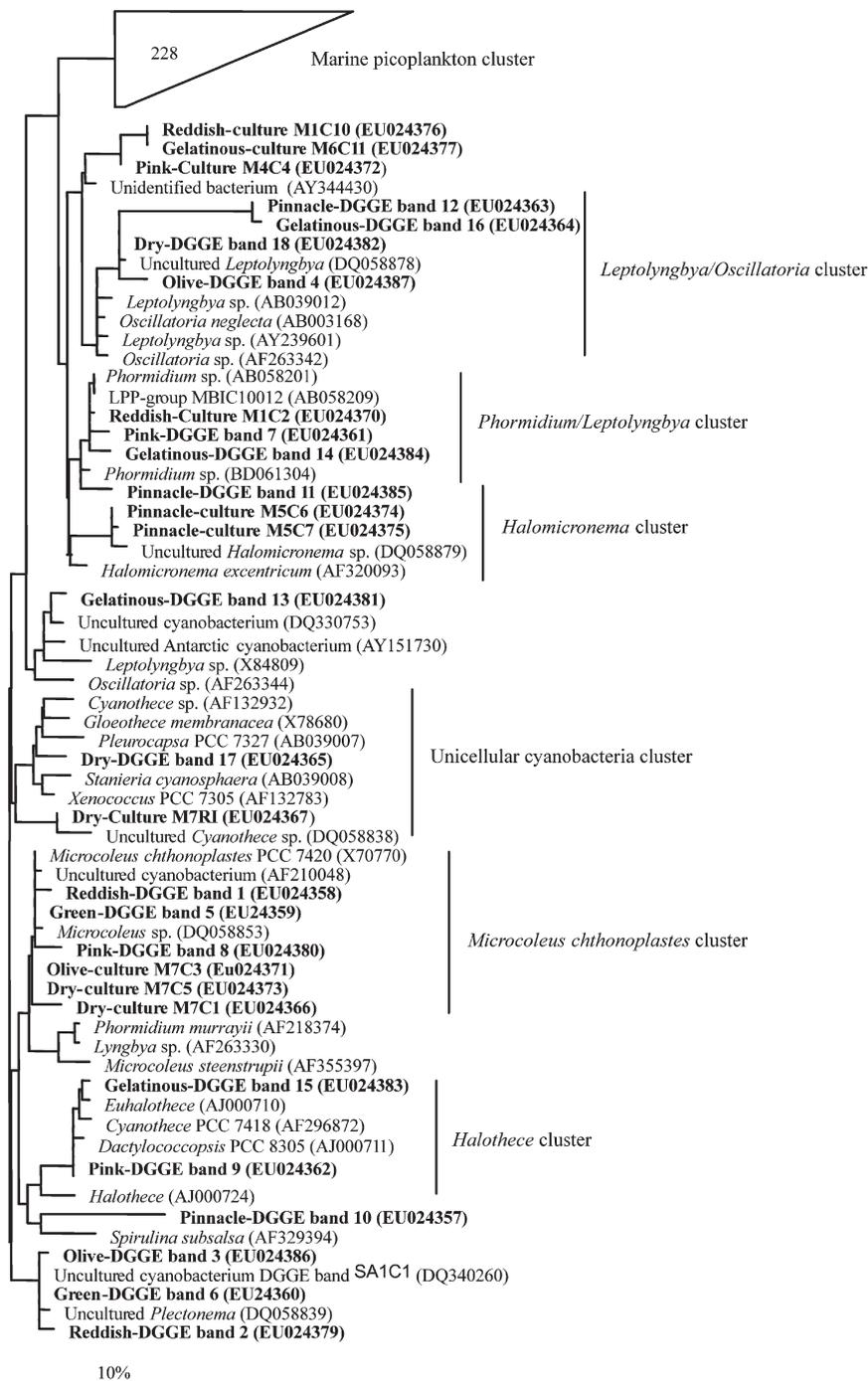


Fig. 7. 16S rRNA gene-based phylogenetic reconstruction based on maximum likelihood methods, showing the affiliation of cyanobacterial sequences retrieved from DGGE and cultures obtained from the studied microbial mats. The partial sequences were inserted into the reconstructed tree by applying parsimony criteria without allowing changes in the overall tree topology. Accession numbers are indicated in parentheses. The scale bar indicates 10% sequence divergence. Data received in this study are shown in bold.

abundant in purple phototrophic bacteria (Fourçans *et al.*, 2004). Most of the detected fatty acids in the studied mats were reported previously in other hypersaline mat systems (Grimalt *et al.*, 1992; Wieland *et al.*, 2003; Rontani & Volkman, 2005).

Many reports suggested that lipids may be involved in protection against environmental stress (Hufleijt *et al.*, 1990; Khamutov *et al.*, 1990; Ritter & Yopp, 1993). Photosynthetic microorganisms tend to synthesize more unsaturated fatty acids on increasing salinity and temperature, which results in increased fluidity of the membrane. Consequently, the Na^+/H^+ antiport system is activated, resulting in protection of photosystems I and II (Singh *et al.*, 2002). Unsaturated fatty acids were estimated to account for 12–39% of the total fatty acids in our mats. The high *trans/cis* ratio (0.6–1.6) of the cyanobacterial fatty acid *n*-18:1 ω 9 in our mats is also evident with the elevated temperature and salinity in the field. An increase in this ratio with an increase in salinity and temperature was demonstrated in bacterial cultures of *Pseudomonas putida* and in soils (Petersen & Klug, 1994; Heipieper *et al.*, 1996).

Cyanobacteria and solar radiation

The elevated levels of UV and solar radiation in the field favored the growth of cyanobacteria that contain scytonemin. *Lyngbya aestuarii*, *Entophysalis* spp. and *Chroococcus* sp., known to possess such sunscreens (Fleming & Castenholz, 2007), were found only in the top layers of all studied mats. Cyanobacteria that lack the ability to produce scytonemin such as *M. chthonoplastes* protected themselves against UV light by inhabiting deeper horizons in the mat. The quantification of scytonemin by HPLC confirmed our microscopic observations. It correlated with the selection of taxa able to produce this protective extracellular pigment and with their response to maximum irradiation. Accordingly, it was higher in air-exposed pinnacle and dry mats, and lower in submerged mats. Dillon *et al.* (2002) demonstrated that temperature and UV-A irradiation caused a synergistic increase in scytonemin production whereas the synthesis rate of scytonemin declined with increasing salinity. This decrease was correlated with the decreasing growth rate of cyanobacteria with increasing salinity.

In our mat system, the optimum production of scytonemin was detected at 10% salinity in the middle tidal mat, whereas 20% salinity in the high tidal mat apparently limited the growth of cyanobacteria and subsequently the synthesis of scytonemin. The limited growth of cyanobacteria in this mat was clearly reflected by the low concentration of Chl *a*. Periodic desiccation was also shown to induce scytonemin synthesis in cultures of *Chroococcidiopsis* and *Nostoc* (Fleming & Castenholz, 2007). This might explain the higher concentrations of scytonemin in the dry mat than

in the inundated (gelatinous) mat from the higher tidal zone. Scytonemin is extremely stable and remains largely intact in the sheaths of desiccated cyanobacteria. This would facilitate recovery of desiccated mats upon rehydration by allocating a large fraction of energy to metabolic processes other than UV damage repair. A scytonemin-like pigment was found preserved on the surfaces of silicified Proterozoic stromatolites (Golubic & Hofmann, 1976), suggesting that protection from solar radiation by extracellular pigments is an ancient adaptation.

Cyanobacterial diversity and resistance to desiccation

Desiccation is among the most important factors exerting selective pressure on bacterial communities. Cyanobacterial diversity and species richness have been shown to decrease as the desiccation frequency increases (Rothrock & Garcia-Pichel, 2005). Interestingly, this was not the case in Abu Dhabi mats, where the most desiccated mat in the studied system exhibited a diverse cyanobacterial community as revealed by microscopy (seven morphotypes), DGGE (three phylotypes) and enrichment cultivation (four strains), suggesting the presence of diverse desiccation-tolerant cyanobacteria in these mats. In the submerged, reddish mat we found five morphotypes, two phylotypes and three strains. Many field cyanobacteria apparently resisted isolation and only a few from each mat outcompeted all others in cultures.

Desiccation damages the cells by causing DNA strand breaks, protein denaturation and membrane leakage upon rehydration (Potts, 1994, 1999), however, several cyanobacteria were shown to tolerate an air-dried state and complete dehydration for prolonged periods. Among such desiccation-tolerant cyanobacteria are *Microcoleus*, *Lyngbya* and *Chroococcidiopsis* (Grilli Caiola *et al.*, 1996; Ohad *et al.*, 2005; Fleming *et al.*, 2007), which were also identified in our dry mat. Desiccation tolerance of these cyanobacteria can be achieved through the production of polyhydroxyl carbohydrates, which replace the water shell around cellular macromolecules, preventing denaturation (Potts, 1994, 1999). Studies on *Chroococcidiopsis* strains have demonstrated their ability to survive prolonged desiccation through efficient repair of the DNA damage that occurred during dehydration (Billi *et al.*, 2000). Desiccated *Lyngbya*-dominated mats from Baja California, Mexico, were shown to recover within 12 h when rehydrated even after more than 1 year of dryness (Fleming *et al.*, 2007). This points to the resilience of *Lyngbya* to long-term desiccation and possibly explains the considerable dominance of this cyanobacterium in our mat system. The production of extensive EPS sheaths in *Lyngbya* spp. and other desiccation-tolerant cyanobacteria enables them to survive complete dryness by enhancing water retention and absorption in their sheaths (Tamaru *et al.*,

2005). The increased level of EPS in the higher tidal mats is possibly a response to prolonged exposure to water evaporation and desiccation.

In conclusion, the multiple extreme environmental conditions in the Arabian Gulf and their impact on the diversity and function of microbial mats render this system worthy of further research. More insights into the adaptations of the mats' microorganisms to simultaneous extreme conditions of salinity, UV, desiccation and temperature and their response with changes in these parameters during tidal regime are needed. The properties of these mats identify them as valid modern analogues of ancient stromatolites, and fossil microbial communities of the Proterozoic (Golubic, 1976), and possibly Archaean times (Nisbet & Sleep, 2001).

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Publication 4

Abundance and community composition of Bacteria and Archaea in the oxic layer of a hypersaline intertidal cyanobacterial mat

**Abundance and community composition of Bacteria and
Archaea in the oxic layer of a hypersaline intertidal
cyanobacterial mat**

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Abstract

The community composition of bacteria and archaea in the uppermost oxic layer (1 to 3,5 mm) of a hypersaline, intertidal cyanobacterial mat from Abu Dhabi was investigated, using cultivation and molecular tools. Using quantitative PCR and catalyzed reporter deposition fluorescent in situ hybridization (CARD-FISH), bacterial and archaeal cell numbers were found to be in the range of 10^8 - 10^{10} and around 10^7 cells g^{-1} mat, respectively. CARD-FISH hybridization of thin mat cross sections showed, that *Chloroflexi*-related bacteria and *Microcoleus chthonoplastes* were dominant in top layer of the mat. Enrichment cultivation resulted in 20 axenic strains distributed among the groups *Actinobacteria*, *Bacteroidetes*, *Firmicutes*, *Gammaproteobacteria* and *Haloarchaea*, but most of them did not belong to the dominant community members. Most abundant bacterial sequences, obtained by 16S rRNA cloning, belonged to *Bacteroidetes*, *Chloroflexi* and *Proteobacteria*, whereas most archaeal sequences were affiliated with the marine benthic group B (MBGB) and Haloarchaea. For a high proportion of obtained 16S rRNA sequences (49%, archaeal sequences; 33% bacterial sequences) no closely related sequences (>97% sequence similarity) were found in existing databases. In conclusion, this study provides the first insight into the novel and unique archaeal and bacterial diversity, and abundance in the oxic part of the gelatinous hypersaline microbial mat from Abu Dhabi. The oxic part of the mat is not only inhabited by a high number of bacteria, but also archaea are abundant. Additionally some interesting microbial groups (e.g. red colored *Chloroflexi*-related bacteria and MBGB crenarchaeota) have been detected, which are not well investigated, since isolation attempts have not yet been successful. These microorganisms live in close association with cyanobacteria, and thus, they probably play an important role in carbon cycling, which can be confirmed by physiological experiments, such as stable isotope probing (SIP), or HISH-nanoSIMS.

Introduction

Carbon cycling, particularly photosynthesis and aerobic respiration, in cyanobacterial mats occurring at the intertidal flats of the Arabian Gulf (UAE), has been studied at different salinities and temperatures using oxygen microsensors (Abed et al., 2006 and 2007a). The ratio of both processes largely determine the net accretion of benthic phototrophic communities. Gross photosynthesis and oxygen consumption remained coupled at all salinities, although both areal rates decreased at salinities above 10%, regardless of the mats' tidal position and ambient average salinity. However, the extent of inhibition of these processes was more pronounced in lower tidal mats than in upper tidal mats, which experience a wider range of salinity (i.e. between 6-20%), (Abed et al., 2007a). Areal rates of gross and net photosynthesis increased with increasing temperature and maximum rates were detected at 45°C. Above 50°C, photosynthesis was completely inhibited, probably due to high sulfide concentrations. In contrast, oxygen consumption in the light showed a decreasing trend between 25 and 45°C, suggesting a decoupling of the two processes (Abed et al., 2006).

The diversity of photosynthetic microorganisms (mainly cyanobacteria) in these mats has been studied previously (Abed et al., 2008), however, little is known about the identity of respiring aerobic heterotrophs. The top few millimeters of cyanobacterial mats, mainly inhabited by cyanobacteria and aerobic heterotrophic prokaryotes (AHP), is the biochemically most active part of the mat, where carbon flux driven by photosynthesis is highest (Ley et al., 2006). AHP are important for the degradation part of the carbon cycle within mats, as their respiratory activity is always coupled to cyanobacterial photosynthesis, probably due to the direct growth on cyanobacterial exudates (Bateson and Ward, 1988; Grötzschel et al., 2002). Using enrichment cultivation and substrate spectra of obtained isolates, AHP were found to belong to different functional guilds, where each contain populations that are specialized in the consumption of specific cyanobacterial exudates. The isolates belonged to the genera *Marinobacter*, *Halomonas*, *Roseobacter* and *Rhodobacter* and *Alcanivorax* (Jonkers and Abed, 2003; Abed et al., 2007b). Despite the significant role of AHP in microbial mats, little research has been performed to investigate their identity and abundance using molecular tools. In particular,

the diversity of archaea in the oxic part of hypersaline microbial mats has received little attention (Jahnke et al., 2008; Robertsen et al., 2009).

This study aimed to identify and quantify the potential aerobic heterotrophic bacteria and archaea in these mats by molecular methods to complement the existing information on their metabolic processes revealed by previous microsensor work.

Material and Methods

Origin of mat samples

Mat samples were collected in June (2005), from the intertidal flats of Abu Dhabi (Arabian Gulf coast, United Arab Emirates) during low tide (Fig 1A and B). Air temperature in this region typically reaches over 50°C during hot summers and between 15 and 35°C in winter. Mat pieces (8x15 cm) were collected from the upper intertidal zone. The salinity of the overlying water fluctuated between 6 and 20‰ as measured during the lower tide using a portable refractometer. The water temperature was 32°C at the time of sampling. Cooled mat samples (4°C) were transferred to the Max Planck Institute for marine Microbiology and were kept at -80°C for further analysis. Some samples were kept alive in aquaria.

Microcoleus chthonoplastes, *Entophysalis major*, *Lyngbya aestuaris*, *Oscillatoria*, and *Halotheca*-like unicellular cyanobacteria were found to be the dominating cyanobacteria in the upper oxic millimeters of the microbial mat analysed in this study (Abed et al., 2008).

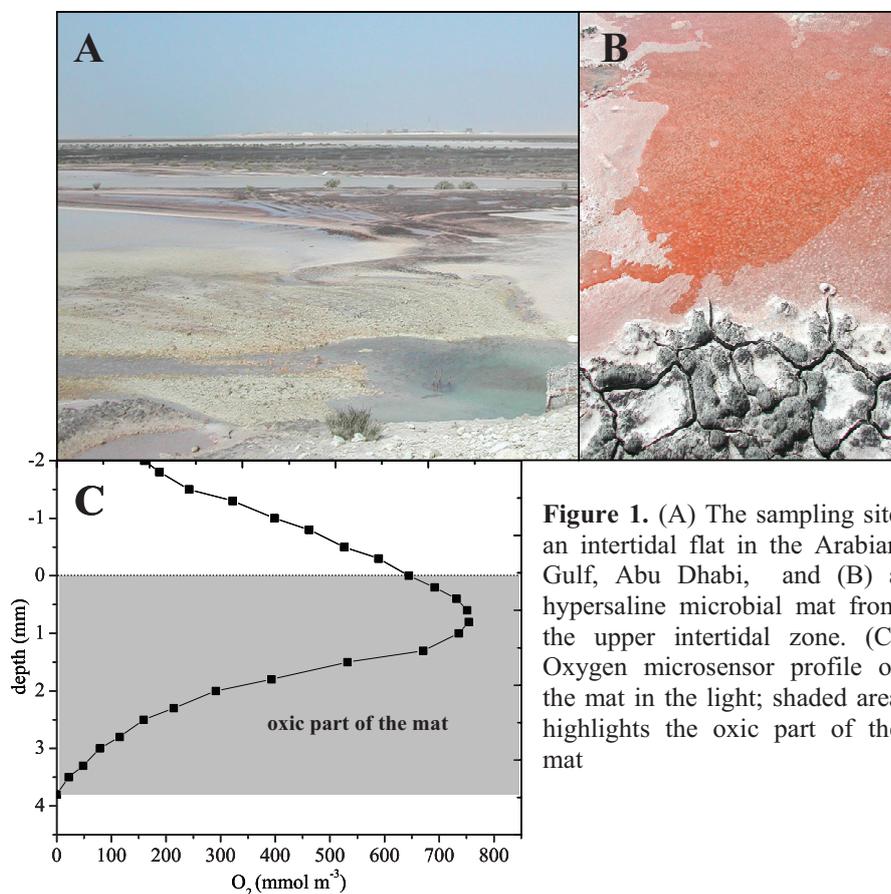


Figure 1. (A) The sampling site an intertidal flat in the Arabian Gulf, Abu Dhabi, and (B) a hypersaline microbial mat from the upper intertidal zone. (C) Oxygen microsensor profile of the mat in the light; shaded area highlights the oxic part of the mat

Oxygen microsensor measurements

Oxygen microprofiles in the mat were measured with a microsensor system described previously (Polerecky et al., 2007, www.microsen-wiki.net). The mat was illuminated with photosynthetically active radiation (PAR) of constant intensity (~400 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$). A two-point linear calibration was performed using the readings in the anoxic zone of the mat and in the overlying aerated seawater (10% salinity). The oxygen solubility at the experimental temperature and salinity was obtained from the table available at www.unisense.dk.

The oxygen microprofile of the mat shows the oxic part of the mat (1-3,5 mm), which was used for most of the further experiments in this study (Fig. 1C).

Quantitative PCR

For sample preparation, two mat pieces were incubated in O.C.T. compound (Plano) at 4°C for 24 hours. The mat pieces were then transferred into cryotrays and left at -20°C for 5 hours. Thin sections (250-500 μm) were prepared using a cryomicrotome (HM 505E, Microm) at -31°C. Each layer was subjected to nucleic acid extraction after Lueders et al. (2004). Quantitative PCR (qPCR) was applied for the quantification of 16S rRNA genes of the total bacteria using TaqMan probe 6-FAM-5'-CGTATTACCGCGGCTGCTGGCAC-3'-dark quencher and the primer sets 331F and 797R at the PCR conditions described by Nadkarni et al. (2002). The total Archaea were quantified using the probe Arch516F and the primer sets ARCH349F and ARCH806R as described by Takai & Horikoshi (2000). The assays were performed on Bio-Rad IQ5 (Bio-Rad, Germany). The PCR reaction was performed in a total volume of 25 μl using the TaqMan Universal PCR Master Mix (Eurogentec), containing 100 nM of each of the universal forward and reverse primers and 1 μL 10-fold dilution of sample DNA solution (10 ng). The standard DNAs were DNA mixtures of different Archaea and Bacteria. Standard curves for each assay were constructed on the basis of serial 10-fold dilutions of DNA mixtures containing around 1×10^{10} copies. Only standard curves with regression coefficient values >0.98 were used. All PCRs were performed in triplicates for each sample. PCR efficiency was calculated from the slope of the standard curves using:

$$Efficiency = \left(10^{\left(\frac{1}{\text{slope}}\right)} - 1\right) * 100$$

CARD-FISH

CARD-FISH oligonucleotide probe mixture (EUB338 I-III) to target all bacteria was used (Amann et al., 1990; Daims et al., 1999). Duplicate mat samples were fixed overnight at 4°C with formaldehyde (4% final concentration). The fixed samples were washed with 1×PBS, and stored in PBS/ethanol (1:1) at –20°C for further processing. Fixed samples were prepared as described in the qPCR section. Mat slices and were homogenized using glass homogenizers and ultrasonication. The samples were subsequently filtrated on polycarbonate filters (GTTP type; pore-size 0.22µm; Millipore, Eschborn, Germany). Permeabilization of the cells, hybridization and tyramide signal amplification were performed as previously described by Pernthaler et al. (2004) and Snaidr et al. (1997). After hybridization, the cells were counterstained with DAPI (1 µM) and analysed using a fluorescence microscope (Axioskop2 mot plus; Axiocam MRM, Axiovision 4.6, Zeiss, Germany). At least 1000 DAPI-stained cells were counted. Hybridization with probe NON338 was performed as a negative control (Wallner et al., 1993).

Thin cross-sections (5 µm) (see qPCR section) of fixed mat sample were placed onto polysine coated slides. CARD-FISH with the probes GNSB-941 and CFX-1223 (Gich et al., 2001; Björnsson et al., 2002) specific for *Chloroflexi* was performed on mat slices as described above.

Enrichment cultivation

Isolation of aerobic heterotrophic bacteria and archaea was performed in artificial seawater medium supplemented with different carbon sources. The medium contained MgCl₂·6 H₂O (5.6 g l⁻¹), MgSO₄·7 H₂O (6.8 g l⁻¹), CaCl₂·2 H₂O (1.47g l⁻¹), KCl (0.66 g l⁻¹) and KBr (0.09g l⁻¹). Hypersaline media of 5 and 20% (w/v) final total salinity were obtained by adding appropriate amounts of NaCl. After autoclaving, KH₂PO₄ and NH₄Cl solutions were added to the medium in final concentrations of 0.15 and 0.2 gl⁻¹, respectively. Sterile filtrated solutions of trace elements mixtures (Widdel et al., 1992), selenite and tungstate and vitamins (Heijthuijsen et al., 1986) were then added (1 ml each l⁻¹). Solid media were prepared with 1% (w/v) agar. Direct plating and serial dilution were used as isolation techniques. Different populations of aerobic bacteria were isolated

at different concentrations of salinity and on different carbon sources. The following enrichments were performed: (a) serial dilutions at two salinities (5% and 20%) using a mixture of 10 mM acetate and 5 mM succinate as a carbon source, (b) enrichments on 0.05% *Spirulina* extract (complex polymeric substances of dead cyanobacteria) (5% and 20%) and (c) enrichment cultivation in marine broth medium (carbon source: yeast extract); 25% salinity. Axenic strains were obtained from high dilutions by plating on agar medium containing the same carbon source. DNA of obtained axenic strains was amplified with primer pairs GM3F/GM4R or 21F/958R (see section 16S rRNA cloning).

16S rRNA cloning

The uppermost layer (1 mm thickness, ca ~100 mg wet weight) of the studied mat sample was subjected to nucleic acid extraction after Lueders et al. (2004). 16S rRNA cloning was carried out using the bacterial primers GM3F and GM4R (Muyzer et al., 1995) and archaeal primers 21F and 958R (DeLong, 1992) at annealing temperatures of 42°C and 55°C, respectively. The PCR products were purified using the QIA quick PCR purification kit (Qiagen, Hilden, Germany) and were cloned using the TOPO TA Cloning Kit (Invitrogen, Karlsruhe, Germany) according to the manufacturer's instructions. The obtained clones were screened for the presence of correct inserts and the positive clones were then sequenced. A total of 131 bacterial and 152 archaeal partial sequences with a length of 500-800 base pairs were obtained.

Phylogeny and diversity calculation

The obtained sequences were aligned and analyzed using the ARB software (Ludwig *et al.*, 1998) version 071207 and the official database (<http://www.arb-silva.de>) of October 2008 for small subunit RNA sequences (ssuref_96_silva_04_10_08_opt.arb) (Prüsse et al. 2009). The phylogenetic trees were calculated by maximum likelihood method, based on long 16S rRNA gene sequences (>1300 bp). The partial sequences were inserted into the pre-established tree using the parsimony ARB tool, while maintaining the overall tree topology without changes.

For determining the number of operational taxonomic units (OTU) similarity matrices among the sequences were calculated with the arb program. One OTU was defined for

sequences which have more than 97% similarity. Rarefaction curves were calculated using the freeware program aRarefactWin (available at <http://www.uga.edu/~strata/software/Software.html>). The coverage of the clone libraries were calculated as previously described by Good (1953).

Results

Quantification and spatial distribution of bacterial and archaeal populations using qPCR and CARD-FISH

Bacterial and archaeal abundances were determined by qPCR from the vertical mat layers. The bacterial abundance showed no distinct distribution pattern along depth (Fig 2A and B). In the oxic part of the mat, values were highly variable, with a mean value of 2.02×10^8 cells g^{-1} . In the anoxic part, the bacterial abundance was more stable, but possessed a similar mean value of 2.04×10^8 cells g^{-1} . The archaeal abundance possessed a more distinct pattern of distribution with a significant increase with depth. It can be noted that the smallest abundance of bacterial and archaeal cells were obtained at the same layer (1500-1750 μm). In the oxic part a mean of 9.06×10^6 cells g^{-1} was detected, whereas in the anoxic part, the archaeal abundance significantly increased with a mean value of 3.63×10^7 cells g^{-1} .

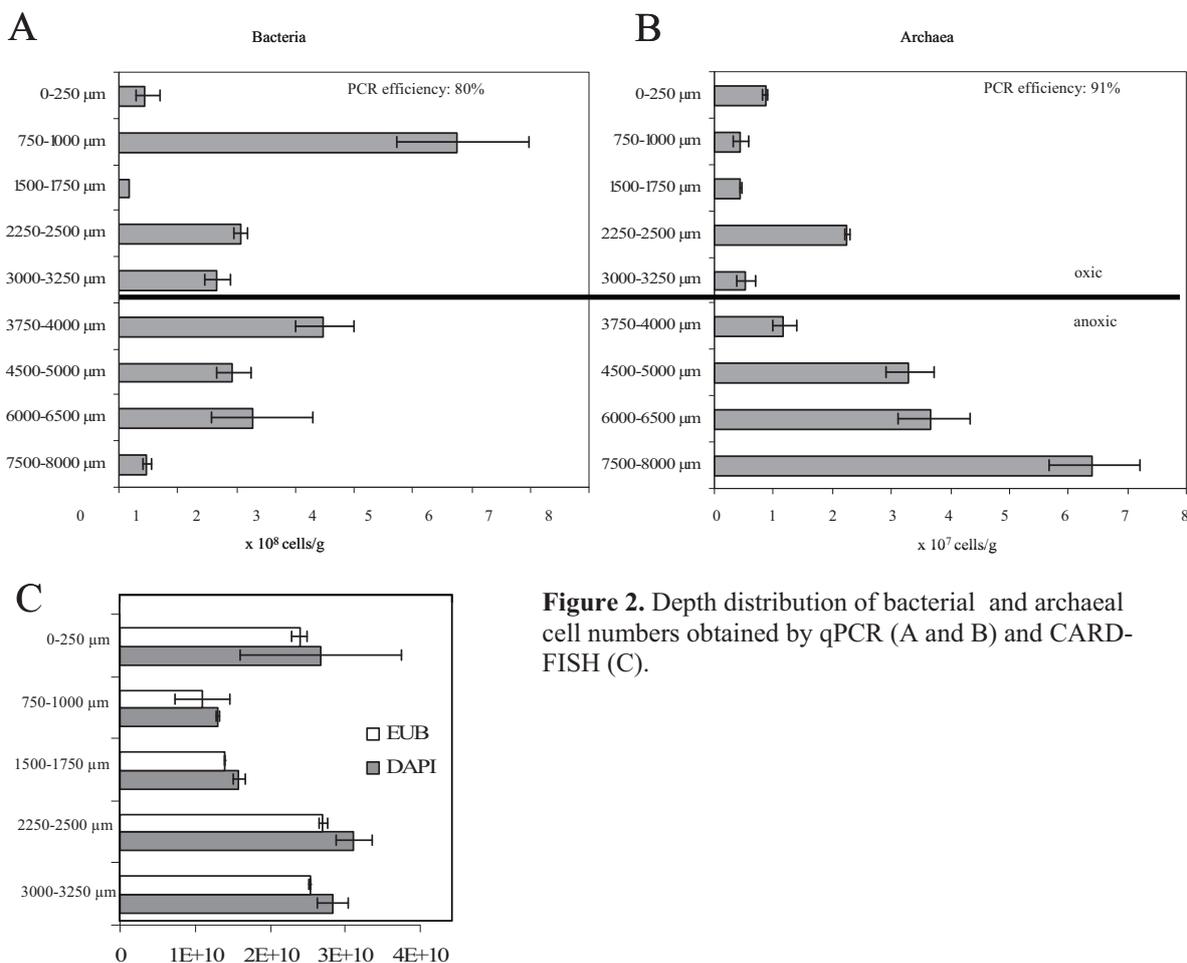


Figure 2. Depth distribution of bacterial and archaeal cell numbers obtained by qPCR (A and B) and CARD-FISH (C).

Bacterial abundance was also determined by CARD-FISH in the oxic part of the mat and revealed much higher cell numbers (Fig. 2C). Cell numbers decreased from the first to the third layer, but increased again in deeper layers with a mean value of 2.23×10^{10} cells g^{-1} . CARD-FISH with *Chloroflexi*-specific probes GNSB-941 and CFX-1223 on 5 μm thin cross-sections of the oxic mat layer showed a dominance of *Chloroflexi*-like bacteria in close proximity to bundles of *Microcoleus chthonoplastes* filaments (Fig. 3). These *Chloroflexi*-like bacteria were not greenish, as for example *Chloroflexus aurantiacus*, but reddish.

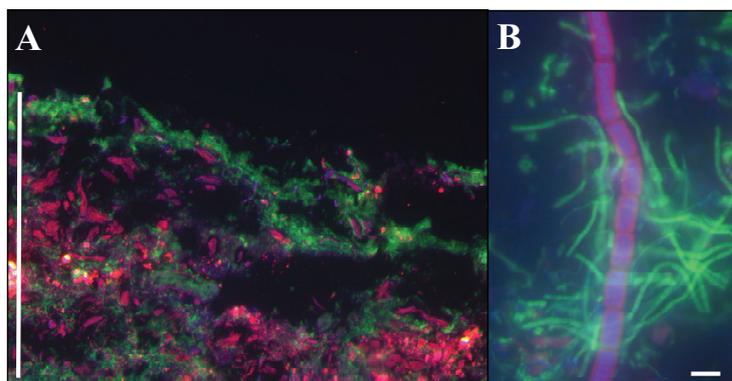


Figure 3. Catalyzed reporter deposition-fluorescence *in situ* hybridization (CARD-FISH) of 5 μm thick cross-section of the microbial mat using the probes (CFX-1223 and GNSB-941) specific for *Chloroflexus*-related bacteria (A) green color shows probe signal; pink color shows cyanobacterial autofluorescence; blue color shows DAPI signals of the remaining cells; (B) a closer view of the same cross-section. Scale bar corresponds to 500 μm and 5 μm , respectively. (modified from Abed et al., 2010)

Cultivation of aerobic microorganisms

Cultivation of aerobic heterotrophic bacteria and archaea using artificial seawater medium (ASM) with different salinities and supplemented with different carbon sources and marine broth medium, respectively, resulted in 20 axenic strains. The obtained isolates were phylogenetically affiliated to *Actinobacteria*, *Bacteroidetes*, *Firmicutes*, *Alphaproteobacteria*, *Gammaproteobacteria* and *Haloarchaea*. Isolates within the *Actinobacteria* were closely affiliated to *Micrococcus* sp. and were isolated on 5% ASM supplemented with acetate and succinate. *Firmicutes*-related isolates clustered with *Bacillus* sp. and were isolated with 20% ASM supplemented with acetate and succinate and with *Spirulina*-extract. Isolates within the *Alphaproteobacteria* were closely related to *Caulobacter* sp. and were isolated with 5% ASM supplemented with *Spirulina*-extract. All strains isolated on marine broth medium at 25% salinity belonged to the *Gammaproteobacteria* and fell next to *Salinivibrio* sp., *Pseudoalteromonas* sp., *Idiomarina* sp., and *Halomonas* sp.. Archaeal isolates were enriched on ASM with

Spirulina-extract at 5% and 20% salinity and were affiliated with *Halobacteria*, *Haloferax* and *Halorubrum* (Fig 4).

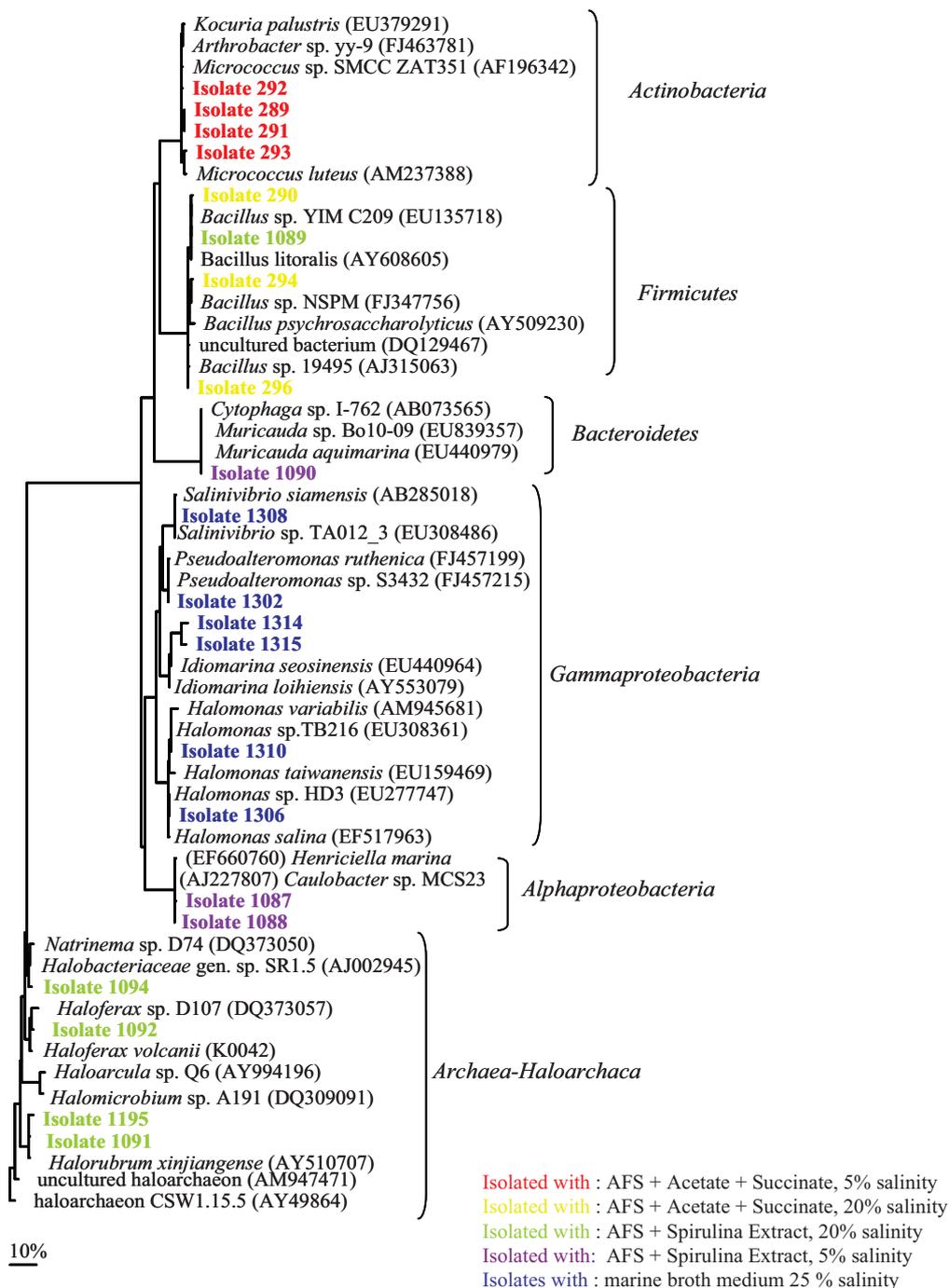
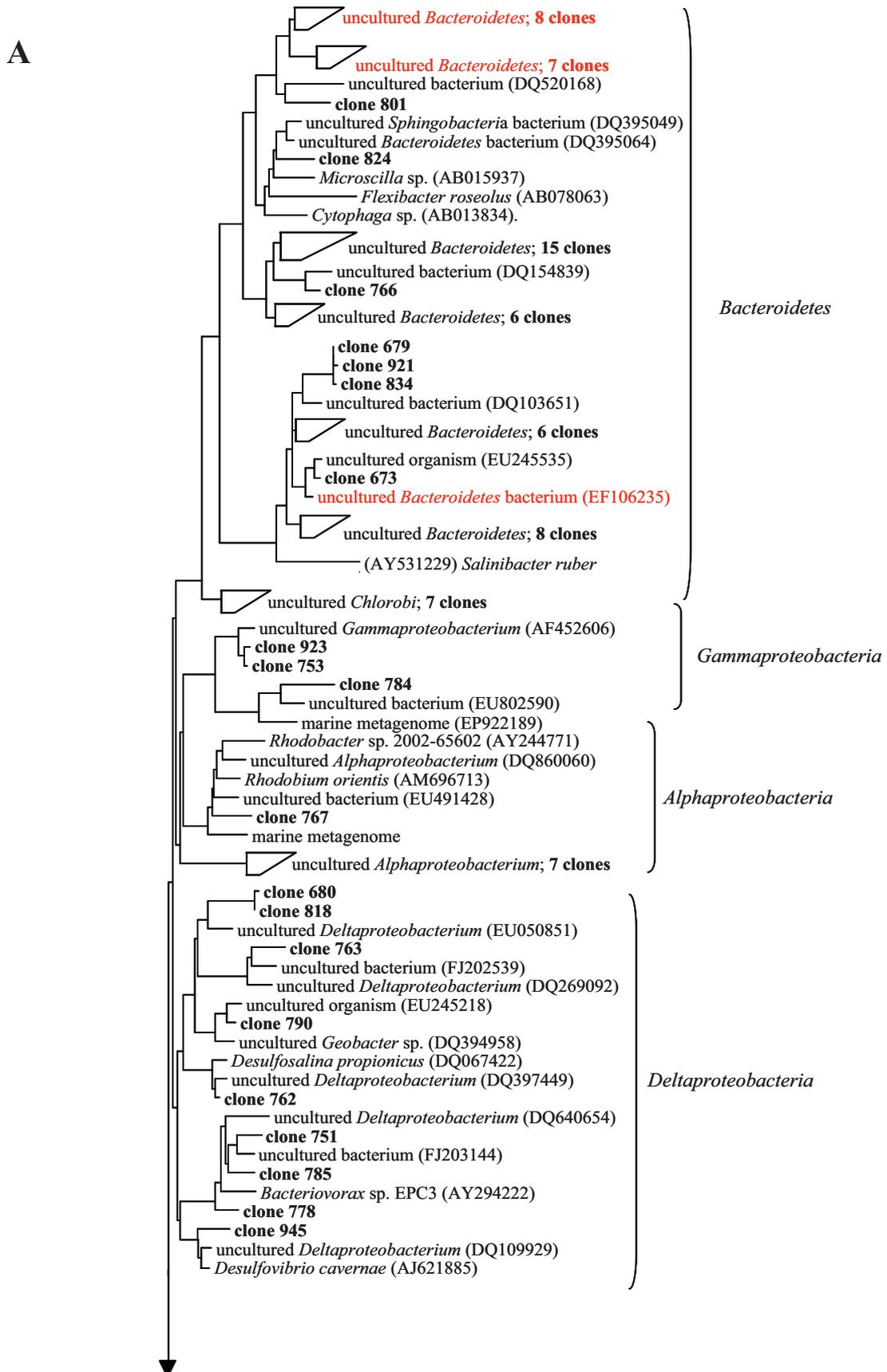


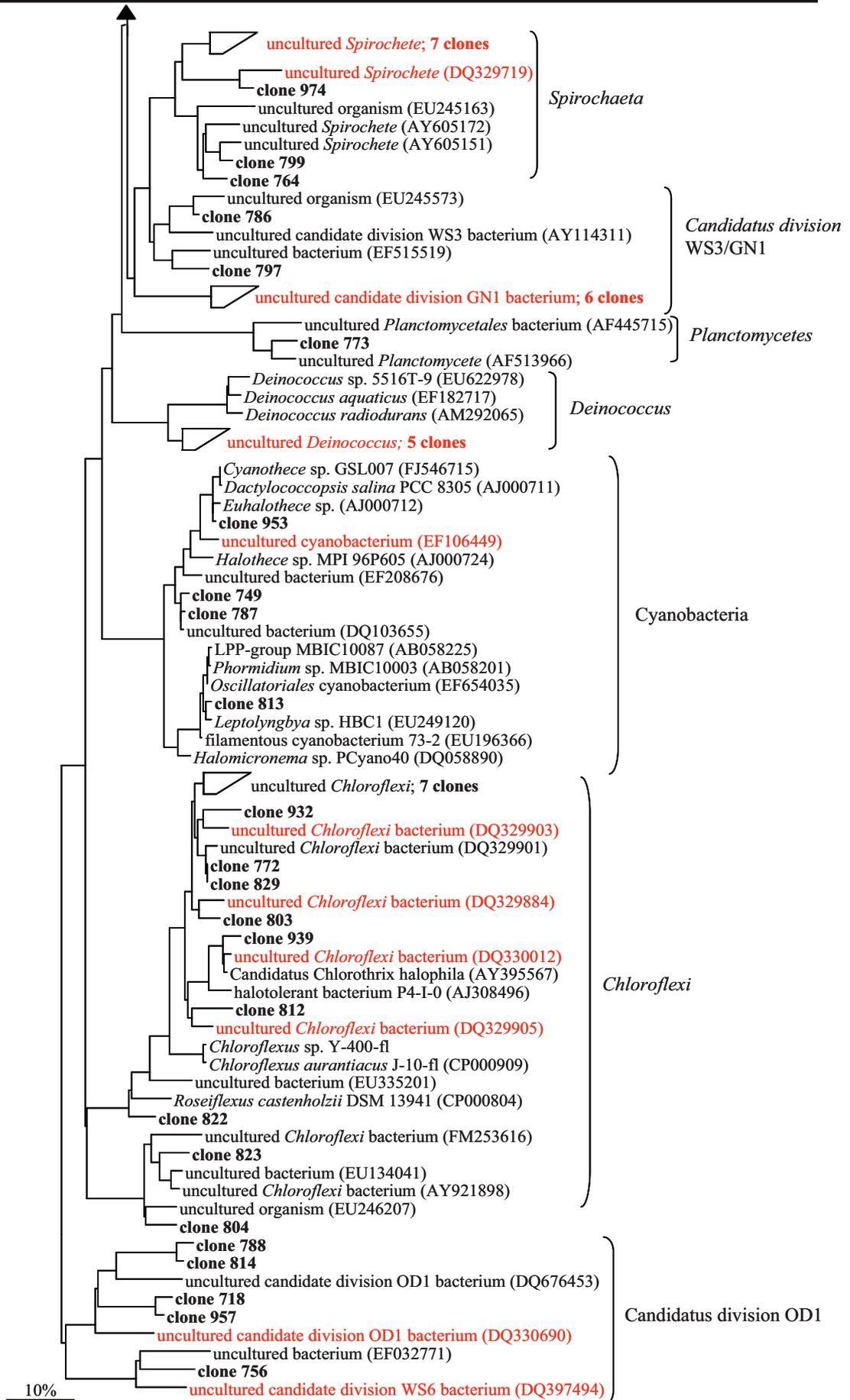
Figure 4. 16S rRNA gene-based phylogenetic reconstruction based on maximum likelihood methods, showing the affiliation of sequences retrieved from isolates obtained from the uppermost layer of the studied microbial mat. The partial sequences were inserted into the reconstructed tree by applying parsimony criteria without allowing changes in the overall tree topology. Accession numbers are indicated in parentheses. The scale bar indicates 10% sequence divergence. Sequences obtained in this study are shown in bold. Different colors indicate different media used for isolation

16S rRNA clone libraries of bacteria and archaea

Based on sequence similarity comparison of bacterial and archaeal clones, 71 distinct phylotypes or operational taxonomic units (OTUs, based on an 97% identity threshold) were identified among 132 bacterial clones sequences, versus 18 distinct phylotypes among 152 clones sequences for Archaea. Statistical evaluation by rarefaction analysis showed that the archaeal diversity was mostly covered (88% coverage) while an insufficient number of clones have been analyzed to cover the major part of bacterial diversity (42% coverage) (Fig. 6C). For a high proportion of archaeal (49% of the clone library) and bacterial sequences (33%), no closely related sequences were found, based on a 97% sequence similarity, in the databases Arb-silva (<http://www.arb-silva.de>) and NCBI (<http://www.ncbi.nlm.nih.gov>).

The major part of the bacterial library (48%) was affiliated with sequences belonged to sequences of uncultured *Bacteroidetes*. Uncultured *Chloroflexi*-related sequences constituted 11% of the library. The remaining sequences belonged to the bacterial groups *Alpha*-(4%), *Gamma*-(3%), and *Deltaproteobacteria* (7%), *Spirochaeta* (8%), *Planctomycetes* (1%), *Deinococci* (4%), uncultured candidate division (11%), and cyanobacteria (3%) (Fig 6A). Archaeal clone library showed less diversity compared to the bacterial clone library, with only 18 phylotypes (152 clones). Most of the sequences were affiliated with sequences of uncultured *Crenarchaeota* (40%), and uncultured *Haloarchaea* (27%), (Fig 6B). Sequences within the uncultured *Crenarchaeota* belonged to the marine benthic group B (MBGB). The rest of the sequences was closely related to *Halorubrum* (18%), *Halorhabdus* (4%), *Halobacteria* (3%), uncultured Euryarchaea (5%) and unknown Archaea (3%). Many of our bacterial and archaeal sequences were affiliated with sequences obtained from microbial mats from Guerro Negro, Mexico (Fig 5A and B).





B

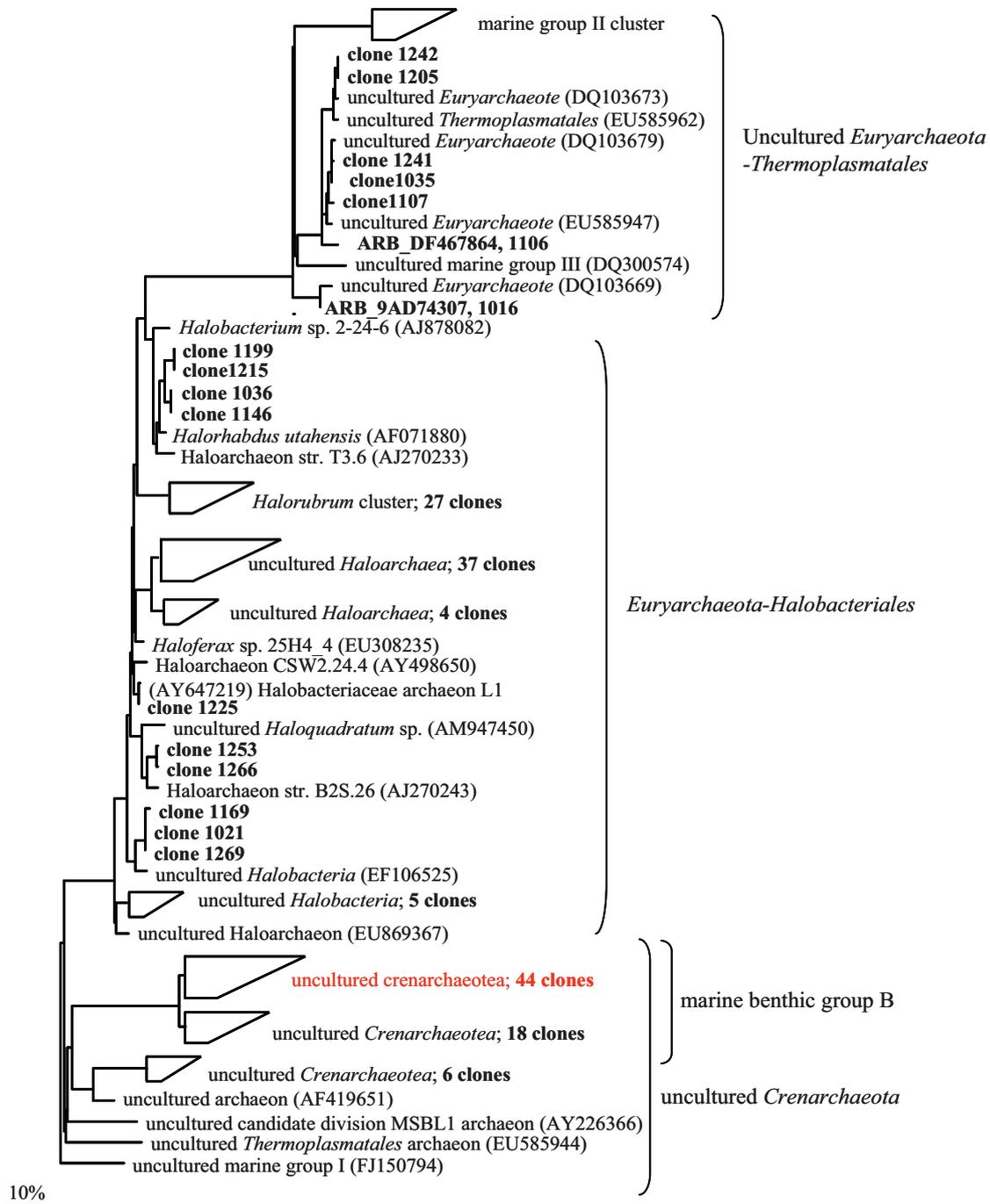


Figure 5. 16S rRNA gene-based phylogenetic reconstruction based on maximum likelihood methods, showing the affiliation of (A) bacterial and (B) archaeal sequences retrieved from 16S rRNA cloning of the uppermost layer of the studied microbial mat. The partial sequences were inserted into the reconstructed tree by applying parsimony criteria without allowing changes in the overall tree topology. Accession numbers are indicated in parentheses. The scale bar indicates 10% sequence divergence. Sequences obtained in this study are shown in bold. Red highlighted sequences were obtained from Guerrero Negro mats (Mexico) (Ley et al., 2007, Robertsen et al., 2009).

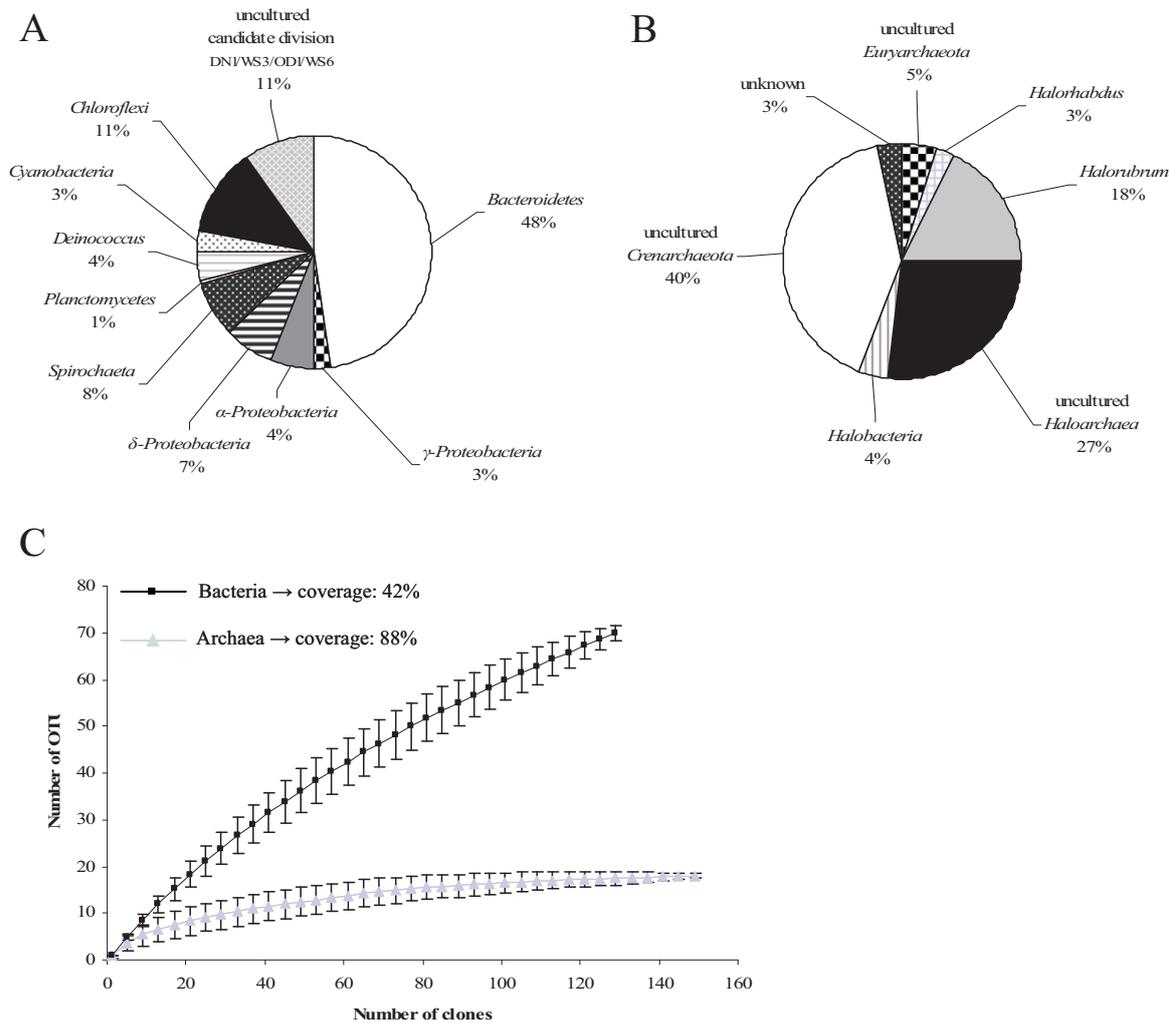


Figure 6. (A) Bacterial and (B) archaeal community composition retrieved by 16S rRNA cloning. Shown fractions indicate the relative percentage to the total number of clones.(C) Calculated rarefaction curves of observed OTUs richness among the clone libraries from bacteria and archaea.

Discussion

The hypersaline mats from Abu Dhabi (UAE) have been extensively studied in order to assess the cyanobacterial diversity (Abed et al., 2008) and the effect of salinity and temperature changes on bacterial diversity and microbial processes like photosynthesis and oxygen consumption (Abed et al., 2006, Abed et al., 2007a). However, little is known about the abundance, identity and diversity of bacteria and archaea from the oxic part of microbial mats, which might play an important role in carbon cycling due to their close proximity to cyanobacteria. In our study, the combination of microbiological and molecular biological methods were used to quantify and characterize the community of aerobic heterotrophic bacteria and archaea in the mat, allowing us to circumvent the limitations of each method.

By cultivation, it was not possible to isolate the dominant members of the bacterial/archaeal communities, since none of them were detected in the clones libraries. One exception was the archaeal *Halorubrum*-related isolate. This discrepancy could be due to the low number of isolates. However, this highlights the difficulty to cover the major diversity and to explore microbial community structure by using classical microbiological methods, as previously stated in other studies (Amman et al., 1995; Felske et al., 1999). Bacterial cell numbers determined with CARD-FISH were two orders of magnitude higher than those obtained by qPCR. These variations are likely due to low PCR efficiencies. Other possible reasons might be variable 16S rRNA operon numbers and/or variable genome copy numbers for different bacterial taxa.

Archaeal cell numbers, of around 10^7 cells/g, determined by qPCR showed that archaea are also abundant in the aerobic layers and thus might also play an important role in carbon cycling. Unfortunately, CARD-FISH for archaea was not successful, most likely due to suboptimal cell permeabilisation.

Many of the bacterial clones and some archaeal clones were closely related to sequences obtained from hypersaline microbial mats from Guerrero Negro (Mexico; Ley et al., 2007; Robertsen et al., 2009). On 16S rRNA level, same phylotypes have often been identified at similar habitats in different geographic areas (Ramette and Tiedje, 2007). Interestingly the mat from Guerrero Negro originates from a pond with constant salinities of 8.5%,

whereas this mat originates from an intertidal flat with salinity fluctuations from 5 to 22% in a diurnal cycle. Consequently, salinity fluctuations seem to be of minor importance as community shaping environmental factor.

Aerobic archaea in microbial mats: a novel diversity and a possible role in carbon cycling

Until now, only few studies have focused on the identity and diversity of archaea in hypersaline mats (Sørensen et al., 2005; Jahnke et al., 2008; Robertsen et al., 2009; Allen et al., 2009). Furthermore, separate data about the oxic layers of microbial mats were only provided by two of them (Jahnke et al., 2008; Robertsen et al., 2009). Similar to our study, these studies showed that most of the archaeal 16S rRNA sequences obtained from the oxic layer of Guerrero Negro mats (Mexico) belonged to *Crenarchaeota*, halophilic Archaea *Thermoplasmatales* and uncultured *Euryarcheota*, respectively.

Interestingly in this study, archaeal clones library presented a high number of sequences affiliated with *Crenarchaeota* belonging to marine benthic group B (MBGB), designated by Vetriani and colleagues (1999). Until now, no member of the MBGB has been isolated. Although *Crenarchaeota* are widespread among the marine habitats (Vetriani et al., 1999) their metabolic activities are still unknown (DeLong et al., 1992; Karner et al., 2001; Schouten et al., 2002; Knittel et al., 2005). Since MBGB were also found in methane seeps it was speculated that they could be involved directly or indirectly in AOM (Knittel et al., 2005; Biddle et al., 2006) or in sulfate reduction (Torres et al., 2002). However in this, and also in other studies (Jahnke et al., 2008; Robertson et al., 2009) they were found in the oxic layer of an hypersaline microbial mat which are completely different habitats than methane seeps. Furthermore, planktonic *Crenarchaeota* have been proposed to have an impact in carbon cycling showing the potential to function either as a strict autotroph, or as a mixotroph utilizing both carbon dioxide and organic material as carbon source. They have the potential for using reduced nitrogen compounds like ammonia (NH₃) as energy sources, fueling autotrophic metabolism, and thus might also belong to ammonium oxidizers (Hallam et al., 2006). Interestingly, within the *Euryarchaeota*, a group of sequences affiliating with uncultured Haloarchaea has been detected, which does not have a very close neighbor with more than 97% 16S rRNA

sequence similarity. This group constituted 27% of the total archaeal clones, and could be a new species or even genus. The fact that no close related sequences has been found previously indicates that this group of organisms might be unique for this habitat.

Potential heterotrophic bacterial key players in the oxic mat layers

The major fraction of the bacterial clones library consisted of sequences related to *Bacteroidetes*, a group where many bacteria are chemoorganotrophic, providing a high capability of digesting large polysaccharides such as chitin, pectin and cellulose (Madigan et al., 2001; Kirchmann, 2001). The high abundance of *Bacteroidetes* might be explained by the presence of a matrix with very high amounts of extra polymeric substances (EPS) in this mat.

The high abundance of *Chloroflexus*-like bacteria coexisting in close neighborhood with cyanobacteria suggests strong relationships between both the groups. Previous studies showed that such bacterial communities perform a transfer of photoautotrophically fixed carbon from cyanobacteria featuring a symbiotic association among these groups (Nold et al., 1996, Ward et al., 1998). Moreover, the mat showed a relatively high diversity within the *Chloroflexus* group which agrees with the suggestion of Nübel and colleagues (2001) that the diversity of *Chloroflexus* relatives in hypersaline environments is higher than previously thought. The significance of *Chloroflexus*-like bacteria in hypersaline microbial mats has also been demonstrated in studies on mats from Guerrero Negro (Mexico) (Nübel et al., 2001, Ley et al., 2006). Their importance for aerobic community respiration was shown in mats from la Salada de Chiprana (Spain) (Bachar et al., 2007). Remarkably, high numbers of reddish coloured *Chloroflexus*-like bacteria were found in the present study. They have also been found to form distinct red layers in hot spring microbial mats from Yellowstone National Park (USA) (Castenholz 1984; Boomer et al., 2002), but were not yet found in hypersaline environments.

Most cultured representatives of the *Deinococci* group are aerobic heterotrophic bacteria. The *Deinococci* group contain bacterial species that are resistant to very high doses of ionizing radiation (e.g. caused by high UV light intensities), as well as to other mutagens (Moseley et al., 1983; Minton et al., 1994; Carpenter et al., 2000). This feature has been presumed to support the *Deinococcus* spp. to repair their DNA. The ability to survive

ionizing radiation seems to be also necessary to survive prolonged periods of desiccation most likely because of their potent DNA-repair capabilities (Mattimore et al., 1996). Consequently, the capability to resist high UV light intensities and long periods of desiccation might provide the Deinococci group with a selective advantage to in these mats.

Although cloning was restricted to the oxic layer of the mats, bacteria related to sulfate-reducing bacteria belonging to the genera *Desulfovibrio* and *Desulfosalina* were detected. Sulfate-reducing bacteria were previously considered as anaerobic bacteria that dominate the deeper anoxic layers of the mats. However, several studies showed that sulfate-reducing bacteria can also be dominant and active in fully oxic zones of microbial mats (Minz et al., 1999; Fourçans et al., 2004; Jonkers et al., 2005; Ley et al., 2006). Furthermore Canfield et al. (1993) and Visscher et al. (1998) detected high sulfate reduction rates in the upper oxic part of a hypersaline mat and stromatolites, respectively, although aerobic respiration is thermodynamically favoured compared to sulfate reduction. Moreover, detected biomarkers from sulfate-reducing bacteria in the top layer of hypersaline microbial mat and their ^{13}C -uptake patterns indicated a close coupling between sulfate-reducing bacteria and cyanobacteria (Bühning et al., 2009). Thus, in accordance with previous studies, our results indicate the presence of heterotrophic sulfate-reducing bacteria, which might contribute to carbon degradation in the oxic part of microbial mats. Besides cyanobacteria and *Chloroflexi*-related bacteria, only few other clones were found which can have an autotrophic metabolism, namely *Chlorobi* (7 sequences) and *Rhodobacter* sp. (1 sequence), both being capable of anoxygenic photosynthesis. Sequences obtained from the upper part of the mat mainly belonged to known aerobic heterotrophes, and no known methanotrophes, nitrifiers, or sulfide oxidizers have been found. This indicates that oxygen and carbohydrates produced by cyanobacteria is mainly respired by AHP (including *Chloroflexi*-related and sulfate-reducing bacteria) in the oxic part of the studied mats.

In conclusion, this study gives an insight into the archaeal and bacterial diversity and abundance in an intertidal hypersaline microbial mat. Many extremophilic bacteria and archaea have been found, supporting previous microsensor studies, which showed that physiological processes like photosynthesis, respiration and sulfide production were

maintained or even enhanced at high temperatures and high salinities (Abed et al., 2006, Abed et al. 2007a). In spite of very harsh environmental conditions, like high temperatures, high UV light intensities, desiccation, high salinity, and strong salinity fluctuations, a rich and novel prokaryotic diversity was found. Several interesting microbial groups (e.g. red colored *Chloroflexi*-related bacteria and MBGB crenarchaeota) have been detected, which are not well investigated, since isolation attempts have not yet been successful. Archaea made up 10% of the prokaryotes in the oxic zone. All detected bacteria and archaea live in close neighborhood with cyanobacteria, thus may favor from either their exudates and/or the produced oxygen. It needs to be investigated which strains are involved in organic carbon degradation and which ones are chemoautotrophic. Therefore, physiologically experiments like stable isotope probing (SIP) and HISH-nanoSIMS are needed.

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Publication 5

Molecular identification of aerobic heterotrophic bacteria in hypersaline microbial mats and their interaction with associated cyanobacteria

**Molecular identification of aerobic heterotrophic bacteria in
hypersaline microbial mats and their interaction with
associated cyanobacteria**

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Abstract

Aerobic heterotrophic bacteria (AHB) are firmly attached to cyanobacteria in microbial mats, thus hindering their isolation in axenic cultures. In this study, a polyphasic molecular approach was used to gain a better insight into the interactions between cyanobacteria and their associated AHB. DNA-stable isotope probing (DNA-SIP) was carried out to identify the main CO₂ fixing cyanobacteria and photosynthate metabolizing AHB that play a significant role in carbon cycling in intact cyanobacterial mats. A surprisingly specific assimilation of ¹³CO₂ by *Cyanothece*, but not *Microcoleus*-related cyanobacteria within a freshly sampled mat was observed. A specific and clear C¹³-labeling of AHB was not evident, most likely due to methodological artifacts. However, the results hinted to a potential importance of *Chloroflexi*-like bacteria, *Bacteroidetes*, and *Proteobacteria* in carbon cycling. Monocultures of cyanobacteria from different microbial mats were used as model systems to identify the associated AHB and their interactions with the cyanobacteria. AHB in these cultures were identified using denaturing gradient gel electrophoresis (DGGE) and their distribution was investigated using catalyzed reporter deposition fluorescent *in situ* hybridization (CARD-FISH). Furthermore, the interactions between cyanobacteria and AHB were studied in co-culture growth experiments. Most of the attached bacteria belonged to the *Bacteroidetes* phylum, which contain bacteria, known to grow on cyanobacterial extrapolymeric substances (EPS). Different cyanobacterial species were found to be associated with different AHB, although *Bacteroidetes*-related sequences were found in most cultures. Using CARD-FISH with a *Bacteroidetes* specific probe, filamentous bacteria were observed to split cyanobacterial filaments and feed on dead cells. The growth of an axenic culture of *Microcoleus chthonoplastes* PCC7420 was stimulated upon the addition of a filtrate containing AHB cells and released substances, obtained by filtering a non-axenic *Microcoleus*-related culture. In contrast, the growth of *Cyanothece* PCC 7418 with a filtrate, obtained from a non-axenic *Cyanothece*-related culture, was completely inhibited. We conclude that the interactions between AHB and cyanobacteria are highly specific, complex and variable.

Introduction

Microbial mats are complex ecosystems, with aerobic heterotrophic bacteria (AHB) and cyanobacteria dominating the uppermost few millimetres in close association (van Gemerden, 1993, Karsten and Kühl, 1996; Abed and Köster, 2005;). The relationships between cyanobacteria and AHB can be competitive for scarce nutrients and other resources, or mutualistic stimulating the survival and persistence of each other (Paerl, 1996). For example, too high oxygen concentrations, evolved during cyanobacterial photosynthesis, can inhibit several metabolic and biosynthetic pathways of cyanobacteria. It is thought that AHB counteract the chemical changes in O₂, CO₂ and pH induced by photosynthesis via respiration activities (Whitton, 1973; Wieland and Kühl 2006). Cyanobacteria and associated heterotrophic bacteria were shown to exchange vitamins, other growth factors as well as nitrogen and carbon sources, leading to enhanced cyanobacterial growth (Burkholder, 1963; Paerl, 1982; Steppe et al., 1996). Unlike these beneficial interactions, some studies reported the lysis of cyanobacterial filaments by associated gliding bacteria (Shilo, 1970; Rashidan and Bird, 2001). Furthermore, AHB are thought to play a significant role in carbon cycling through direct utilization of cyanobacterial exudates and regeneration of CO₂ required for cyanobacterial photosynthesis (Lange, 1967; Bauld and Brock, 1974; Paerl, 1976; Herbst and Overbeck, 1978; Cole, 1982; Bateson and Ward, 1988; Baines et al., 1991; Wang et al., 1994; Epping et al., 1999; Kirkwood et al., 2006). Most of the identified AHB from hypersaline microbial mats belonged to the genera *Marinobacter*, *Halomonas*, *Roseobacter*, *Rhodobacter*, *Marinobacter* and *Alcanivorax* and were assigned to different functional guilds based on their growth spectrum on different organic substrates (Jonkers and Abed, 2003; Abed et al., 2007). These studies were cultivation-based, where the isolated strains do not necessarily represent the *in situ* diversity, and the growth of AHB on cyanobacterial exudates was not validated directly in field samples. Still, little is known about the identity, distribution and species-specificity of AHB, which are directly involved in the uptake of organics from cyanobacteria under *in situ* conditions. Moreover, the actual functioning of AHB in the carbon cycling of mats is not unambiguously clarified.

Here, we have combined a number of molecular and cultivation-based approaches to unravel potential multitrophic interactions within a marine cyanobacterial mat. We applied DNA-stable isotope probing (DNA-SIP) on intact mats, incubated under an atmosphere enriched in $^{13}\text{CO}_2$ in order to identify the AHB populations that are involved in the *in situ* uptake of cyanobacterial exudates. We also identified AHB communities in different monoclonal cyanobacterial cultures obtained from the same microbial mats, using denaturing gradient gel electrophoresis (DGGE), with the aim to find out whether different cyanobacteria have similar or different associated AHB. The distribution of AHB around cyanobacterial filaments was further studied using catalyzed reporter deposition fluorescent *in situ* hybridization (CARD-FISH) and the interaction between the two groups was investigated using growth experiments under different conditions.

Material and Methods

Origin of mat samples

Mat samples were collected in June 2005, from the intertidal flats of Abu Dhabi (Arabian Gulf coast, United Arab Emirates) during lower tide. Air temperature in this region typically reaches above 50°C during hot summers and between 15 and 35°C in winter. Mat pieces were collected from the upper intertidal zone (gelatinous mat; Abed et al., 2008). The salinity of the overlying water was measured during the lower tide using a portable refractometer and was found to fluctuate between 6 to 20‰ due to tidal regime but during inundation, all mats had 6‰ salinity. The water temperature was 32°C at the time of sampling.

DNA-SIP

Intact mat cores (~3 cm diameter, ~4 cm depth) were sub-sampled, transferred into 250 ml serum bottles, and embedded upright in a matrix of artificial seawater medium (10‰ salinity) with 0.5 % agar, leaving the top ~3 mm uncovered. After agar solidification, the mat was covered with a final ~2 cm of seawater water medium, and the bottles were closed under a headspace of ambient air using rubber stoppers. For ¹³C labeling, 9 replicate mat cores were incubated under an atmosphere of ¹³CO₂, which was regularly flushed and replenished with a syringe. At successive time points of incubation (0.5 to 14 d) under a 12 h / 12 h light-dark cycle, single incubations were terminated, the top ~5 mm of the mat sliced off and frozen for DNA extraction.

DNA extraction, SIP ultracentrifugation, as well as quantitative (qPCR) and qualitative (T-RFLP of bacterial rRNA genes) evaluation of density-resolved DNA fractions was done as previously described (Kunapuli et al., 2007; Rasche et al., 2009). “Heavy” and “light” DNA correspondents to ¹³C-labeled and non-labeled DNA, respectively. Processing and alignment of T-RFLP data was done using T-REX (Culman et al., 2009) with the following settings: std. dev. multiplier for noise filtering using peak heights: 1.5; definition of T-RFs by aligning peaks with a clustering threshold of 1.5; omission of T-RFs occurring in less than 5 (of 40) fingerprints.

16S rRNA cloning

For cloning of representative “heavy” and “light” DNA gradient fractions (Hh02 and Hh13, after 14 d of incubation), bacterial 16S rRNA genes were PCR amplified using the primers GM3F and GM4R (Muyzer et al., 1995) at an annealing temperature of 42°C. The PCR products were purified using the QIA quick PCR purification kit (Qiagen, Hilden, Germany) and were cloned using the TOPO TA Cloning Kit (Invitrogen, Karlsruhe, Germany) according to the manufacturer's instructions. The obtained clones were screened for the presence of inserts and the positive clones were then sequenced. 113 partial sequences with a length of 500-800 base pairs were obtained.

DGGE

The cyanobacterial cultures were subjected to nucleic acid extraction after Lueders et al. (2004). Bacterial 16S rRNA genes were amplified using primers GM5F (with GC-clamp) and 907R (Muyzer et al., 1995). DGGE was carried out using a Bio-Rad D-Code system and run at 60°C and a constant voltage of 200V for 3.5 hours. DGGE bands were excised and sequenced as described before (Abed et. al 2008).

Phylogeny

The sequences obtained from 16S rRNA cloning as well as from DGGE bands were aligned and analyzed using the ARB software (Ludwig *et al.*, 1998) version 071207 and the official database (<http://www.arb-silva.de>) of October 2008 for small subunit RNA sequences (ssuref_96_silva_04_10_08_opt.arb) (Prüsse et al. 2009). Phylogenetic trees were calculated by maximum likelihood, based on long 16S rRNA gene sequences (>1300 bp). The partial sequences were inserted into the pre-established tree using the parsimony ARB tool, while maintaining the overall tree topology without changes.

FISH and CARD-FISH

Fluorescence *in situ* hybridization of the cyanobacterial cultures was done with mono-labelled probe EUBI-III (Amann et al., 1990; Daims et al., 1999) to target all bacteria, and HRP-labeled oligonucleotide probe Cf319a to target most members of the phylum *Bacteroidetes* (Manz et al., 1996). Culture samples were fixed for 2 h with formaldehyde

(1% final concentration) and filtered on polycarbonate filters (GTTP type; pore-size 0.22 μm ; Millipore, Eschborn, Germany). Permeabilization of the cells, hybridization and tyramide signal amplification were performed as previously described (Pernthaler et al. 2004). After hybridization, the cells were counterstained with DAPI (1 μM) and analysed using a fluorescence microscope (Axioskop2 mot plus; AxioCam MRM, Axiovision 4.6, Zeiss, Germany).

Growth experiments

The cyanobacterial isolates investigated in this study were already described in Abed et al. 2008. These isolates were non-axenic and were obtained from different microbial mats from Abu Dhabi (UAE). Due to the difficulty to purify our strains from firmly attached heterotrophs, two axenic cultures of *Cyanothece* PCC 7418 and *Microcoleus chthonoplastes* PCC 7420 were ordered from the Pasteur Culture Collection for Cyanobacteria (Paris). *Cyanothece* PCC 7418 was grown in ASN III medium (Rippka et al., 1979), while *Microcoleus chthonoplastes* PCC 7420 was grown in MN medium (Rippka et al., 1979). Pure culture of *Flexibacter tractuosus* was ordered from the German Collection of Micro-organisms and Cell Cultures (DSMZ, Braunschweig), and was grown at 25 °C in 100 ml flasks (Nunc, Roskilde, Denmark) in *Cytophaga* marine medium (www.dsmz.de; medium 172).

Using these cultures, several experiments were carried out to find out:

- i) The influence of *Flexibacter tractuosus* on cyanobacterial growth. Since *Microcoleus chthonoplastes* PCC 7420 does not grow homogeneously, for each time point 3 sterile 10 ml tubes (Greiner Bio-One, Frickenhausen, Germany) with 5 ml MN medium were inoculated with 100 μl axenic *Microcoleus chthonoplastes* culture and 100 μl of a *Flexibacter tractuosus* culture. At each time point, the 3 tubes were collected and stored at -20°C until pigment extraction (see below).
- ii) The influence of an environmental heterotrophic community obtained from isolated unialgal cyanobacterial cultures on similar axenic cyanobacterial cultures. Filtrates obtained from culture M7CRI and M2C3 were added to axenic cultures of *Cyanothece* sp. and *Microcoleus chthonoplastes*, respectively.

For filtration, Isopore™ Membrane filters (Millipore, Massachusetts, USA) of pore size 5µm (filtrate with bacterial cells) and 0.2µm (filtrate without bacterial cells) were used. For the growth experiment with *Cyanothece*, 500 µl of the axenic culture was added to 18 ml ASN III medium in 100ml flasks (Nunc, Roskilde, Denmark). Then 500 µl of filtrate with and without cells was added. At each time point, 1 ml culture of each flask was sampled and stored at -20°C until pigment extraction. For the growth experiment with *Microcoleus chthonoplastes*, 5 ml MN medium was added to sterile 10 ml tubes (Greiner Bio-One, Frickenhausen, Germany), 100 µl of the axenic culture and 100 µl of filtrate M7RI (with and without cells) was added. At each time point, 3 tubes were collected and stored at -20°C until pigment extraction.

iii) Upon adding filtrate M7RI to *Cyanothece* PCC 7480, the growth of the cyanobacterium was completely inhibited. Hence, to investigate the nature and origin of this growth inhibiting substance the filtrate was treated in different ways: centrifugation, heating, further filtration step (Fig. 1).

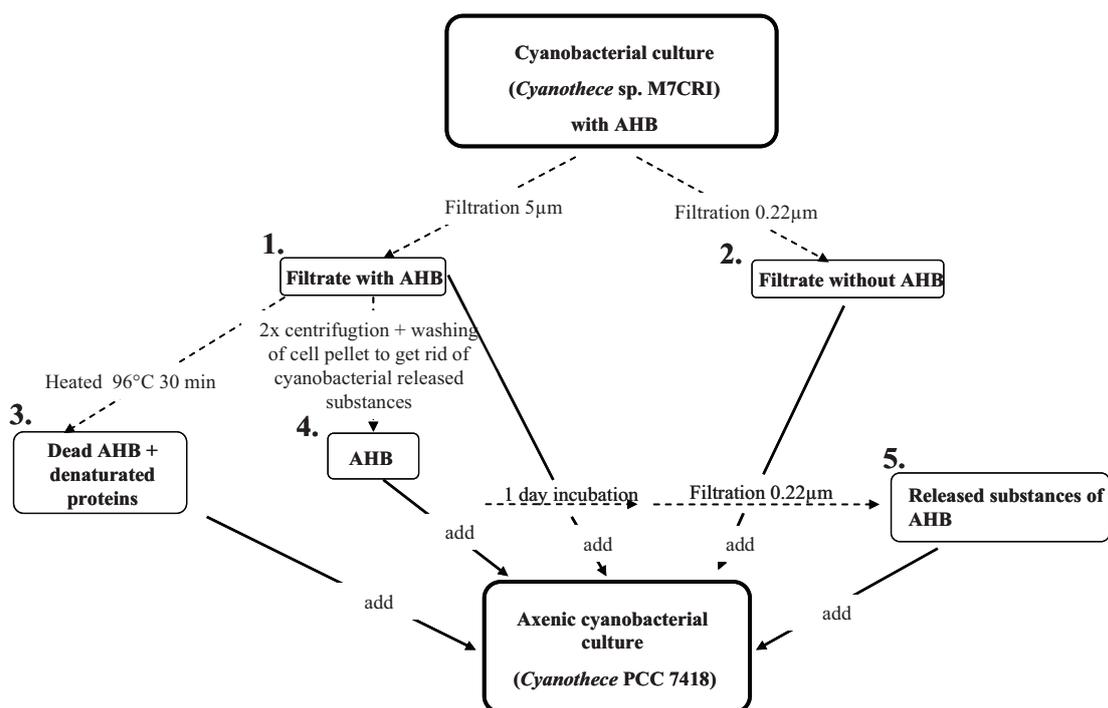


Figure 1. Scheme of growth experiments of *Cyanothece* PCC 7480 with addition of filtrates obtained from M7CRI (see Figure 8 B and C)

All growth experiments were performed in triplicates and a control experiment without addition of other bacteria or filtrates was performed for each axenic cyanobacterial culture. All cultures were grown at 25°C with a day-night cycle of 12 hours each and a light intensity of 240 μ E. Inoculation was done with logarithmic-phase cultures.

Chlorophyll *a* (Chl *a*) extraction and spectrophotometric measurement.

The collected culture samples were thawed and centrifuged at 16,000 rpm, 4°C for 5 minutes. The supernatant was discarded and 1 ml of 99.8% ice-cold methanol (Merck, Darmstadt, Germany) was added to the pellet, followed by incubation for 2 days at -20°C. After 2 days, the samples were again centrifuged at 16,000 rpm, 4°C for 10 minutes and the methanol containing the extracted pigments was collected. An additional extraction was done by adding 0.5 ml methanol on the pellet, followed by sonication. The samples were incubated overnight at -20 °C and the second fraction was collected the next day after centrifugation at 16,000 rpm, 4°C for 10 minutes. Chl *a* was measured at a wavelength of 665nm and corrected for turbidity by subtracting the absorbance at 750 nm (Riemann and Ernst, 1982) using Perkin Elmer UV-Vis Spectrometer Lambda 20 (Buckinghamshire, England).

Results

DNA-SIP of fresh mat samples

DNA-stable isotope probing (DNA-SIP) was carried out to identify the AHB that play a significant role in carbon cycling in the upper millimetres of an intact cyanobacterial mat. Quantitative PCR (qPCR) of fractionated gradients detected maximal DNA banding in the “light” fractions (buoyant densities (BDs) between 1.70 and 1.71 g ml⁻¹) of all successive gradients. Only for the “heavy” fractions (i.e. BDs > 1.74 g ml⁻¹) of the late ¹³CO₂ DNA gradients (after 4.2, 8 and 14 d of incubation), qPCR detected increased amounts of bacterial DNA (Fig. 2). Thus, substantial amounts of ¹³C-labeling of microbial mat DNA was not accomplished under our incubation conditions.

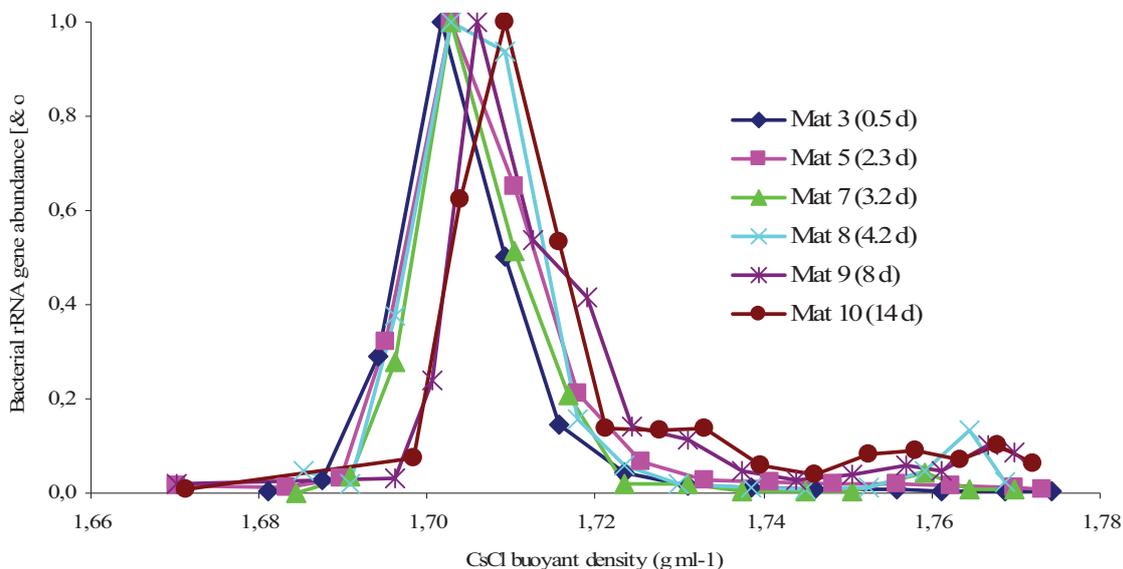


Figure 2. Quantitative distribution of bacterial DNA in ultracentrifugation gradients at successive time points after incubation with ¹³CO₂. Bacterial 16S rRNA genes were quantified by qPCR of DNA from gradient fractions and normalized to maximum detectability for each gradient.

Bacterial 16S rRNA gene targeted T-RFLP fingerprinting revealed clear labelling, when comparing “light” and “heavy” gradient fractions over time (Fig. 3). After 3.2 d of incubation, especially the relative abundance of the 492 bp terminal restriction fragments (T-RF) increased in the “heavy” fractions, thus showing clear evidence of ¹³C-labeling. After 8 d, also the 201 bp T-RF showed ¹³C-labeling. Other important T-RFs in the mat community were the 148 and 113 bp fragments, however no further specific labeling was observed.

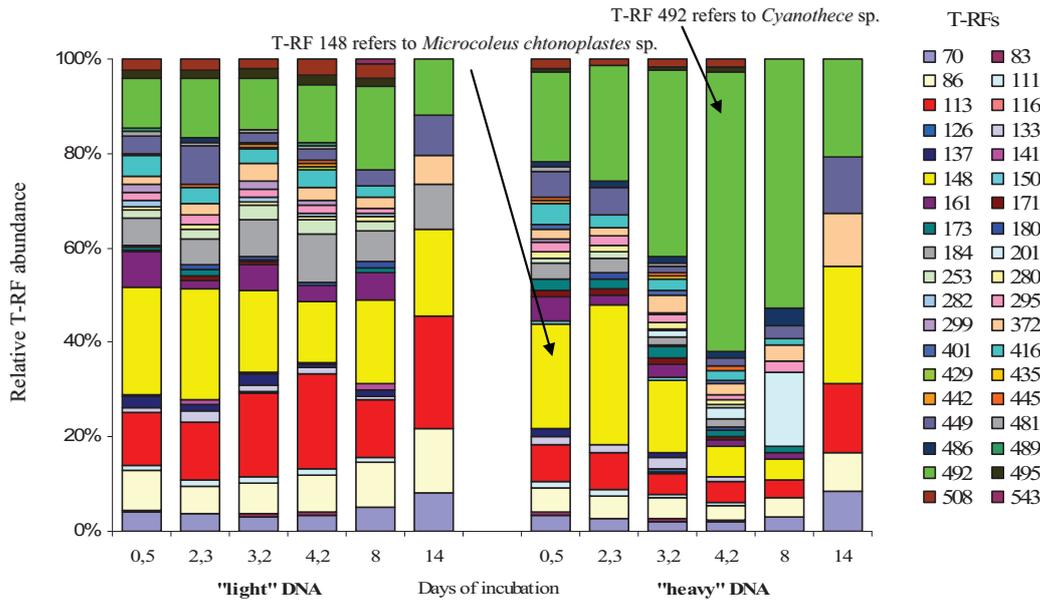
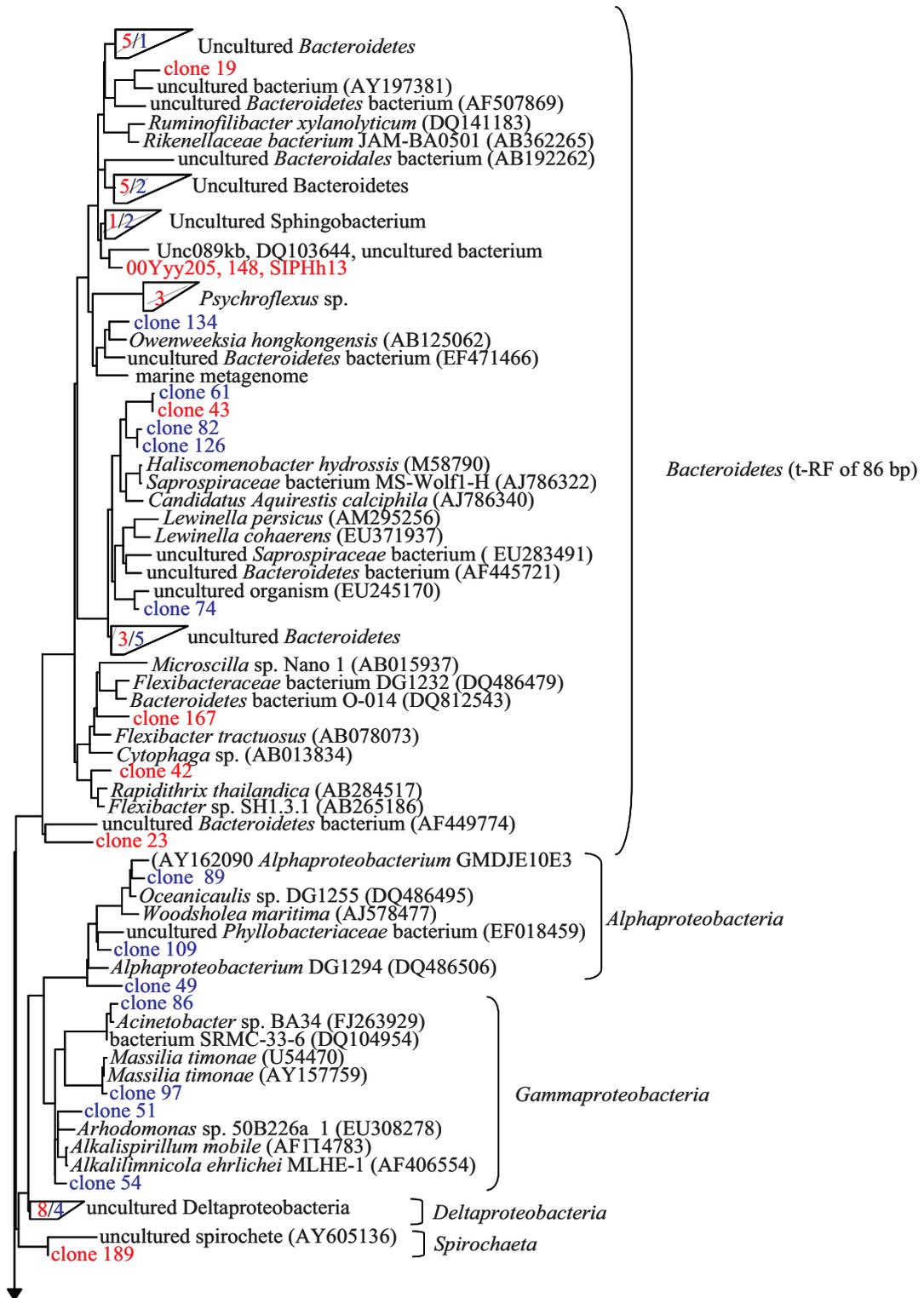


Figure 3. Relative abundance of bacterial T-RFs amplified from representative “light” and “heavy” DNA gradient fractions at successive time points after incubation with $^{13}\text{CO}_2$. The average buoyant density of shown “light” fractions was $1.704 \pm 0.003 \text{ g ml}^{-1} \text{ CsCl}$, that of “heavy” fractions 1.755 ± 0.006 ,

Identification of these T-RFs was done by sequencing of bacterial 16S rRNA gene clones from a representative “light” and “heavy” gradient fraction (day 14 DNA gradient, Fig. 4). The 492 bp fragment was clearly affiliated to a cluster of clones related to *Cyanothece* sp. (predicted T-RF of 493 bp), while the 148 bp T-RF belonged to *Microcoleus* sp. (150 bp predicted). Interestingly, from two dominating cyanobacterial population present in the mat, only *Cyanothece* sp. showed strong uptake of $^{13}\text{CO}_2$. No specific ^{13}C -labeling of other populations than those represented by the 492 and 201 bp T-RFs was observed. Unfortunately, the latter could not be identified via cloning. Further identified T-RFs belonged to members of the *Chloroflexi* (372 and 449 bp, 375 and 447 bp predicted) and *Bacteroidetes* (86 bp, 90 bp predicted). Moreover, 16S rRNA cloning revealed that sequences within *Chloroflexi*-related bacteria and *Alpha*- and *Gammaproteobacteria* were preferentially allocated to the heavy fraction, whereas most of the other groups possessed a balanced ratio between sequences in light and heavy fraction.



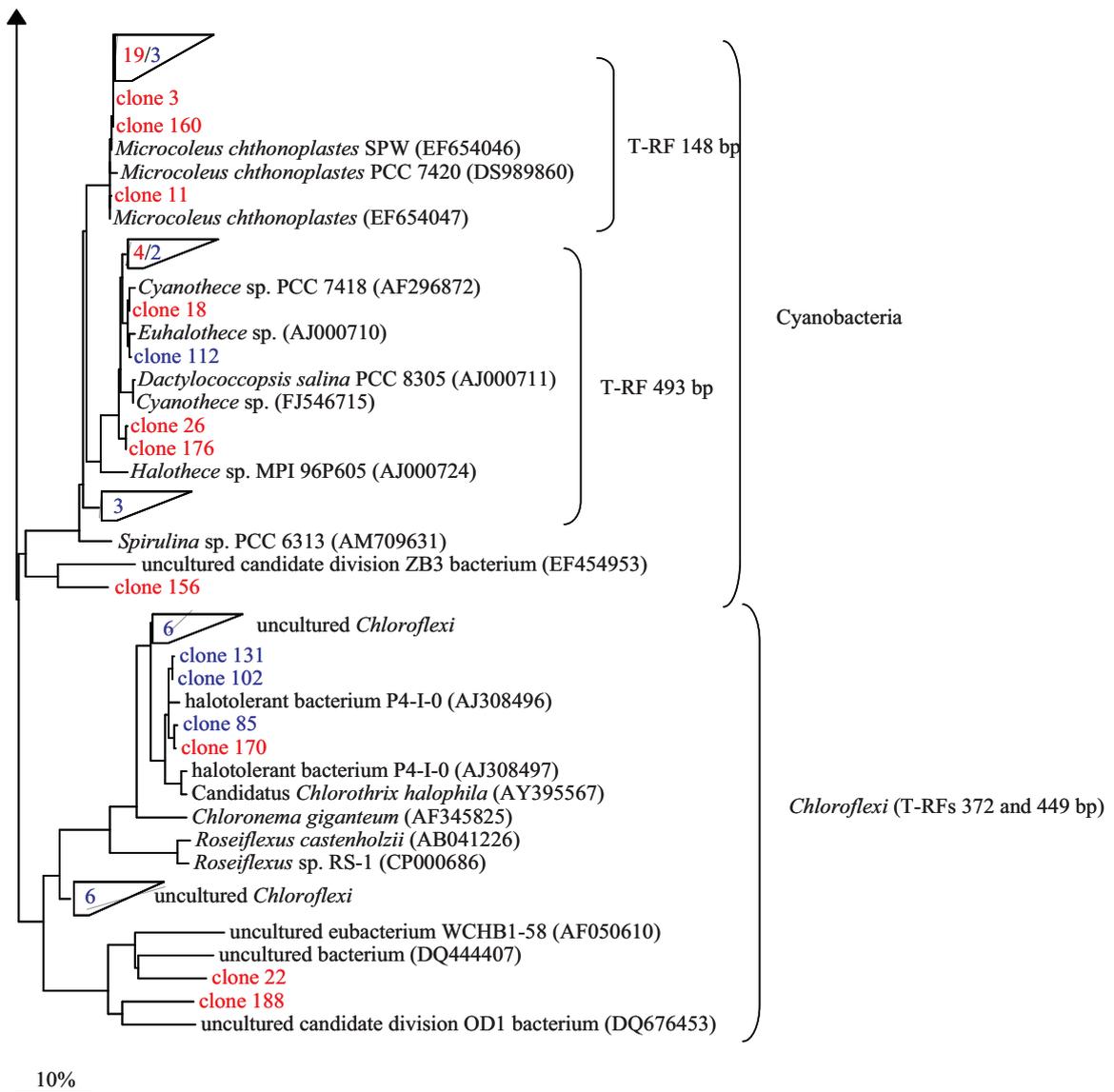


Figure 4. 16S rRNA gene-based phylogenetic reconstruction using maximum likelihood methods, showing the affiliation of sequences retrieved from 16S rRNA gene clones from representative “light” (red) and “heavy” (blue) SIP gradient fractions (day 14 DNA gradient). Partial clone sequences were inserted applying parsimony criteria without allowing changes in overall tree topology. Accession numbers are indicated in parentheses.

DGGE of cyanobacterial cultures

The AHB, associated with different strains of cyanobacteria, were identified to find out whether their identity is cyanobacteria-specific. The phylogeny of the used cyanobacterial strains was described in Abed et al. (2008). DGGE showed that different cyanobacterial species were associated with different populations of AHB (Fig. 5). The two strains of *Microcoleus* (i.e. M7C3 and M7C5) possessed same types of associated heterotrophs, while these bacteria were different in the other cyanobacterial cultures (*Microcoleus* sp. M7C1 and *Cyanothece* sp. M7CR1), obtained from the same mat. The *Halomicronema* strains M5C6 and M5C7, which were isolated from the same mat, showed a similar DGGE pattern of associated bacteria except for two bands (band 45 and 46), which were only present in the culture M5C7. Interestingly, *Leptolyngbya* strains M6C11 and M1C10, which were derived from different mats, varied clearly in the DGGE patterns of accompanied bacteria, although some bands were common.

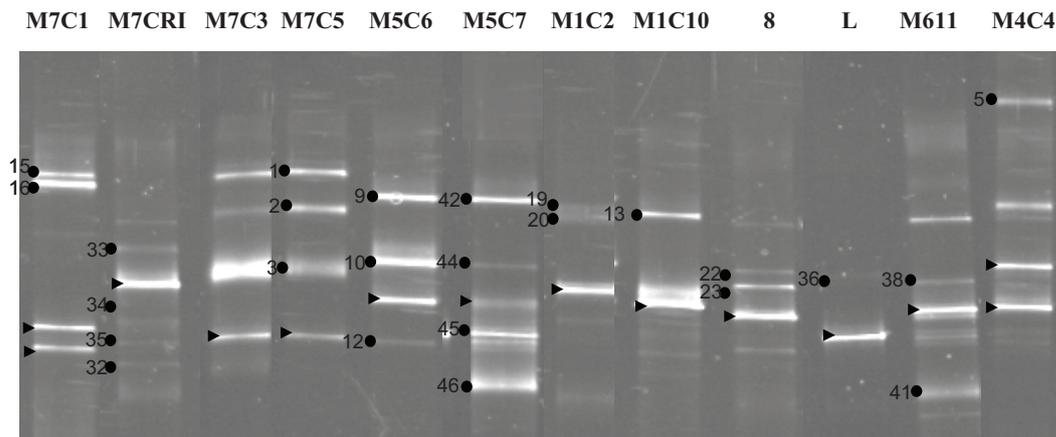


Figure 5. DGGE pattern of cyanobacterial cultures with their associated bacteria using bacterial primers GM5-GC and 907RC. Sequences retrieved of associated bacteria are numbered and highlighted with a circle. Bands belonging to cyanobacteria are marked with triangle.

Phylogenetic analysis of sequenced DGGE bands (Fig. 6) revealed that most of the associated heterotrophs belonged to the phylum *Bacteroidetes*, but *Alpha-*, *Gammaproteobacteria* and *Chloroflexi*-related bacteria were also detected. *Bacteroidetes*-related bacteria were found in most cultures, regardless of their origin. The identity of associated heterotrophs differed between filamentous and unicellular cyanobacteria.

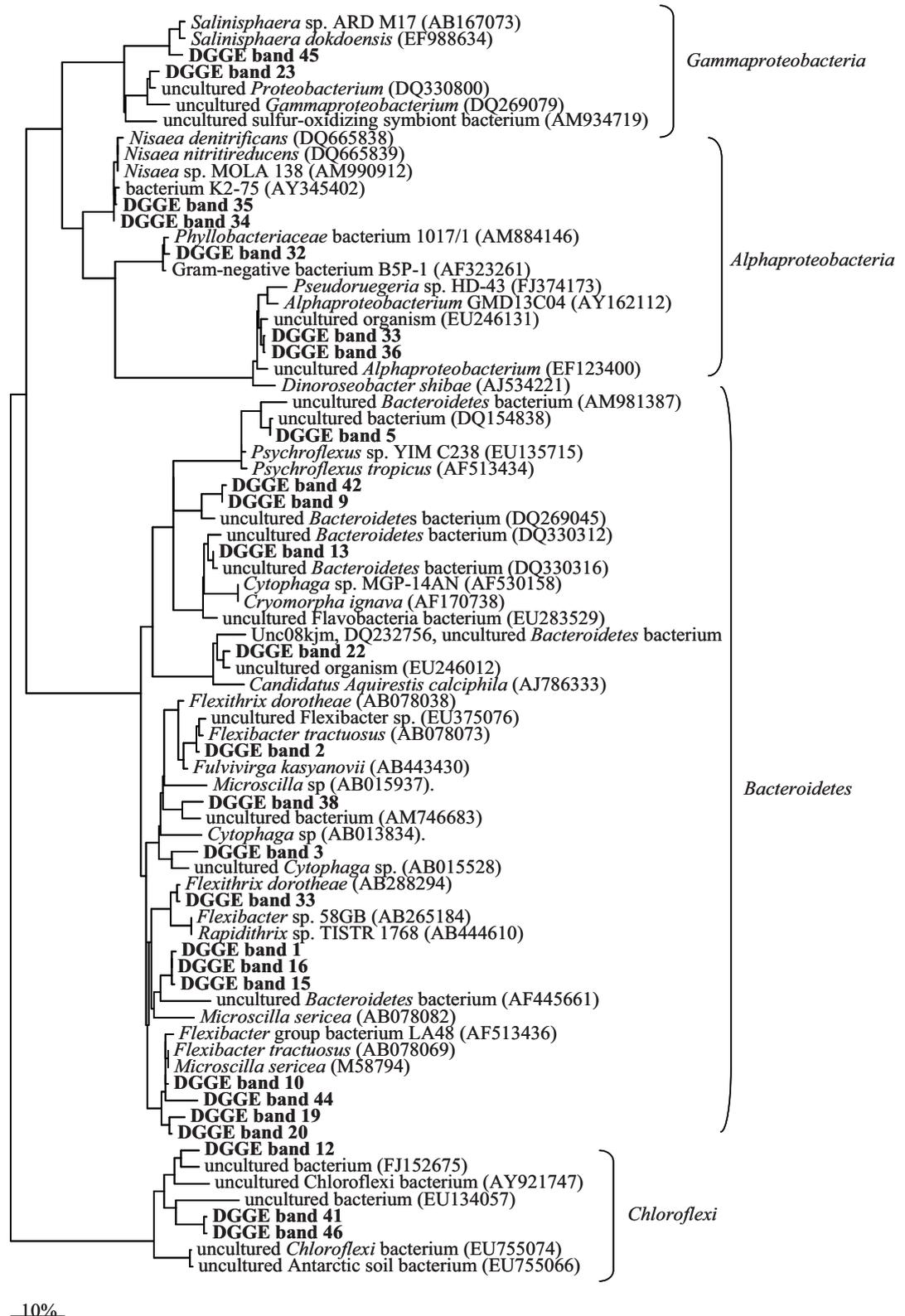


Figure 6. 16S rRNA gene-based phylogenetic reconstruction based on maximum likelihood methods, showing the affiliation of sequences retrieved from DGGE bands obtained from different unialgal cyanobacterial cultures. The partial sequences were inserted into the reconstructed tree by applying parsimony criteria without allowing changes in the overall tree topology. Accession numbers are indicated in parentheses.

Within the *Bacteroidetes* group, *Flexibacter*-related sequences were most dominant although sequences belonging to the genera *Microscilla*, *Cytophaga* and *Psychroflexus* were also encountered. Bacteria related to *Microscilla sericea* were found in most of the cyanobacterial cultures. Sequences affiliated to *Alphaproteobacteria* were closely related to *Bacillus* and *Nisaea*, while sequences within the *Gammaproteobacteria* belonged mainly to *Salinisphaera*. Associated bacteria closely related to *Chloroflexi*, were found in three cultures (i.e. *Halomicronema* spp. M5C6 and M5C7, and *Leptolyngbya* spp. M6C11). In the unicellular culture *Cyanothece* spp. M7CRI, associated bacteria were affiliated with the denitrifying bacteria, *Nisaea denitrificans* and *Nisaea nitritireducens*.

FISH and CARD-FISH of cyanobacterial cultures

FISH and CARD-FISH hybridisations were carried out in order to study the distribution of AHB around cyanobacterial cells. Hybridization of *Leptolyngbya* sp. M6C11 with the universal probe for bacteria EUB I-III (Fig. 7C) showed coccoid cells, which were surrounded by a DAPI-stained ring. These cells could be either bacterial cells that are not targeted by the probe or extracellular DNA embedded in an EPS matrix. Also some DAPI signals of long, filamentous bacteria, possibly *Chloroflexi*-related bacteria, were not targeted by this probe. Since most of the associated bacteria in the cultures belonged to *Bacteroidetes*, CARD-FISH hybridizations were carried out using the specific probe Cf319a which targets around 38% of this phylum (www.microbial-ecology.de/probebase). Hybridization of culture M6C11 with Cf319a, showed rod shaped bacteria, which were typically attached to cyanobacterial dead cell material in most cases (Fig. 7C). Hybridization of *Phormidium* sp. M1C2 and *Microcoleus* sp. M7C5 with this probe (Fig. 7A and B) showed filamentous and coccoid bacteria either firmly attached to the cyanobacterial filaments or free-living in the culture. Some of these bacteria were also associated to dead cyanobacterial cells (Fig 7A and B; see arrows), and seemed involved in the cleavage of cyanobacterial filaments (Fig.7B).

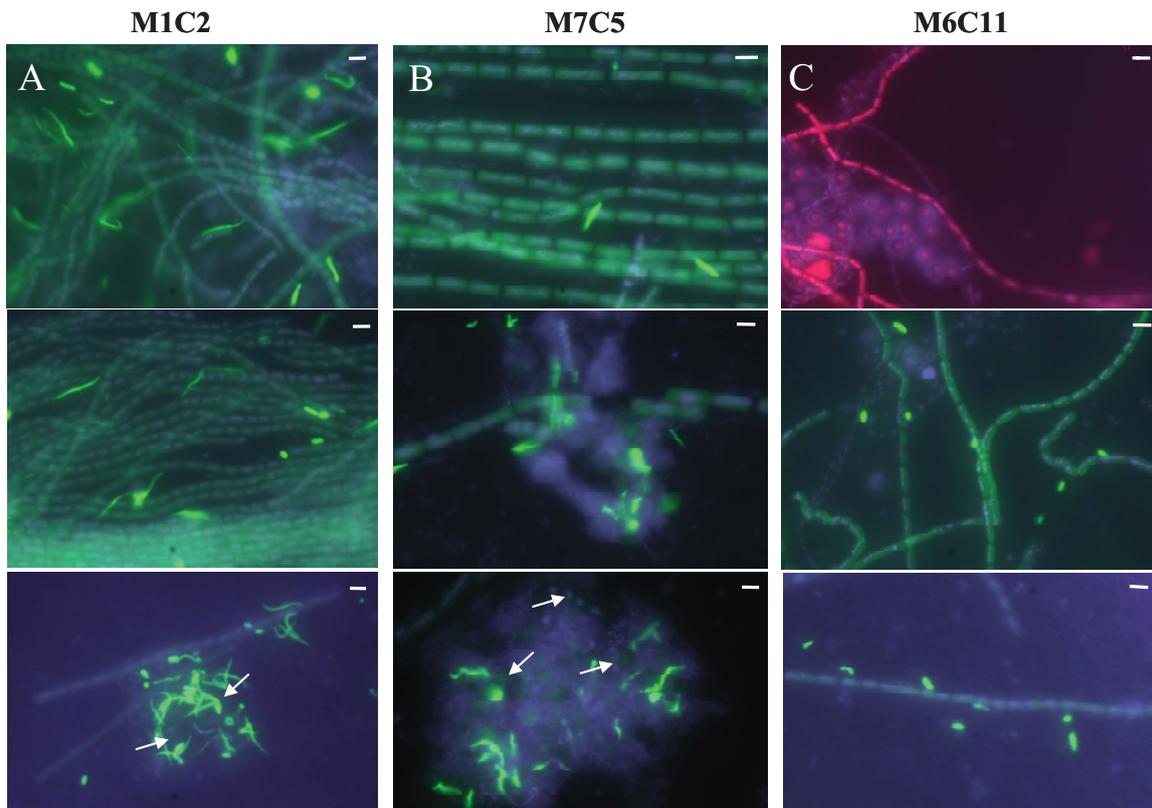


Figure 7. Cyanobacterial cultures (*Phormidium* sp. M1C2 (A), *Microcoleus* sp. M7C5 (B), *Leptolyngbya* sp. M6C11(C)) isolated from mats of Abu Dhabi with their attached and free living aerobic heterotrophic bacteria, visualized with FISH (probe EUB I-III, targets all bacteria) and CARD-FISH (probe Cf319, targets *Bacteroidetes*). Scale bar= 2 μ m. Green (Cf319) and red (EUB I-III) color show the probe signal, blue color shows the DAPI signal. Arrows indicate dead cyanobacterial cells.

Growth experiments

Different growth experiments were carried out, in order to get a better understanding of the specific interactions between cyanobacteria and AHB (Fig. 8). The growth of the axenic culture of *Microcoleus chthonoplastes* PCC 7420 was enhanced upon addition of filtrates with and without bacterial cells, obtained from the closely related cyanobacterial culture *Microcoleus* sp. M2C3 (Fig. 8A). In contrast, after adding similar filtrates obtained from a non-axenic unicellular cyanobacterial culture (i.e. *Cyanothece* spp. M7CRI), the growth of *Cyanothece* PCC 7418 was completely inhibited (Fig. 8B and C). These filtrates had the same growth inhibitory effect to other tested cyanobacterial strains like *Leptolyngbya* PCC 8103, *Xenococcus* PCC 7304, *Microcoleus* PCC 7420 (data not shown). To explore the nature and origin of the inhibiting substances, the filtrates were treated in different ways (Fig.1). Filtrate 3, which was heated to destroy proteins, had

initially an inhibitory effect on the *Cyanothece* PCC 7814 axenic culture. The culture with filtrate 3 showed a lag phase of about 35 days longer, compared to the control (Fig 8C). The M7CRI associated AHB (filtrate 4; Fig.1) as well as their released substances only (filtrate 5; Fig.1), inhibited the growth of the cyanobacterium *Cyanothece* PCC 7814 completely (Fig 8C). When adding *Flexibacter tractuosus* to *Microcoleus chthonoplastes* PCC 7420, the cyanobacterium first grew normally until the growth was suddenly stopped after day 28 of the experiment, followed by a rapid decrease in Chl *a* concentrations.

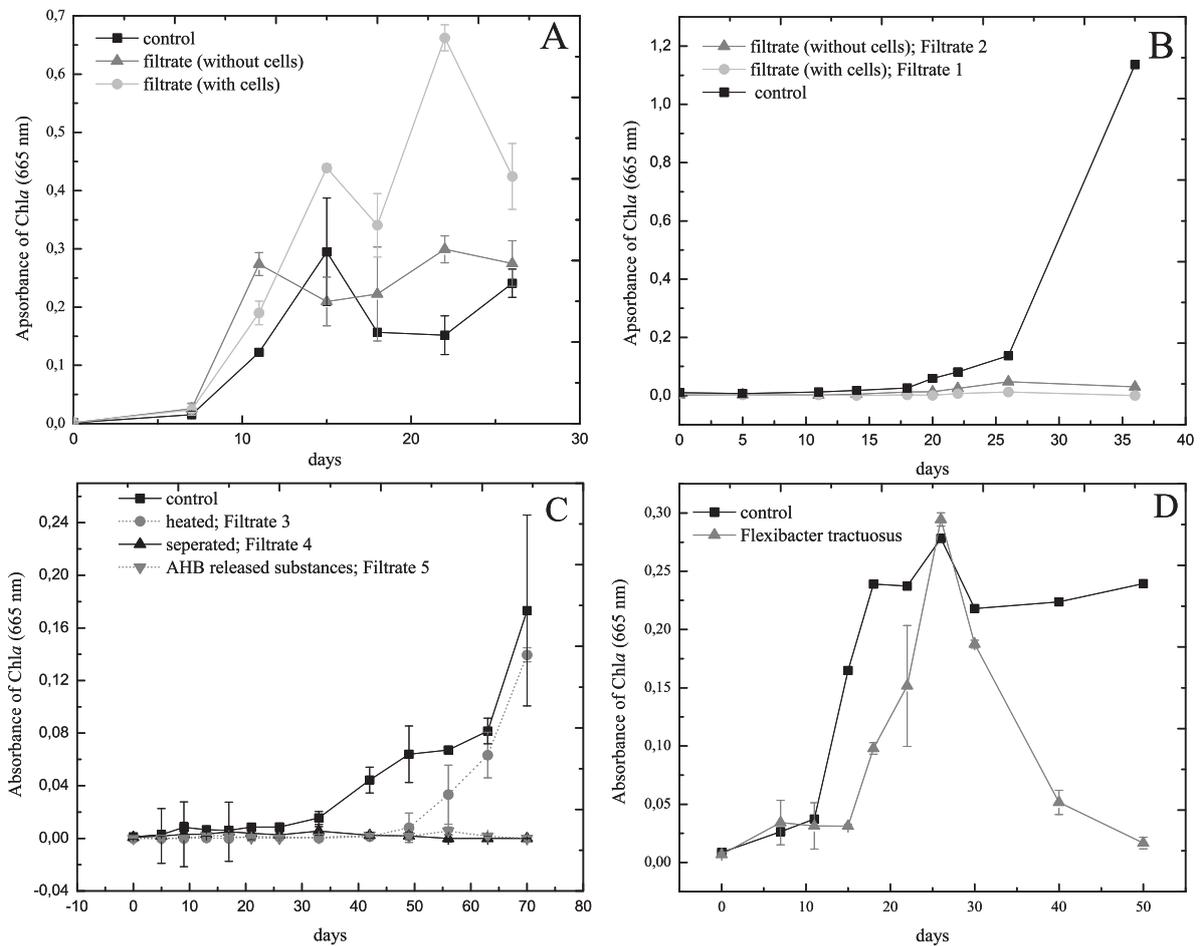


Figure 8. Growth experiments of axenic cyanobacterial cultures (A) *Microcoleus chthonoplastes* PCC 7420 with addition of filtrates (with and without bacteria) obtained from culture *Microcoleus* spp. M2C3. (B -C) *Cyanothece* PCC 7480 with addition of filtrates obtained from culture *Cyanothece* spp. M7CRI (numbers of filtrates refer to Figure 1). (E) *Microcoleus chthonoplastes* PCC 7420 with addition of *Flexibacter tractuosus*.

Discussion

DNA-SIP on an intact microbial mat indicated that unicellular cyanobacteria were much more active in $^{13}\text{CO}_2$ assimilation than filamentous cyanobacteria closely related to *Microcoleus* species. Indeed, unicellular cyanobacteria are known to be extremely halotolerant and halophilic and usually dominate hypersaline environments (Brock 1976; Walsby et al. 1983; MacKay et al. 1984; Javor 1989, Garcia-Pichel F., 1998; Oren A. 2002). Hence, they might be more active at higher salinities than filamentous cyanobacteria. Although clone libraries indicated a preferential allocation of certain lineages to the “heavy” fractions (Fig. 4), this was not consistent with T-RF abundances (Fig. 3), indicative for PCR and cloning bias. The successively increased detectability of bacterial DNA in “heavy” fractions (i.e. BDs $> 1.74 \text{ g ml}^{-1}$) after 4.2, 8 and 14 d of incubation under $^{13}\text{CO}_2$, indicate that ^{13}C -label had been passed on from the primary assimilating *Cyanothece*-relatives to the remaining mat microbial community. However, this appeared more related to a general cross-feeding, than to a selective labeling of specific secondary mat AHB, since the entire mat community appeared labelled after a certain time. This illustrates an insufficient resolution and sensibility of our SIP experiment, which might be due to the fact that DNA-SIP instead of RNA-SIP was performed. The sensitivity of RNA-SIP is significantly higher than DNA-SIP, because of the rapid turnover of rRNA in the cell. An additional advantage RNA-SIP is that the active population does not require replication to generate labeled RNA, whereas the recovery of labeled DNA requires at least one cell division (Dumont, 2006; Whiteley, 2006). Unfortunately, RNA extraction for this mat was not successful although different protocols were tried out.

Investigation of AHB-cyanobacterial relationships in monocultures indicated, that the community structure of AHB associated with cyanobacteria strongly depends on the specific environment from which the cyanobacterium was isolated, and also strongly on the identity of cyanobacterial species. The importance of *Bacteroidetes*-related bacteria in cyanobacterial cultures was also stressed by Hube and colleagues (2009), who showed that besides *Alphaproteobacteria*, *Bacteroidetes* were most dominant in two marine cyanobacterial cultures. Many of the bacteria within the *Bacteroidetes* are chemoorganotrophic, providing a high capability of digesting large polysaccharides such

as chitin, pectin and cellulose (Madigan et al., 2001; Kirchmann, 2002). They might benefit from cyanobacterial exudates as well as from dead cell material. The fact that they were often found attached to dead cell material suggests that they may rather on decaying cyanobacterial biomass, than on exudates from living cyanobacteria. The high abundance of *Chloroflexi*-like bacteria in the mats and cultures suggests a strong interaction between both groups. Similar observations were previously reported by Ley et al. (2006) for microbial mats from Guerrerro Negro (Mexico). They suggested a co-metabolism of sulfur, where the *Chloroflexi* bacteria reduce H₂S, that is stressful for the cyanobacteria. Cyanobacteria transfer oxygen and possibly organic carbon to the *Chloroflexi*-related bacteria. Since *Microcoleus* filaments were often disrupted near *Chloroflexi* cells, they suggested that *Chloroflexi* bacteria kill cyanobacteria. We could not confirm this observation and suggest the association is of mutual advantage.

The detection of known denitrifying and nitrite reducing bacteria (i.e., *Nisaea denitrificans* and *Nisaea nitritireducens*) and the presence of the *nifH* gene (data not shown) in the *Cyanothece* culture (M7CRI), hints to a possible role of AHB in nitrogen cycling. The associated bacteria might form nitrogen out of nitrate by denitrification, which could then be fixed by other bacteria or even by the host-cyanobacterium. Some bacteria are known to attach to heterocysts of *Anabena* species, promoting nitrogen fixation by respiring the oxygen around the heterocysts, thus protecting the oxygen sensitive nitrogenase from high oxygen levels (Paerl 1976 and 1982, Paerl and Keller 1978). Steppe and colleagues (1996) proposed a mutualistic N₂-fixing consortium, where non N₂-fixing *Microcoleus* spp. provide a habitat for good growth conditions for epiphytic diazotrophic bacteria, which supply the cyanobacteria with fixed nitrogen. Interestingly, in a recent study it was shown that the cyanobacterium *Leptolyngbya nodulosa* contains a functional nitrogenase which is not expressed in the absence of heterotrophic bacteria (Zonghkui et al., 2010).

Growth experiments revealed that the released substances of an AHB community apparently promote the growth of its host cyanobacterium, but inhibit the growth of other potentially competitive cyanobacteria. The enhancement of *Microcoleus chthonoplastes* PCC 7420.growth upon the addition of a filtrate obtained from a closely related non-axenic cyanobacterial culture (*Microcoleus* spp. M2C3) could be due to the release of

certain growth factors and vitamins (Paerl, 1996). The addition of vitamin B₁₂ to the cyanobacterial culture also resulted in enhancement of its growth (data not shown). Interestingly, it was shown that cyanobacterial isolates from marine muds produced and excreted B vitamins, which were utilized by vitamin requiring bacteria (Burkholder 1963). Additionally, the growth of cyanobacteria could be stimulated by oxygen removal and H₂S removal, and CO₂ supply by the AHB, thereby protecting the cyanobacteria from photo-oxidative stress (Abeliovich et al., 1972; Eloff et al., 1976; Paerl and Kellar, 1978). In contrast to these beneficial relationships, *Flexibacter* spp. e.g. is known to lyse cyanobacteria (Marshall, 1989). This could be also shown in this study using CARD-FISH and growth experiments. However, in this interaction a direct attachment of the cells seems to be required for the cyanobacterial lysis, which is carried out by the release of lysozyme-like substances (Stewart and Brown, 1969; Sallal, 1994). Furthermore, the filtrate of a unicellular culture (M7CRI) showed a strong bactericidal potential on other cyanobacteria. Viruses as the source of growth inhibition or killing can be excluded, since they are known to be highly host specific (Sullivan et al. 2003). A given type of virus usually has a restricted range of hosts, often a single species (Fuhrman 1999). Here all tested cyanobacterial genera were killed except the cyanobacterial host. This indicated that an inhibiting substance, e.g. an antibiotic, was released by the cyanobacteria-associated bacteria into the medium. This might be protective for the cyanobacteria enabling them to compete against other cyanobacteria, while the associated bacteria, in return, feed on the cyanobacterial exudates. Indeed antibiotic compounds provide a competitive advantage to the bacteria producing them over non-producing strains isolated from the same habitat (Lemos et al., 1991). The production of antibiotics, toxins, signalling molecules and other secondary metabolites, as effective competition and defence strategies by microorganisms represents an important resource for the discovery and development of new drugs and bioactive substances, with potential applications in medicine, industry and environmental settings. However, the real identity of the growth-inhibiting substance in this study remains unclear and needs further investigations.

In conclusion, the interactions between cyanobacteria and AHB are very complex, manifold, highly specific and cannot be generalized. Some of the released substances might have high potential for biotechnical purposes, due to unknown released substances.

Thus, further experiments are required to focus on the identity and function of released substances in these cultures. Furthermore, optimized RNA-SIP experiments, nanoSIMS and MAR-FISH are needed in order to identify the key-players in carbon cycling in microbial mats.

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Eidesstattliche Erklärung

Gem. § 6(5) Nr.1-3 PromO

Ich erkläre hiermit, dass ich

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