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Research Article

RESEARCH ARTICLE

Abundance and diversity of aerobic heterotrophic microorganisms and their interaction with cyanobacteria in the oxic layer of an intertidal hypersaline cyanobacterial mat

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One sentence summary: This study used a suite of culture-dependent and independent approaches to study diversity and abundance of aerobic heterotrophs and their interaction with cyanobacteria.

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ABSTRACT

Aerobic heterotrophic microorganisms (AH) play a significant role in carbon cycling in cyanobacterial mats; however, little is known about their abundance, diversity and interaction with cyanobacteria. Using catalyzed reporter deposition fluorescence *in situ* hybridization (CARD-FISH), bacterial counts in the mat's oxic layer reached a mean of $2.23 \pm 0.4 \times 10^{10}$ cells g^{-1}. Cultivation of AH yielded strains belonging to *Actinobacteria*, *Bacteroidetes*, *Firmicutes*, *Gammaproteobacteria* and *Haloarchaea*. 16S rRNA bacterial sequences retrieved from the mat's oxic layer were related to *Bacteroidetes*, *Chloroflexi* and *Proteobacteria*, whereas archaeal sequences belonged to *Crenarchaeota* and *Haloarchaea*. Monocultures of cyanobacteria from the same mat were associated with different AH, although *Bacteroidetes* were found in most cultures. CARD-FISH showed that *Bacteroidetes*- and *Chloroflexi*-related bacteria were closely associated with filaments of *Microcoleus chthonoplastes*. The growth of an axenic culture of *M. chthonoplastes* PCC7420 was stimulated on the addition of a filtrate obtained from a non-axenic *Microcoleus* culture and containing only AH and released substances. In contrast, a similar filtrate from a non-axenic *Cyanothece*-related culture killed *Cyanothece* PCC 7418. We conclude that a diverse community of AH exist in close association with cyanobacteria in microbial mats and the interactions between AH and cyanobacteria are species-specific and involve the release of substances.

Keywords: cyanobacterial mats; aerobic heterotrophs; archaea; qPCR; CARD-FISH; Cyanobacteria

INTRODUCTION

The uppermost oxic layer of cyanobacterial mats is mainly inhabited by cyanobacteria and aerobic heterotrophic microorganisms (AH), and is the most active part of the mat in volumetric carbon cycling (Ley *et al.* 2006). AH are important for the degradation part of the carbon cycle within mats (van Gemerden 1993). Their respiratory activity is closely coupled to

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cyanobacterial photosynthesis even under varying conditions of salinity, temperature and irradiance (Wieland and Kühl 2000; Grötzschel *et al.* 2002; Abed *et al.* 2006; Abed, Kohls and de Beer 2007). This is probably due to their ability to grow on cyanobacterial exudates (Bateson and Ward 1988; Grötzschel *et al.* 2002). Using enrichment cultivation and substrate spectra of obtained isolates, different functional guilds of AH specialized in the consumption of specific cyanobacterial exudates have been identified (Jonkers and Abed 2003; Abed *et al.* 2007). Most probable number counts showed a numerical abundance of AH in the range of 1.15–8.13–10^6 cells g^{-1} and obtained isolates belonged to the genera *Marinobacter*, *Halomonas*, *Porphyrobacter*, *Roseobacter*, *Rhodobacter* and *Alcanivorax* (Jonkers and Abed 2003; Abed *et al.* 2007; Hube, Heyduck-Söller and Fischer 2009). Despite the significant role of AH in microbial mats, little research has been performed to investigate their abundance (depth distribution) and diversity using culture-independent molecular techniques. 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; Wong *et al.* 2016).

The relationships between cyanobacteria and AH can be competitive for scarce nutrients and other resources, or mutualistic stimulating the survival and persistence of each other (Paerl 1996). For instance, too high oxygen concentrations, evolved during cyanobacterial photosynthesis, can inhibit several metabolic and biosynthetic pathways of cyanobacteria. It is thought that AH counteract the chemical changes in O_2, CO_2 and pH induced by photosynthesis via respiration activities (Carr and Whitton 1973; Wieland and Kühl 2006). Cyanobacteria and associated AH 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, AH are thought to play a significant role in carbon cycling through direct utilization of cyanobacterial exudates and regeneration of CO_2 required for cyanobacterial photosynthesis (Lange 1967; Bauld and Brock 1974; Paerl 1976; Herbst and Overbeck 1978; Cole 1982; Bateson and Ward 1988; Baines and Pace 1991; Wang and Priscu 1994; Epping, Khalili and Thar 1999; Kirkwood, Nalewajko and Fulthorpe 2006). Indeed, recent studies have clearly demonstrated, using stable isotope imaging based on nanometer scale secondary ion mass spectrometry (NanoSIMS), the ability of AH to assimilate carbon and nitrogen fixed by cyanobacteria (Behrens *et al.* 2008; Pett-Ridge and Weber 2012). All these studies suggest a complex and manifold relationship between AH and cyanobacteria; thus, it becomes clear that a gap still exists in our knowledge of the microbial communities surrounding cyanobacteria.

This study aimed at identifying and quantifying the bacterial and archaeal populations in the fully oxic layer of a hypersaline cyanobacterial mat from Abu Dhabi (UAE) using culture-dependent and independent approaches. We also identified AH in different monoclonal cyanobacterial cultures obtained from the same microbial mat, using denaturing gradient gel electrophoresis (DGGE), with the aim to find out whether different cyanobacteria have similar or different (species-specific) associated AH. The distribution of AH 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 co-cultivation growth experiments under different conditions.

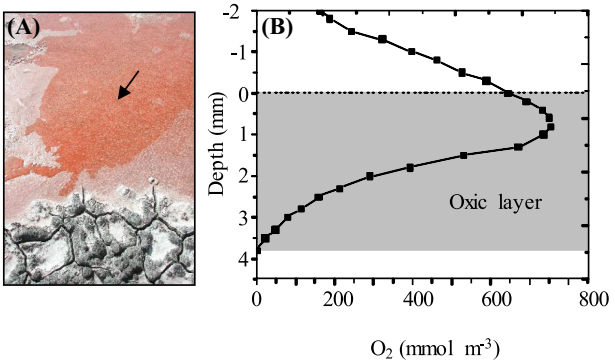

Figure 1. (**A**) A field photograph showing (arrow) the hypersaline microbial mat from the upper intertidal zone used in this study, (**B**) oxygen microsensor profile of the mat in the light; shaded area highlights the oxic part of the mat.

MATERIAL AND METHODS

Mats origin and determination of the oxic layer

Mats pieces (8 × 15 cm) were sampled from the upper (termed as gelatinous mat in Abed *et al.* 2008) intertidal flats of Abu Dhabi (Arabian Gulf coast, United Arab Emirates) during low tide (Fig. 1A and B). Air temperature in the region typically reaches over 50°C during hot summers and between 15°C and 35°C in winter. The salinity of the mat's overlying water fluctuated between 6% and 20% as measured during low and high tide using a portable refractometer. The water temperature was 32°C at the time of sampling. Collected samples were stored at –80°C for further molecular analysis, but some samples were kept alive in aquaria for cultivation of microorganisms. We used only the oxic layer of the mats for our study, which was found to be 3.5 mm thick as determined by an oxygen microelectrode (Fig. 1B). This layer was excised under a dissecting microscope with a clean scalpel blade and sterile forceps. This delimited the analyses to populations that are potentially active as AH.

Quantification by qPCR

Depth distribution and abundance of bacterial and archaeal 16S rRNA genes in the excised oxic layer of the mats were studied using qPCR. The anoxic layer of the mat was also included in the analysis for comparison. Mat pieces were incubated in O.C.T. compound (Plano) at 4°C for 24 h. The mat pieces were then transferred into cryotrays and left at –20°C for 5 h. 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, Manefield and Friedrich (2004). qPCR for the bacterial 16S rRNA genes was performed 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 archaeal 16S rRNA genes were quantified using the probe Arch516F and the primer set ARCH349F and ARCH806R as described by Takai and Horikoshi (2000). The assays were performed on a 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 each of the universal forward and reverse primers and 1 μl 10-fold dilution of sample DNA solution (10 ng). The standard was a DNA mixture of different archaeal and bacterial strains. 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

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

Catalyzed reporter deposition fluorescent *in situ* hybridization

Since qPCR quantification provided information only on the depth distribution pattern of 16S rRNA copy numbers and that the used bacterial primers can also pick up cyanobacterial DNA, bacterial cells in the oxic layer were specifically counted by CARD-FISH. Oligonucleotide probe mixture (EUB338 I–III) targeting all bacteria was used (Amann *et al.* 1990; Daims *et al.* 1999). 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. Mat slices were homogenized using glass homogenizers and ultrasonication. The samples were subsequently filtrated 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 described by Pernthaler, Pernthaler and Amann (2002) and Snaidr *et al.* (1997). After hybridization, the cells were counterstained with DAPI (1 μM) and analyzed using a fluorescence microscope (Axioskop2 mot plus; Axiocam MRM, Axiovision 4.6, Zeiss, Germany). At least 1000 DAPI-stained cells were counted. Hybridization with the probe NON338 was performed as a negative control (Wallner, Amann and Beisker 1993). Thin cross-sections (5 μm; see the qPCR section) of fixed mat sample were placed onto polysine coated slides. CARD-FISH was additionally performed on mat slices with the *Chloroflexi*-specific probes GNSB-941 and CFX-1223 (Gich, Garcia-Gil and Overmann 2001; Björnsson *et al.* 2002), as described above.

Isolation of AH

Isolation of AH (bacteria and archaea) from the oxic layer of the mat was performed in artificial seawater medium supplemented with different carbon sources. The medium contained $MgCl_2 \cdot 6\,H_2O$ (5.6 g l^{-1}), $MgSO_4 \cdot 7\,H_2O$ (6.8 g l^{-1}), $CaCl_2 \cdot 2\,H_2O$ (1.47 g l^{-1}), KCl (0.66 g l^{-1}) and KBr (0.09 g l^{-1}). Hypersaline media of 5 and 20% (w/v) salinity were obtained by adding appropriate amounts of NaCl. After autoclaving, KH_2PO_4 and NH_4Cl solutions were added to the medium in final concentrations of 0.15 and 0.2 g l^{-1}, respectively. Sterile filtrated solutions of trace elements mixture (Widdel and Bak 1992), selenite and tungstate and vitamins (Heijthuijsen and Hansen 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 AH were isolated at different concentrations of salinity and on different carbon sources as follows: (i) serial dilutions at two salinities (5% and 20%) using a mixture of 10 mM acetate and 5 mM succinate as a carbon source, (ii) enrichments on 0.05% *Spirulina* extract (complex polymeric substances of dead cyanobacteria) at 5% and 20% salinities and (iii) enrichment cultivation in marine broth medium (carbon source: yeast extract) at 25% salinity. Axenic strains were obtained from high dilutions by plating on agar medium containing the same carbon source. The 16S rRNA genes of the obtained strains were sequenced and phylogenetically analyzed as described before (Abed *et al.* 2007).

16S rRNA cloning

The uppermost oxic layer (3.5 mm thickness, ca. ~100 mg wet weight) of the studied mat was subjected to nucleic acid extraction after Lueders, Manefield and Friedrich (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 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. The obtained sequences were aligned and analyzed using the ARB software (Ludwig *et al.* 1998) and the SILVA database (Pruesse *et al.* 2009). Phylogenetic trees were constructed based on long 16S rRNA sequences (>1300 bp) by applying different methods integrated in the ARB software such as maximum likelihood, maximum parsimony and neighbor joining. The latter calculation was based on a matrix of evolutionary distances determined using the Jukes–Cantor or Felsenstein equations and subject to bootstrap analysis (1000 replicates). All treeing approaches yielded similar relationships. Maximum-likelihood trees were chosen for presentation. The 16S rRNA sequence of *Escherichia coli* was included in the calculations as an outgroup sequence. The partial sequences were aligned to the sequences in the ARB database using the alignment tool of the ARB software package and then inserted into the pre-established tree using the parsimony ARB tool and 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 was calculated as previously described by Good (1953).

Cyanobacteria-associated AH

The AH, associated with different monoclonal strains of cyanobacteria, were identified to find out whether their identity is cyanobacterium-specific. The phylogeny of the used cyanobacterial strains can be found in Abed *et al.* (2008). Briefly, four strains of *Microcoleus* (M7C1, M7C3, M7C5 and S8), two strains of *Halomicronema* (M5C6 and M5C7), three strains of *Leptolyngbya* (M1C10, M6C11 and M4C4) and one strain each of *Cyanothece* (M7R1) and *Phormidium* (M1C2) were used. The non-axenic cyanobacterial cultures were subjected to nucleic acid extraction after Lueders, Manefield and Friedrich (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 200 V for 3.5 h. DGGE bands were excised and sequenced as described before (Abed *et al.* 2008). The partial sequences of DGGE bands were inserted into a maximum-likelihood pre-established tree using the parsimony ARB tool as described above.

FISH of the cyanobacterial cultures was carried out to visualize and study the distribution of AH around cyanobacterial cells, using the HRP-labeled probe Cf319a to target most

members of the phylum *Bacteroidetes* (Manz *et al.* 1996), since most of the associated bacteria in the cultures belonged to *Bacteroidetes*. 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). Permeabilization of the cells, hybridization and tyramide signal amplification were performed as previously described (Pernthaler, Pernthaler and Amann 2002). After hybridization, the cells were counterstained with DAPI (1 μM) and analyzed using a fluorescence microscope (Axioskop2 mot plus; Axiocam MRM, Axiovision 4.6, Zeiss, Germany).

Co-culture growth experiments

Two non-axenic cyanobacterial strains (i.e. *Cyanothece* sp. M7R1 and *Microcoleus* sp. M7C3) from the above-mentioned strains were selected to study the interactions between AH and cyanobacteria in co-cultivation experiments. Due to the difficulty to purify these strains from firmly attached heterotrophs, the closest related (>98% sequence similarity) 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 *M. chthonoplastes* PCC 7420 was grown in MN medium (Rippka *et al.* 1979). Pure culture of *Flexibacter tractuosus* was ordered from the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig), and was grown at 25°C in 100 ml flasks (Nunc, Rosklide, Denmark) in *Cytophaga* marine medium (www.dsmz.de; medium 172). Using these cultures, the following experiments were carried out to find out:

I. The influence of *F. tractuosus* on the growth of the axenic *M. chthonoplastes* PCC 7420. Since this cyanobacterium does not grow homogenously, three sterile 10 ml tubes (Greiner Bio-One, Frickenhausen, Germany) with 5 ml MN medium were incubated for each time point and each tube was inoculated with 100 μl of each strain. At each time point, the three tubes were collected and stored at –20°C until pigment extraction (see below).
II. The influence of the AH obtained from the isolated non-axenic cyanobacterial cultures on similar axenic strains. Filtrates (filtrates 1 and 2 in Fig. S1, Supporting Information) obtained from *Cyanothece* sp. M7R1 and *Microcoleus* sp. M7C3 cultures were added to the axenic cultures of *Cyanothece* sp. PCC7418 and *M. chthonoplastes* PCC7420, respectively. Filtration was carried out using Isopore Membrane filters (Millipore, MA, USA) of pore size 5 μm and 0.2 μm to obtain filtrate with and without bacterial cells, respectively. For the experiment with *Cyanothece* sp. PCC7418, 500 μl of the axenic culture was added to 18 ml ASN III medium in 100 ml flasks (Nunc). Then, 500 μl of filtrate with and without AH cells (filtrates 1 and 2, Fig. S1, Supporting Information) was added. One milliliter culture of each flask was sampled at each time point, and stored at –20°C until pigment extraction. For the experiment with *M. chthonoplastes* PCC 7420, 5 ml MN medium was added to sterile 10 ml tubes (Greiner Bio-One), 100 μl of the axenic culture and 100 μl of filtrate M7RI (with and without cells) were added. At each time point, three tubes were collected and stored at –20°C until pigment extraction.
III. Upon adding filtrate M7RI to *Cyanothece* PCC 7418, the growth of the cyanobacterium was completely inhibited. Hence, further experiments were carried out to investigate the nature and origin of this growth inhibiting substance(s) using differently treated filtrates (i.e. centrifugation, heating, further filtration step) (Fig. S1, Supporting Information).

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 h each and a light intensity of 240 μmol photons m^{-2} s^{-1}. Inoculation was done with logarithmic-phase cultures.

Chlorophyll *a* measurement

Chlorophyll a (Chl) *a* was quantified in the previous experiments and used as a proxy for cyanobacterial growth. The collected culture samples were thawed and centrifuged at 16 000 rpm, 4°C for 5 min. 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. The samples were then centrifuged again at 16 000 rpm, 4°C for 10 min, 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 min. Chl *a* was measured at a wavelength of 665 nm 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

Quantification and spatial distribution using qPCR and CARD-FISH

qPCR showed no distinct distribution pattern of bacterial and archaeal abundances with depth (Fig. 2A and B). The copy number of bacterial 16S rRNA genes in the oxic layer reached its maximum value of $2.1 \pm 1.7 \times 10^9$ copies g^{-1} at the depth of 0.75–1 mm. The mean values of bacterial 16S rRNA copy numbers in the oxic and anoxic layer were comparable (i.e. 7.3×10^8 copies g^{-1}). Archaeal 16S rRNA copy numbers were significantly higher in the anoxic than in the oxic layer ($P < 0.005$). In the anoxic layer, these numbers significantly increased with depth from $0.16 \pm 0.1 \times 10^8$ to $1.05 \pm 0.2 \times 10^8$ copies g^{-1} of the mat. The lowest abundances of bacterial and archaeal cells were obtained at the same layer at the depth of 1.5–1.75 mm. The mean bacterial abundance in the oxic layer as determined by CARD-FISH was $2.23 \pm 0.4 \times 10^{10}$ cells g^{-1} (Fig. 1C). The highest bacterial abundance was encountered in the layers between 2.25 and 3.25 mm.

Diversity of AH using cultivation and molecular approaches

A total of 20 axenic bacterial and archaeal strains were obtained from enrichment cultivation. These strains were phylogenetically affiliated with *Alphaproteobacteria*, *Gammaproteobacteria*, *Actinobacteria*, *Bacteroidetes*, *Firmicutes* and *Haloarchaea* (Fig. S2, Supporting Information). Alphaproteobacterial isolates were closely related to *Caulobacter* spp. and were isolated on *Spirulina* extract at 5% salinity. All strains isolated on marine broth medium at 25% salinity belonged to the genera *Salinivibrio*, *Pseudoalteromonas*, *Idiomarina* and *Halomonas* within the class *Gammaproteobacteria*. Actinobacterial isolates grouped with *Micrococcus* spp. and were isolated on 5% ASM supplemented with

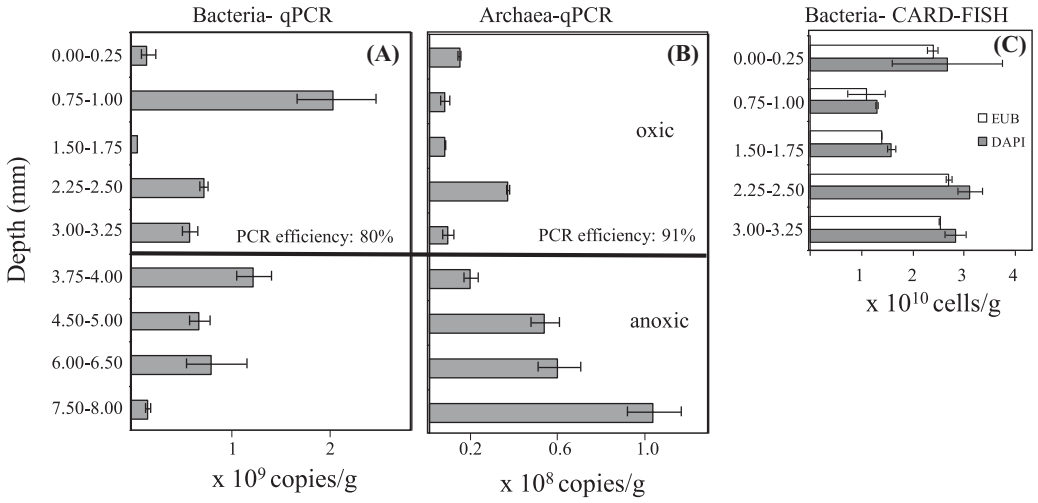

Figure 2. (**A** and **B**) Depth distribution of bacterial and archaeal 16S rRNA gene copy numbers (copies/g) in the mat obtained by qPCR. (**C**) Bacterial cell numbers (cells/g) as revealed by CARD-FISH. Error bars represent ± standard deviation (n = 3).

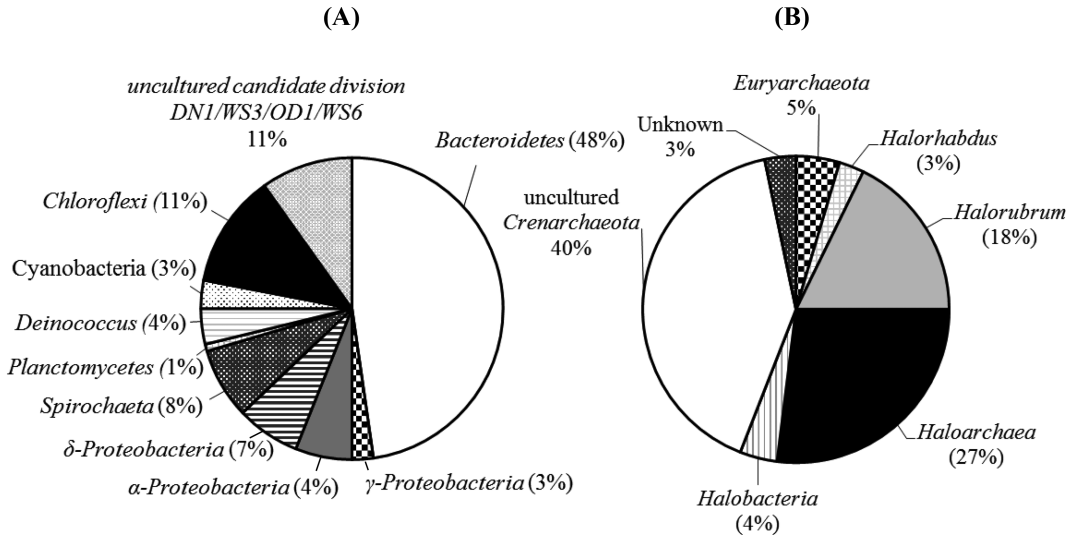

Figure 3. (**A**) Bacterial and (**B**) archaeal community composition retrieved by 16S rRNA cloning. Shown fractions indicate the relative percentage to the total number of clones.

acetate and succinate. *Firmicutes*-related strains were related to *Bacillus* spp. and were isolated at 20% salinity both on acetate and succinate as well as on *Spirulina* extract. Three archaeal isolates were enriched on ASM with Spirulina extract at 20% salinity and were affiliated to the genera *Halobacteria*, *Haloferax* and *Halorubrum* (Fig. S2, Supporting Information).

Based on 16S rRNA sequence similarity, 71 and 18 distinct OTUs (based on an 97% identity cut off) were identified among 132 bacterial and 152 archaeal clones, respectively. Rarefaction curves showed that the archaeal diversity was mostly covered (88% coverage) while an insufficient number of sequences was analyzed to cover the whole bacterial diversity (42% coverage) (Fig. S3, Supporting Information). In the bacterial clone library, 48% of the sequences were affiliated to *Bacteroidetes* whereas 11% were related to *Chloroflexi*-related sequences (Fig. 3A). The remaining sequences belonged to *Alpha*- (4%), *Gamma*-(3%) and *Deltaproteobacteria* (7%), *Spirochaeta* (8%), *Planctomycetes* (1%), *Deinococci* (4%), uncultured candidate division OD1 and WS6 (11%) and cyanobacteria (3%) (Fig. 3A). Sequences within the *Deltaproteobacteria* were related to known sulfate-reducing bacteria (SRBs) of the genera *Desulfovibrio* and *Desulfosalina*. Among the archaeal sequences, 40% were affiliated to sequences of *Crenarchaeota* and 27% to *Haloarchaea* (Fig. 3B). Sequences within the *Crenarchaeaota* belonged to the marine benthic group B (MBGB). The remaining sequences were closely related to *Halorubrum* (18%), *Halorhabdus* (4%), *Halobacteria* (3%), uncultured *Euryarchaea* (5%) and archaea without known relatives (3%).

AH associated with cyanobacterial cultures

DGGE showed that different cyanobacterial species were associated with different populations of AH (Fig. 4). The two *Microcoleus* strains M7C3 and M7C5 possessed the same associated AH, while these bacteria were different in the other cyanobacterial cultures (*Microcoleus* sp. M7C1 and *Cyanothece* sp. M7CR1), isolated from the same mat. The *Halomicronema* strains M5C6 and M5C7, which were also isolated from the same mat, showed a similar DGGE pattern of associated bacteria except for two bands (bands 45 and 46), which were only present in M5C7 strain

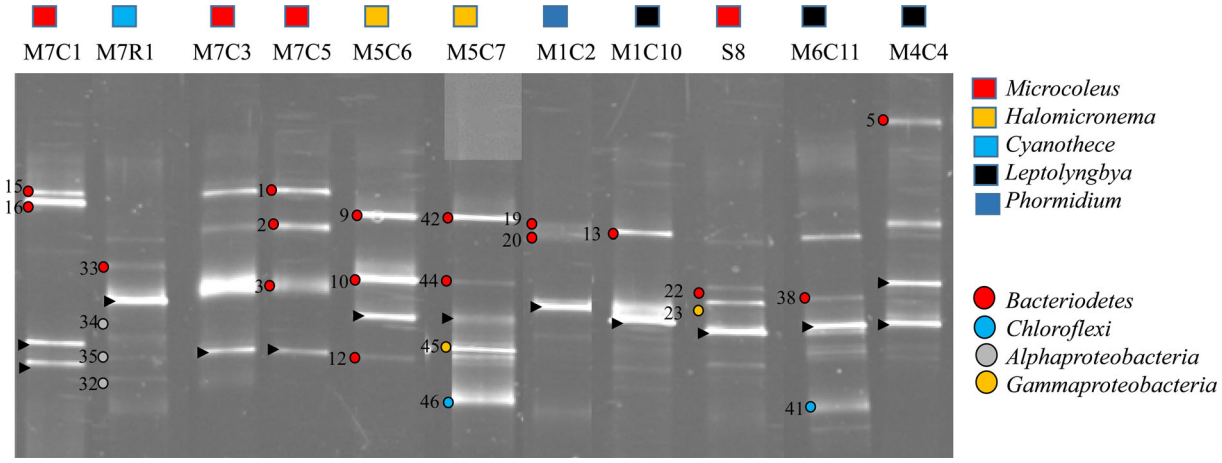

Figure 4. DGGE pattern of monoclonal cyanobacterial cultures with their associated AH using the bacterial primers GM5-GC and 907RC. Sequences retrieved from the associated bacteria are numbered and highlighted with a circle. Bands belonging to cyanobacteria are marked with triangle.

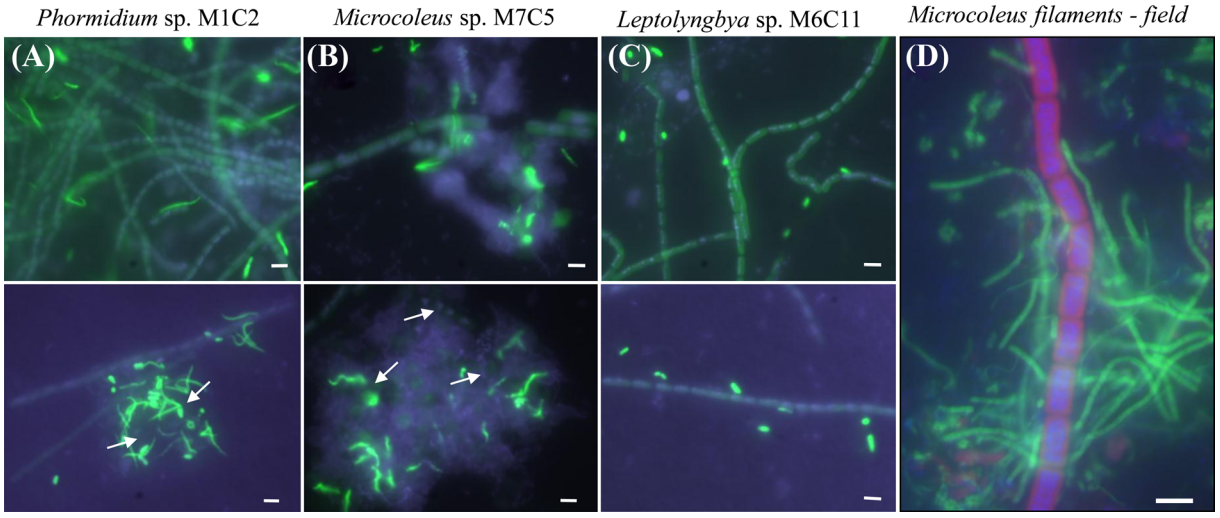

Figure 5. FISH (probe EUB I-III, targets all bacteria) and CARD-FISH (probe Cf319, targets *Bacteroidetes*) images of the cyanobacterial cultures *Phormidium* sp. M1C2 (**A**), *Microcoleus* sp. M7C5 (**B**) and *Leptolyngbya* sp. M6C11 (**C**) isolated from the mats with their attached and free-living AH. Scale bar = 2 μm. Green (Cf319) and red (EUB I-III) color show the probe signal; blue color shows the DAPI signal. The arrows indicate dead cyanobacterial cells. (**D**) Shows CARD-FISH of 5-μm thick cross-section of the microbial mat using probes CFX1223 and GSNB-941 specific for *Chloroflexus*-related bacteria. Green shows probe signal; pink: cyanobacterial autofluorescence; blue: DAPI signals of remaining cells. Scale bars correspond to 5 μm.

(Fig. 4). *Leptolyngbya* strains M6C11 and M1C10 varied clearly in the DGGE patterns of accompanied bacteria, although some bands were common (Fig. 4).

Phylogenetic analysis of sequences from amplifiable DGGE bands (Fig. S4, Supporting Information) revealed that most of the associated heterotrophs belonged to the phylum *Bacteroidetes*, but *Alphaproteobacteria*, *Gammaproteobacteria* and *Chloroflexi*-related bacteria were also detected. *Bacteroidetes* were detected in all cyanobacterial isolates, and sequences of this group fell mainly within the genera *Flexibacter*, *Microscilla*, *Cytophaga* and *Psychroflexus* (Fig. S4, Supporting Information). While alphaproteobacterial sequences were closely related to *Nisaea* and other environmental sequences, gammaproteobacterial sequences belonged mainly to *Salinisphaera*. Associated bacteria closely related to *Chloroflexi* were found in *Halomicronema* sp. M5C7 and *Leptolyngbya* sp. M6C11. In the unicellular culture *Cyanothece* spp. M7RI, associated bacteria were affiliated with the denitrifying bacteria, *Nisaea denitrificans* and *N. nitritireducens* (Fig. S4, Supporting Information).

FISH and CARD-FISH of cyanobacterial cultures

Hybridization of *Phormidium* sp. M1C2 and *Microcoleus* sp. M7C5 with the specific probe Cf319a, which targets sequences of the *Bacteriodetes* phylum (Fig. 5A and B), showed filamentous and coccoid bacteria either firmly attached to the cyanobacterial filaments or free living in the culture (Fig. 5). Some of these bacteria were also associated with dead cyanobacterial cells (Fig. 5A and B; see arrows), and seemed involved in the breakdown of cyanobacterial filaments (Fig. 5B). Hybridization of culture M6C11 with Cf319a, showed rod-shaped bacteria attached to the cyanobacterial filaments (Fig. 5C). CARD-FISH with the *Chloroflexi*-specific probes GNSB-941 and CFX-1223 performed on 5-μm thin cross-sections of the oxic mat layer showed a

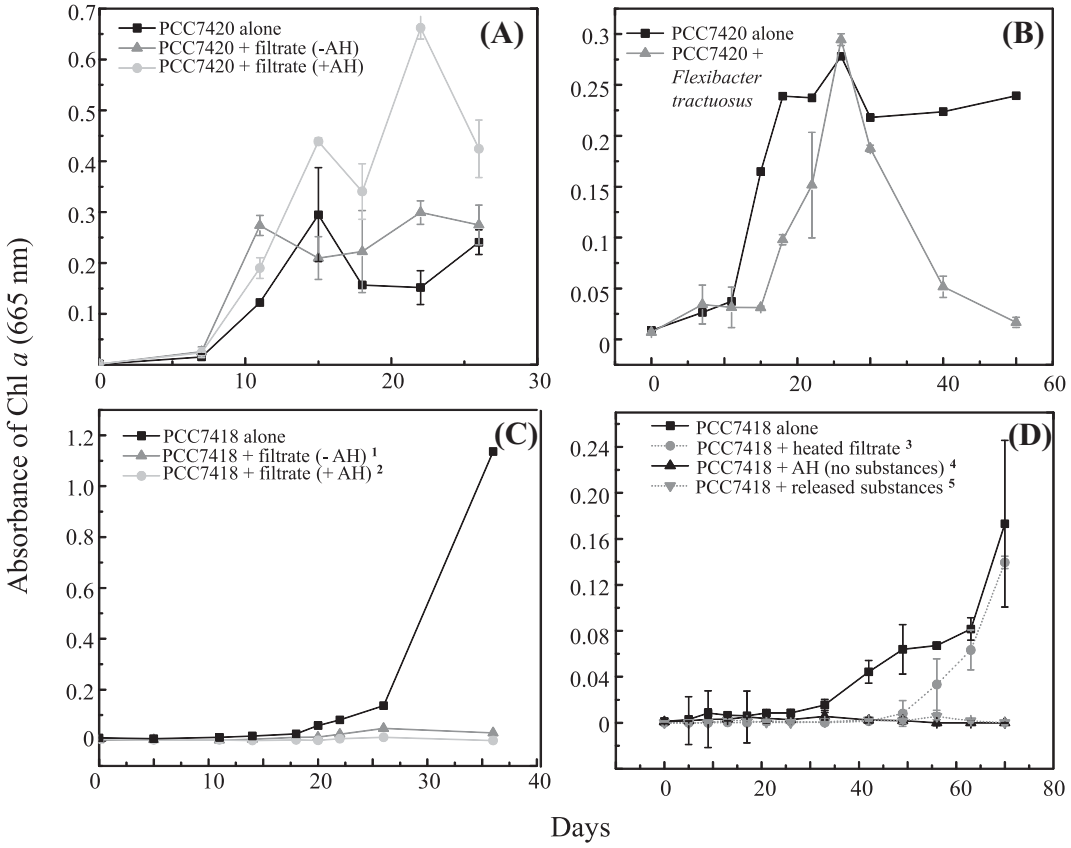

Figure 6. (**A**) Growth experiments of the axenic cyanobacterial culture of *M. chthonoplastes* PCC 7420 with the addition of filtrates (with and without AH) obtained from culture *Microcoleus* sp. M2C3. (**B**) Growth of *M. chthonoplastes* PCC 7420 in the presence of *F. tractuosus*. (**C** and **D**) Growth of *Cyanothece* PCC 7480 with addition of filtrates obtained from the *Cyanothece* sp. M7RI culture. Superscripted numbers refer to filtrate number in Fig. S1 (Supporting Information).

dominance of *Chloroflexi*-like bacteria in close proximity to bundles of *M. chthonoplastes* filaments (Fig. 4D). These *Chloroflexi*-like bacteria were not greenish, as for example *Chloroflexus aurantiacus*, but reddish.

Co-cultivation experiments

Different growth experiments were carried out in order to get a better understanding of the specific interactions between cyanobacteria and AH (Fig. 6). The growth of the axenic culture of *M. chthonoplastes* PCC 7420 was enhanced upon addition of filtrates with and without AH cells, obtained from the closely related cyanobacterial culture *Microcoleus* sp. M2C3 (Fig. 6A). On the contrary, when *F. tractuosus* was added to *M. 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 (Fig. 6B). Remarkably, filtrates obtained from the non-axenic *Cyanothece* sp. M7RI strain completely inhibited the growth of the axenic strain *Cyanothece* PCC 7418 (Fig. 6C). These filtrates had the same growth inhibitory effect on other tested cyanobacterial strains such as *Leptolynbya* PCC 8103, *Xenococcus* PCC 7304 and *Microcoleus* PCC 7420 (data not shown). To explore the nature and origin of the inhibiting substances, the filtrates were treated in different ways (Fig. S1, Supporting Information). The addition of the filtrate after heating delayed growth of *Cyanothece* PCC 7418, as could be inferred from the longer lag phase (ca. 35 days) (Fig. 6D). Thus, heating contributed to the destruction of the inhibiting substrate(s). The M7RI associated AH (filtrate 4; Fig. S1, Supporting Information) as well as the released substances alone (filtrate 5; Fig. S1, Supporting Information) completely inhibited the growth of *Cyanothece* PCC 7418 (Fig. 6D).

DISCUSSION

Bacterial and archaeal communities in the oxic layer of the studied cyanobacterial mat were diverse and numerically abundant. Most of the isolated strains from the mat were absent in the clone libraries, supporting the prevalently described discrepancy between cultivation and molecular techniques (e.g. Amann, Ludwig and Schleifer 1995; Felske *at al.* 1999). Using qPCR, the bacterial 16S rRNA copy numbers in the mat did not exhibit a distinct distribution pattern with depth. This finding is in contrast with the distribution of bacterial biomass (estimated using ATP) and pigments in the Guerrero Negro mats, which suggested a decrease in bacterial abundance from oxic to anoxic layers (Ley *et al.* 2006). However, it should be kept in mind that different quantification methods may yield different distribution patterns, as could be clearly observed when our qPCR and CARD-FISH depth patterns in the same mat are compared. Bacterial cell numbers determined with CARD-FISH were around two to four orders of magnitude higher than those reported by cultivation methods (Jonkers and Abed 2003; Abed *et al.* 2007). This variation highlights the need to use direct counting by molecular techniques. qPCR results suggested a higher dominance of bacteria than archaea in the oxic layer, whereas the latter exhibited their highest abundance in the anoxic layer. Although such higher abundance of bacteria than archaea has been previously observed in other mats such as Guerrero Negro mats (Jahnke *et al.*

2008), archaea were more abundant than bacteria in the hypersaline mat from Salar de Atacama, Chile (Fernandez *et al.* 2016). In the latter mats, the relative abundance of aerobic heterotrophic archaea decreased with depth while methanogens showed an opposite trend.

Bacterial AH in the mat

Many of the bacterial sequences from the oxic layer of our mat were closely related to sequences obtained from other hypersaline microbial mats (Jonkers *et al.* 2005; Ley *et al.* 2006; Abed, Kohls and de Beer 2007; Robertsen *et al.* 2009). The major fraction of the bacterial sequences was related to *Bacteroidetes*, a group of chemoorganotrophs with the ability to digest large polysaccharides such as chitin, pectin and cellulose (Kirchman 2002; Madigan, Martinko and Parker 2001). The high abundance of *Bacteroidetes* might be due to the presence of excessive extrapolymeric substances in this mat (Abed, Kohls and de Beer 2007). *Chloroflexus* group was also predominantly encountered in this mat, which is in agreement with previous findings in hypersaline mats from Guerrero Negro (Nübel *et al.* 2001; Ley *et al.* 2006). *Chloroflexus*-like bacteria were mostly found close to cyanobacterial filaments, suggesting a strong relationship between both groups. Previous studies have also suggested a symbiotic association between cyanobacteria and *Chloroflexus*-like bacteria with the possibility of the latter group to assimilate photoautotrophically fixed carbon from cyanobacteria (Nold and Ward 1996; Ward *et al.* 1998). Moreover, *Chloroflexi* were found to be responsible for the entire respiration in the top approximately 1 mm of a hypersaline microbial mat from la Salada de Chiprana, Spain (Polerecky *et al.* 2007). The reddish *Chloroflexus*-like bacteria, found dominant in our mats, were only reported in hot spring microbial mats from Yellowstone National Park, USA (Castenholz 1984; Boomer *et al.* 2002) but never in hypersaline environments. *Deinococci*-related bacteria, which are mostly aerobic heterotrophic bacteria, were also found in the studied mat. This genus contains bacterial species that are resistant to very high doses of UV radiation and to prolonged periods of desiccation because of their potent DNA-repair capabilities (Moseley 1983; Minton 1994; Mattimore and Battista 1996; Carpenter, Lin and Capone 2000). These features provide these bacteria with a selective advantage to live under the extreme conditions of our mats.

Although cloning was restricted to the oxic layer of the mats, sequences related to SRBs of the genera *Desulfovibrio* and *Desulfosalina* were detected. Several studies have shown that SRBs 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). Detected biomarkers from SRBs in the top layer of a hypersaline microbial mat and their ^{13}C-uptake patterns indicated a close coupling between SRBs and cyanobacteria (Bühring *et al.* 2009). Thus, our results hint to a possible role of SRBs in carbon degradation in the oxic part of microbial mats.

Archaeal AH in the mat

The high 16S rRNA copy numbers of archaea in the oxic layer of the mat indicate their abundance and a possible role in carbon cycling. Archaea could not be detectable by CARD-FISH, most likely because of suboptimal cell wall permeabilization. Few studies reported on diversity of Archaea in oxic layers of hypersaline mats (Jahnke *et al.* 2008; Robertson *et al.* 2009; Fernandez *et al.* 2016; Wong *et al.* 2016). Archaea in the oxic layer of our mat belonged to *Crenarchaeota*, *Thermoplasmatales* and *Euryarcheota*. These groups were also encountered in Guerrero Negro mats, Mexico (Jahnke *et al.* 2008), and in mats from Shark Bay, Australia (Wong *et al.* 2016). Interestingly, we detected a high number of sequences affiliated with *Crenarchaeota* belonging to MBGB, as designated by Vetriani *et al.* (1999). To the best of our knowledge, no member of this group has been isolated until now. Although *Crenarchaeota* are widespread among marine habitats (Vetriani *et al.* 1999; Fernandez *et al.* 2016), their exact metabolic activities are not fully understood (DeLong 1992; Karner, DeLong and Karl 2001; Schouten *et al.* 2002; Knittel *et al.* 2005). Because of the detection of MBGB in methane seeps, it was speculated that they could be involved in anaerobic methane oxidation (Knittel *et al.* 2005; Biddle *et al.* 2006; Cho *et al.* 2017) or sulfate reduction (Torres *et al.* 2002). However, it is yet to be demonstrated whether these anaerobic processes can occur in the fully oxic layer of hypersaline microbial mats. Indeed, high rates of methane production concomitant with high abundance of anaerobic archaea were recently shown to occur in the fully oxic layer of microbial mats from Shark Bay, Australia (Wong *et al.* 2016). This suggests the possibility of surface anoxic niches in microbial mats. Planktonic *Crenarchaeota* were proposed to play a role in carbon cycling with their potential to function either as strict autotrophs or as mixotrophs utilizing both CO_2 and organic material as carbon sources (Hallam *et al.* 2006). They have also the potential to use reduced nitrogen compounds like ammonia (NH_3) as energy sources, fueling autotrophic metabolism, and thus might also belong to ammonium oxidizers (Hallam *et al.* 2006). We believe that further research is still needed to elucidate the exact role of archaea in the oxic layer of microbial mats and to isolate representative novel strains of *Crenarchaeota* and *Euryarchaeota*.

Cyanobacteria-associated AH

Investigation of AH–cyanobacterial relationships in monocultures indicated that the community structure of AH depends on the identity of the associated cyanobacterial species. The dominance of *Bacteroidetes*- and *Chloroflexi*-related bacteria in the cyanobacterial cultures, as revealed by DGGE and CARD-FISH, is consistent with their abundances in the 16S rRNA clone library. This points out to a significant role of these two bacterial groups in the oxic layer of microbial mats. The close association of *Bacteroidetes*-related bacteria, besides *Alphaproteobacteria*, with marine cyanobacterial cultures has also been previously shown (Hube, Heyduck-Söller and Fischer 2009). With their ability to grow chemoorganotrophically on complex polymeric substrates (Madigan, Martinko and Parker 2001; Kirchman 2002), *Bacteroidetes*-related bacteria might benefit from cyanobacterial exudates and from dead cell material. Since CARD-FISH revealed that these bacteria were often found attached to dead cell material, we speculate that they may rather grow on decaying cyanobacterial biomass than on exudates from living cyanobacteria. The beneficial association of *Chloroflexi* bacteria with cyanobacteria has also been previously described in microbial mats from Guerrero Negro, Mexico (Ley *et al.* 2006). It was postulated that *Chloroflexi* bacteria reduce H_2S, which is inhibitory for oxygenic photosynthesis, via anoxygenic photosynthesis while cyanobacteria transfer oxygen and possibly organic carbon to the *Chloroflexi*-related bacteria.

The detection of known denitrifying and nitrite-reducing bacteria (i.e. *N. denitrificans* and *N. nitritireducens*) and the presence of the *nifH* gene (data not shown) in the *Cyanothece* culture (M7RI) hints to a possible role of AH 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 heterocysts, and thus protecting the oxygen-sensitive nitrogenase from high oxygen levels (Paerl 1976, 1982; Paerl and Keller 1978). Steppe *et al.* (1996) proposed a mutualistic N_2-fixing consortium, where non–N_2-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).

The identity of cyanobacteria-associated AH yielded insights about the breadth of their physiological capabilities and their potential contribution to the O_2, C, S and N cycles in natural cyanobacterial mats. Their activities and vertical distribution according to known physical, chemical and biological gradients suggest a role in the stratification and structuring of mats. Several taxa of *Proteobacteria* and *Bacteriodetes* have been mainly detected in the top 1–2 mm of the mat (this study; Ley *et al.* 2006), where the most mat-dominating cyanobacterium *M. chthonoplastes* is found and where maximal oxygenic photosynthesis is taking place. The respiration activities of these AH combined with cyanobacterial photosynthesis lead to the development of an O_2 gradient in the mat. On the other hand, *Chloroflexi* bacteria are expected to reside at the O_2-H_2S interface layer (i.e. 3–4 mm), where they receive O_2 and light from the top layer and H_2S from the bottom layer. The depth location of *Chloroflexi* bacteria and their anoxygenic photosynthesis activity contribute also to the development of O_2 and H_2S gradients in the mats. Thus, these variable physiological activities of AH and their complex interactions with cyanobacteria hint to a potential role in the complexity and structuring of cyanobacterial mats.

Cyanobacteria–AH relationship

Growth experiments revealed that the released substances of AH apparently promoted the growth of its host cyanobacterium, but inhibited the growth of other potentially competitive cyanobacteria. The enhancement of *M. chthonoplastes* PCC 7420 growth upon the addition of a filtrate obtained from the closely related non-axenic culture of *Microcoleus* sp. M2C3 could be due to the release of certain growth factors and vitamins (Paerl 1996). Indeed, we observed an increase in the growth of this cyanobacterium upon the addition of vitamin B_{12} (data not shown). AH could also remove O_2 and H_2S and increase CO_2 supply, thereby protecting cyanobacteria from photooxidation and increasing their growth (Abeliovich and Shilo 1972; Eloff, Steinitz and Shilo 1976; Paerl and Kellar 1978).

Unlike these beneficial relationships, some AH such as *Flexibacter* spp. have been shown to lyse cyanobacteria (Marshall 1989). This is congruent with our CARD-FISH and growth experiments data. For the lysis of cyanobacterial filaments, direct attachment of AH cells and the release of lysozyme-like substances seem to be required (Stewart and Brown 1969; Sallal 1994). The filtrate of *Cyanothece* sp. M7R1 also showed a strong bactericidal potential on *Cyanothece* PCC 7418 as well as on other cyanobacteria. The follow-up experiments with the heated and treated filtrates suggested the release of substance(s) by AH into the medium that inhibit the growth of cyanobacteria. This might be a protective mechanism for some cyanobacteria to compete against other cyanobacteria. On the other hand, it is also plausible that the filtrate may contain viruses that are capable of lysing cyanobacterial cells. Although viruses are known to be highly host-specific, their ability to cross infect closely related cyanobacteria has also been demonstrated (Fuhrman 1999; Sullivan, Waterbury and Chisholm 2003). This could explain why all tested cyanobacterial genera were killed by the filtrate of *Cyanothece* M7R1 except the host itself. Indeed, recent studies have shown the occurrence of a very high abundance of viruses in intertidal microbial mats and suggested a possible role in microbial mortality and nutrient cycling (Pacton *et al.* 2014; Carreira *et al.* 2015). Hence, further studies are needed to elucidate the specific interaction between viruses and cyanobacteria in microbial mats.

We conclude that a diverse community of AH exist in close proximity to cyanobacteria in microbial mats and thus benefit from either their exudates and/or the produced oxygen. Most of the cyanobacteria-associated bacteria belong to *Bacteriodetes* and *Chloroflexi*. The interactions between cyanobacteria and AH are complex, specific and involve the release of substances that stimulate or inhibit the growth of each other. The production of antibiotics, toxins, signaling molecules and other secondary metabolites as effective competition and defense 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(s) in this study remains unclear and needs further investigations.

SUPPLEMENTARY DATA

Supplementary data are available at *FEMSEC* online.

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