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Cyanobacterial mats from hot springs produce antimicrobial compounds and quorum-sensing inhibitors under natural conditions

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Abstract Polar (water) and non-polar (ethyl acetate) extracts from the cyanobacterial layer (top 1–3 mm) of four hot spring microbial mats in the Sultanate of Oman were tested for their antibacterial, antidiatom and quorum-sensing inhibitory activities under natural conditions. The chemical composition of the active extracts was analysed using gas chromatography–mass spectrometry (GC-MS). Cyanobacteria within these mats were identified by direct microscopy while the total bacterial community composition was compared using automated ribosomal intergenic spacer analysis (ARISA). Only the extracts from Bowshar and Nakhl mats showed antibacterial properties against *Bacillus* sp., *Micrococcus luteus*, *Shigella sonnei*, *Salmonella enterica* and *Klebsiella pneumoniae*. All tested extracts inhibited the growth of the benthic diatom *Amphora coffeaeformis*. Extracts from Bowshar, Rustaq and Nakhl inhibited quorum-sensing of the reporter strains *Chromobacterium violaceum* CV017 and *Agrobacterium tumefaciens* NTL4. The highest bioactivity was recorded for ethyl acetate extracts from Nakhl mats, which had the lowest number of operational taxonomic units (OTUs). Using GC-MS, 74 chemical compounds were obtained, however with different distribution among the four mat extracts (similarity<43%). Various cyanobacteria, belonging mainly to *Chroococcus*, *Phormidium*, *Leptolyngbya*, *Spirulina* and *Lyngbya* were detected in the different mats, and each mat had its unique bacterial community, as confirmed by ARISA profiles. We conclude that antimicrobial and quorum-sensing inhibitory compounds can be produced by hot spring mat microorganisms under natural conditions and the differences in these compounds could be attributed to the differences in the mats' bacterial composition as well as the physical–chemical conditions of the springs.

Keywords Cyanobacteria · Microbial mats · Bioactivity · Hot springs · Microbial diversity · ARISA

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Introduction

Microorganisms have been recognised as a major source of bioactive metabolites with antibacterial (Volk and Furkert 2006), antifungal (Bottone and Peluso 2003; Lavermicocca et al. 2000), antiviral (Deng and Cliver 1995), anticancer (Williams 2009), insecticidal (Sathiyamoorthy and Shanmugasundaram 1996), algicidal (Blom et al. 2006) and immunosuppressive properties (Burja et al. 2001). Most of these compounds have been obtained from terrestrial actinomycetes and filamentous fungi (Zjawiony 2004; Fenical and Jensen 2006; Newman and Cragg 2007) but more recently from aquatic bacterial groups with a more diverse physiology

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like cyanobacteria (see reviews, Burja et al. 2001; Dahms et al. 2006; Abed et al. 2009). Microorganisms forming microbial mats in hot springs, Antarctic lakes, deep sea and caves have been recently identified as a good source of bioactive compounds for different biotechnological applications (Harvey 2000; Fenical and Jensen 2006; Biondi et al. 2008). Seven out of 48 cyanobacterial isolates (mostly *Pseudophormidium* sp., *Phormidium priestleyi* and *Nostoc* sp.) from Antarctic microbial mats were shown to possess strain-specific antibacterial, antifungal and cytotoxic activities (Biondi et al. 2008). Thermophilic bacteria, like *Bacillus*, *Clostridium* and *Therrnus* strains, from hot spring mats produced thermostable enzymes, such as lipases, proteases, amylases and cellulases (Kristjansson 1989; Harvey 2000). Most of the studies reporting bioactive compounds from cyanobacterial mats have been performed on isolated cyanobacteria or aerobic heterotrophic bacterial strains (Volk 2006; Piccardi et al. 2000; Biondi et al. 2008; Rojas et al. 2009). In nature, however, microorganisms of mats rarely grow in isolation but rather as communities where they can change their physiology and biochemistry as a result of interaction with other bacteria (Paerl and Pinckney 1996; Smith and Thanh Doan 1999). Thus, it is expected that these microorganisms would produce compounds under in situ conditions that may encourage or discourage the growth and function of neighbouring cells. On the other hand, many microorganisms are difficult to cultivate in the laboratory (Eilers et al. 2000) and the behaviour of isolated microorganisms in cultures may not be comparable to their behaviour under field conditions. So far, few studies have been performed to check for the ability of mixed microbial communities to produce bioactive compounds, such as antimicrobial and quorum-sensing inhibitory compounds, under in situ conditions. Recently, it was demonstrated that a diverse array of *N*-acylhomoserine lactones (AHLs), known as autoinducers of quorum-sensing were produced in intact cyanobacterial mats and biofilms (Huang et al. 2007; Decho et al. 2009). Screening of mixed cyanobacterial communities may provide a new strategy to discover novel bioactive compounds and may be used as an initial step before extensive and time-consuming isolation of individual microorganisms is done.

In this study, we tested cyanobacterial layers, dominated mainly by cyanobacteria and aerobic heterotrophic bacteria, of hot spring microbial mats for the production of antimicrobial, antidiatom and quorum-sensing inhibitory compounds. The main goals of this study were: (1) to find out if cyanobacterial mats produce antibacterial, antidiatom and quorum-sensing inhibitory compounds under in situ conditions and (2) whether differences in the bacterial composition of the mats and the physical and chemical conditions of the spring water determine the bioactivity and the types of detected chemical compounds.

Material and methods

Four different hot springs in the villages of Hammam, Bowshar, Nakhl and Rustaq, Sultanate of Oman (UTM grid ^{5}435–^{6}428; ^{25}875–^{26}035) were studied. At each spring, water and air-shade temperatures, pH, electrical conductivity and flow velocity were measured in the field using a mercury-in-bulb thermometer, a pH meter, a salinity-conductivity-temperature meter and a current meter. Water samples were collected in 250 mL glass bottles and fixed in the field with manganese sulphate and alkaline iodide-azide solutions for the estimation of dissolved oxygen (DO) using Winkler's titrimetric method (Winkler 1888). Water samples for the analysis of all major cations and anions were collected in new 500 mL polyethylene bottles after rinsing them twice with the spring water. The bottles were transported to the laboratory in a cool box and stored in a freezer at −20°C till analysis. All samples were analysed within a week using recommended routine methods (Clescerl et al. 1999).

From each spring, approximately 200 g of the cyanobacterial layer (1–3 mm top layer) of microbial mats were scraped from the walls of water channels (Hammam and Bowshar) or stones (Nakhl and Rustaq) and immediately used for the extraction of chemical compounds (see below). Three or four replicate mat samples were collected, transferred on ice to the laboratory and stored at −20 ° C for analysis of bacterial communities (see below).

Preparation of extracts

One gram wet weight of the cyanobacterial layer of each mat was extracted subsequently with 50 mL of ethanol, acetone and ethyl acetate immediately after collection. All obtained extracts for each location were combined and filtered on a Whatmann (No. 1) paper. Combined extracts were evaporated till dryness by a rotary evaporator (Büchi, Switzerland) under reduced pressure at 40°C. Extracts were separated by liquid–liquid extraction using 1:1 distilled water:ethyl acetate. The polar and non-polar fractions were collected separately and evaporated by a rotary evaporator under reduced pressure at 40°C. All fractions were weighed and used for bioassays (see below). Before bioassays extracts were stored in glass vials in a dark at −20°C.

Antibacterial and antidiatom activity

For screening of antibacterial activities, polar and non-polar extracts were pipetted onto circular paper discs (Whatman No. 1; disc diameter=1 cm) at a concentration of 1 μg disc^{-1}. An additional set of discs loaded with ethyl acetate was used as control. Pathogenic and environmental bacterial strains (Table 2), obtained from the Sultan Qaboos

University hospital culture collection, were used in this experiment. Prior to the bioassay, all bacteria were cultivated in a nutrient broth (3% yeast and 5% peptone in sterile water) till they reached exponential growth phase, which was determined by a spectrophotometer. The bioassay was conducted according to Dobretsov and Qian (2002). Briefly, each strain (0.1 mL of 10^6 CFU mL^{-1} culture) was inoculated onto agar plates and the dried discs, with either the tested extracts or the control solutions, were placed onto the agar. The agar plates were incubated for 24 h at 28°C until the bacteria developed a visible film. The observed zones of growth inhibition between the disc and the bacterial film were measured to the nearest 0.2 mm. The experiment was run in triplicates, three times and the mean inhibition zones were calculated.

Anti-diatom activity was investigated in the laboratory using the axenic culture of the diatom *Amphora coffeaeformis* (*Bacillariophyceae*) isolated from a natural biofilm (Ortlepp et al. 2008), according to a protocol previously developed (Dobretsov and Qian 2002). Prior the assay, extracts were evaporated and re-dissolved in dimethyl sulfoxide (DMSO, Sigma). The amount of chlorophyll *a* (μg L^{-1}) in attached diatoms was determined by the technique of Lorenzen (1966) using a spectrophotometer. DMSO was used as a control.

Inhibition of bacterial quorum-sensing

Quorum-sensing inhibitory activities of the extracts were tested using *Chromobacterium violaceum* CV017 (Chernin et al. 1998) and *Agrobacterium tumefaciens* NTL4 pZLR4 (Cha et al. 1998) according to Dobretsov et al. (2010). *C. violaceum* CV017 produces a purple pigment violacein in response to short side-chain AHLs and *A. tumefaciens* NTL4 pZLR4 produces a blue colour in the presence of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) and medium side-chain AHLs. Prior the experiment, bacterial reporters cryopreserved as glycerol stocks were grown in LB medium (Difco, USA; for CV017) or *Agrobacterium* medium (MPI, USA). Three times dilutions of all polar and non-polar extracts were made and 30 μL of extracts were applied to sterile 96-well microtiter plates (Nunc, Denmark). Empty wells and wells with 30 μL of ethyl acetate were maintained as controls. Samples were evaporated to dryness and 100 μL of the reporter mixed with soft agar were applied to each well. The plates were incubated overnight at 30°C. Inhibition of coloration in the assay demonstrates inhibition of QS. These bioassays were repeated three times and the mean minimal inhibitive concentration was calculated. Toxicity assays were performed in order to test the effect of compounds on the growth of the reporter strains according to Dobretsov et al. (2010). Briefly, extracts at the highest tested concentration were applied onto glass fibre discs (diameter=1 cm) and dried. The discs were placed onto LB agar (Difco, USA) inoculated with *C. violaceum* CV017 or *A. tumefaciens* NTL4 strains. Growth inhibition around the disc corresponds to antibacterial activity of the compound against the reporter strains. Ethyl acetate was used as a control.

Chemical composition of extracts

One milligramm of ethyl acetate fractions of the extracts were dried under vacuum, re-dissolved in 1 mL hexane and analysed by a coupled gas chromatography–mass spectrometry (GC-MS). The separation of compounds and their analysis was performed using a Perkin–Elmer Clarus 600 Series GC-MS analyzer with electron impact ionization. GC-MS separations were done in a HP Ultra 2 capillary column (5% phenyl, 95% methylpolysiloxane, length=25 m, diameter=200 μm, film thickness=0.33 μm, Hewlett–Packard, cat. No. 19091B-102). The total GC run time was 53 min, and the carrier gas was helium. The initial oven temperature was held at 80°C for 2 min and then increased by 10°C min^{-1} till it reached 290°C, after which it was held at this temperature for 30 min. The injector temperature was 290°C and the split ratio was 1:30. NIST Mass Spectral library was used to identify the compounds in the extracts using hexane as a control. The closest match with the highest probability in the library was recorded, while compounds with probability below 75% were considered unknown.

Microbial community analysis

The cyanobacterial layer of the mats (1–3 mm) was excised under a dissecting microscope with a clean scalpel blade and sterile forceps, torn apart, mounted in water on a microscope glass slide and observed using transmitted light, phase contrast and fluorescence microscopy. Different morphotypes were identified and photographed. Three cores from each mat sample were observed microscopically to ensure a good overall representation of resident morphotypes.

The bacterial communities in the studied spring cyanobacterial mat layers were compared using ARISA technique (Ramette 2009). Triplicate samples (ca. 300–500 mg each) of the cyanobacterial layers were subjected to DNA extraction using the UltraClean soil DNA isolation kit (MO BIO laboratories, Inc., USA) following the manufacturer's instructions. Polymerase chain reaction (PCR) amplification was performed in triplicates using the universal primer ITSF and the FAM-labelled eubacterial ITSReub primer (Cardinale et al. 2004) at an anealing temperature of 55°C. The PCR products were purified using Sephadox G-50 Superfine (Sigma–Aldrich). 150 ng of DNA was then mixed with 0.5 μL of internal size standard MapMarker®1000 ROX (50–1,000 bp; BioVenures

Inc., USA) and the amplified fragments were discriminated by capillary electrophoresis on an ABI PRISM 3130*xl* Genetic Analyzer (Applied Biosystems, USA). The ARISA profiles were analyzed using GeneMapper software v 3.7 (Applied Biosystem, USA). The total peak area per sample was normalised to one and only fragments between 100 and 1,000 bp were considered. A "fixed window" binning strategy with a bin size of 2 bp was applied to the ARISA data (Böer et al. 2009), and the binning frame that offered the highest pairwise similarities among samples was subjected to multivariate analyses (see below).

Statistical analysis

Results of antibacterial, anti-diatom and quorum-sensing bioassays were square root transformed in order to ensure normality and homogeneity of variance. The normality assumption was verified with the Shapiro–Wilk's test (Shapiro and Wilk 1965). Differences between solvent control and the treatments were determined by one-way analysis of variance (ANOVA) followed by a Dunnet post hoc test. In all cases, the threshold for significance was 5%. Statistical analysis and comparison of chemical extracts and ARISA fingerprints of microbial communities were carried out using the PRIMER 6 software package (PRIMER-E Ltd, Ivybridge, UK). For cluster analysis of chemical extracts Bray–Curtis similarities were used to produce a matrix based on the total number of peaks observed in all extracts and the presence or absence of these peaks in individual extracts. Multivariate analysis of microbial communities from all sites was performed using multidimensional scaling (MDS) based on Bray–Curtis similarities. Ordination of the Bray–Curtis similarities was performed using non-metric MDS, with 100 random restarts and the results were plotted in two dimensions. Agglomerative, hierarchical clustering of the Bray–Curtis similarities was performed using the CLUSTER method of the PRIMER software, and this information was superimposed onto the two-dimensional MDS plot at similarity levels of 20% to 80%. Analysis of similarities (ANOSIM) was carried out to test for significant differences between microbial communities exhibiting differential antimicrobial activity. ANOSIM produces a sample statistics (R), which represents the degree of separation between test groups.

Results

Physical-chemical characteristics of the springs

The physical and chemical characteristics of water in the four springs are summarised in Table 1. The highest range of water temperature was recorded in Hammam (67–60°C), while the lowest was in Bowshar (38–35°C). The pH at Nakhl (7.9) was more alkaline when compared with that of others (6.9–7.0). The conductivity should be higher in waters with higher temperatures and vice versa. Although this was the case in Hammam, Rustaq and Nakhl, the value recorded in Bowshar (1,100 μS) was an anomaly (Table 1). The concentrations of total dissolved solids (TDS) also showed the same pattern because conductivity and TDS are related parameters. The lowest flow velocity (1.0 m s^{-1}) was measured in Bowshar and Hammam, while Rustaq and Nakhl had higher flow velocities (1.7–2.4 m s^{-1}). Water temperature and DO are inversely related. Hammam with the highest temperature range had the lowest DO (1.6 mg L^{-1}) and Nakhl and Bowshar with overlapping temperature ranges had higher DO (4.5–3.8 mg L^{-1}). The ionic dominance of major cations and anions in all four springs were Ca>Na>Mg>K and Chloride>Sulphate>Nitrate>Fluoride, respectively. The concentrations of trace elements (Sr, Fe and Mn) and bicarbonate (HCO_3) were similar in all springs.

Antibacterial and anti-diatom activities

Only extracts from the cyanobacterial mats collected at Bowshar and Nakhl springs showed antibacterial properties (Table 2). The growth of four out of seven pathogenic bacteria (i.e. *Micrococcus luteus*, *Shigella sonnei*, *Salmonella enterica* and *Klebsiella pneumoniae*) and one out of two environmental isolates (i.e. *Bacillus* sp.) was significantly inhibited (ANOVA, Dunnet, $P<0.05$) by these extracts. Polar extracts of Nakhl mats had the highest antibacterial activity. The largest inhibition zone was observed for the polar extract from Nakhl mats against *K. pneumoniae*. Antibacterial activity of non-polar extracts was lower but this activity was detected for both Nakhl and Bowshar mats.

All tested extracts significantly inhibited (ANOVA, Dunnet, $P<0.05$) growth of the diatom *A. coffeaeformis* (Table 2). The highest growth inhibition was observed in the presence of the non-polar extracts from Rustaq mats, while the polar extracts from Hammam mats had the lowest growth inhibition. In general, non-polar extracts were inhibiting growth of the diatom stronger than polar extracts.

Inhibition of bacterial quorum-sensing

Non-polar extracts of Nakhl and Rustaq mats had the lowest inhibitive quorum-sensing (QS) concentrations in the case of *C. violaceum* CV017 bioassay (Table 2). This suggests that these extracts were having the highest QS inhibitory activity. Non-polar extracts from Hammam mats and polar extracts from Nakhl mats were toxic to the reporter strain (data are not shown). In the *A. tumefaciens* NTL4 bioassay, polar extracts from Bowshar, Rustaq and Nakhal and non-polar extracts from Rustaq and Nakhal

Table 1 Physical-chemical characteristics of water in the studied hot springs

	Nakhl	Rustaq	Hammam	Bowshar
UTM grid ref.	$^{5}852\ ^{25}875$	$^{5}435\ ^{25}880$	$^{6}353\ ^{25}965$	$^{6}428\ ^{26}035$
Water temperature (°C)	37–40	46–47	60–67	35–38
Air-shade temperature (°C)	30	39	35	28
PH	7.9	6.93	6.98	7.05
DO (mg L^{-1})	4.5	2.9	1.6	3.8
Conductivity (μS)	800	1,300	1,900	1,100
Flow velocity (m S^{-1})	2.4	1.7	1	1
TDS (mg L^{-1})	376	523	813	533
Ca (mg L^{-1})	62	78	114	82
Mg (mg L^{-1})	27	27	33	32
Na (mg L^{-1})	42	83	129	65
K (mg L^{-1})	3.4	5	6.4	4.2
Sr (mg L^{-1})	0.75	1.3	1.7	0.98
Fe (mg L^{-1})	<0.05	<0.05	<0.05	<0.05
Mn (mg L^{-1})	<0.05	0.05	<0.05	<0.05
HCO3 (mg L^{-1})	231	234	203	238
Cl (mg L^{-1})	70	131	187	105
S (mg L^{-1})	47	75	239	121
N (mg L^{-1})	1.4	1.1	0.2	0.6
NO3 (mg L^{-1})	6	4.8	1	2.5
F (mg L^{-1})	0.4	0.8	0.8	0.5

mats inhibited bacterial QS. The non-polar extracts from Nakhl mats had the lowest inhibitive QS concentrations, thus exhibiting the highest QS inhibitory activity (Table 2). Polar extract from Hammam mats was toxic to the reporter strain *A. tumefaciens* NTL4 (data not shown).

Chemical comparison of the extracts

Chemical composition of non-polar extracts of all cyanobacterial mats was compared using GC-MS. A total of 74 chemical compounds were found in the non-polar extracts from all mats (Table 3). The distribution of these compounds varied from one spring to the other with the highest number of compounds detected in Bowshar (42 compounds) and the lowest in Rustaq mats (28 compounds). The chemical profiles of crude non-polar extracts from Bowshar, Hammam and Rustaq mats shared less than 43% similarity with each other and they were highly different from Nakhl spring mats (Fig. 1, Table 3). The identity of most peaks could be determined by direct comparison to NIST GC-MS chemical library, however 38% of the peaks (i.e., peaks with retention times 17.30, 18.22, 18.40 and 18.93 min) remained unknown. The MS spectra of these peaks did not match with commercially available databases, suggesting the novelty of these compounds. The metabolites indicated by peaks with retention times 11.94, 14.21, 18.22, 21.92, 23.08 and 28.56 min were unique and were present only in extracts of particular mats but absent in others. The peak with the retention time 22.33 min, identified as phytol, a common plant terpenoid, was present in all mats (Table 3). All extracts contained a suite of short chain C_{14}–C_{20} fatty acids, polyalcohols and few wax esters. In many cases, peaks represented secondary metabolites of organisms, while in some cases some plasticizes and pollutants like caprolactam, possibly trapped inside the biofilm matrix, were detected.

Comparison of microbial communities

Chroococcus, Phormidium, *Leptolyngbya*, *Spirulina* and *Lyngbya*–like cyanobacteria were among the most common types observed in the studied mats by direct microscopy. These cyanobacteria were distributed differently among the different springs with the highest richness observed in Hammam (7 morphotypes) and the lowest in Rustaq (2 morphotypes). Hammam spring was dominated by *Chroococcus*, *Phormidum*, *Leptolyngbya* and *Spirulina* whereas Rustaq was mainly dominated by *Spirulina*. Thin filamentous *Phormidium* and *Leptolyngbya* constituted most of the cyanobacterial layer in Nakhl and Bowshar with more *Chroococcus* observed in Nakhl.

ARISA profiles of amplicons generated by PCR of bacterial ITS sequences yielded a total number of 260

Table 2 Bioactivity of the polar (W) and non-polar (EA) fractions of cyanobacterial mats

Activities of extracts	Extracts from cyanobacterial mats							
	Nakhl		Hammam		Bowshar		Rustaq	
	W	EA	W	EA	W	EA	W	EA
Antibacterial (mm)[a]								
Bacillus sp.[b]	0	3.3±0.6	0	0	0	2.7±0.5	0	0
Cytophaga sp.[b]	0	0	0	0	0	0	0	0
Micrococcus luteus	0	3.7±0.6	0	0	0	3.3±0.6	0	0
Staphylococcus epidermidis	0	0	0	0	0	0	0	0
Bacillus subtilis	0	0	0	0	0	0	0	0
Shigella sonnei	5.3±0.6	0	0	0	0	0	0	0
Salmonella enterica	5.7±0.6	0	0	0	0	0	0	0
Escherichia coli	0	0	0	0	0	0	0	0
Klebsiella pneumoniae	8.0±1.0	0	0	0	0	1.3±0.5	0	0
Anti-diatom (%)[c]	75.3± 15.6	79.3± 11.6	33.3± 12.5	50.0± 7.5	37.3± 12.5	54.0± 7.9	62.7± 20.4	83.3± 3.1
Quorum-sensing inhibition								
Inhibitive concentration IC50 (mg mL^{-1})[d]								
Chromobacterium violaceum CV017	0	0.8±0.4	0	0	0	5.3±2.3	6.7±1.9	0.9±0.5
Agrobacterium tumefaciens NTL4	14.7±2.6	5.2±2.6	0	0	9.04±5.2	0	10.0±4.0	8.0±3.0

W water soluble, *EA* ethyl acetate soluble, *QS* quorum-sensing, IC_{50} 50% inhibitive concentrations

[a] Antibacterial activity was measured as mean diameter±SD of inhibition zone

[b] Strains of bacteria isolated from microbial mats

[c] Anti-diatom activity was expressed as inhibition of mean of chlorophyll *a* concentration±SD in comparison with the control

[d] QS inhibition was expressed as the mean IC_{50}±SD. The lower the IC_{50} concentration is the higher the QS inhibitory activity; (0) absence of bioactivity

unique operational taxonomic units (OTUs; i.e. binned ARISA peaks) distributed among the four studied hot spring mats. The highest number of OTUs was detected in samples from Hammam mats (mean±SD, 131±24), while the lowest one was found in Nakhl mats (mean ± SD, 69±38). Cluster analysis of similarity matrices revealed that microbial communities from different sites were highly different from each other (Fig. 2a, b). While communities from Bowshar, Nakhl and Rustaq mats shared some similarity, samples from Hammam cyanobacterial mats were always different. Moreover, comparison of antibiotic producing (Bowshar, Nakhl) and not producing (Hammam, Rustaq) mats showed that they had significantly different (P=0.0005) microbial communities (ANOSIM, R=0.71, Fig. 2c).

Discussion

Unlike previous investigations that relay on screening bioactivity of single isolates under laboratory conditions (Biondi et al. 2008; Rojas et al. 2009), the bioactivity of mixed cyanobacterial and aerobic heterotrophic bacterial community forming the top mat layer was tested here. These cyanobacteria-dominat mat layers were shown to exhibit in vivo antibacterial, anti-diatom, and quorum-sensing inhibitory activities. This approach takes advantage of in situ cell-to-cell relationship in multispecies communities that does not exist in pure cultures, as well as the isolation of compounds that might be produced by uncultured microorganisms. Recently, a metagenomic approach was proposed to explore novel bioactive compounds from uncultured marine microbes (Zhang et al. 2005) and resulted in the discovery of new genes encoding enzymes, and other natural products (Marco 2010). Therefore, screening natural communities for the production of bioactive compounds, either directly as it was done in this study or by looking for potential genes, may reveal different types of compounds that cannot be otherwise produced by individual isolates. In spite of that, it remains difficult in this approach to identify the cyanobacteria or other microorganisms that are responsible for the observed bioactivity.

The production of antibacterial, anti-diatom and quorum-sensing inhibitory compounds under natural conditions by

Table 3 Composition of non-polar extracts of the studied cyanobacterial mats revealed by gas chromatography mass spectrometry (GC-MS)

Peak retention time (min)	Extracts of microbial mats				Close match
	Hammam	Bowshar	Nakhl	Rustaq	
4.22	–	0.26	–	0.11	Octanoic acid
4.52	–	0.06	0.05	0.05	Ethylmethylmaleimide,[1]H-pyrrole-2,5-dione, 3-ethyl-4-methyl
4.63	0.34	0.34	–	0.22	Caprolactam
4.85	0.09	0.06	–	–	Nonanoic acid
4.94	0.02	–	–	–	*n*-Undecanoic acid
5.19	–	–	0.11	–	Decane, 2-methyl
5.61	–	–	0.14	–	UI
8.17	–	0.37	–	0.47	2([4]H)-Benzofuranone, 5,6,7,7atetrahydro-4,4,7a-trimethyl
8.27	–	0.14	–	–	UI
9.17	–	1.21	–	–	Dodecanoic acid
9.56	–	0.33	–	–	UI
10.52	–	0.17	–	0.1	UI
11.13	–	–	–	0.25	4-Norcadin-5-en-4-on isomer B
11.47	–	0.37	–	–	Tridecanoic acid
11.94	–	1.13	–	–	UI
12.84	0.33	–	–	2.11	1-Tridecanol
12.86	–	–	4.59	–	Pentadecane
12.94	3.82	–	–	14.8	Heptadecane
14	–	4.7	1.1	–	Tetradecanoic acid
14.11	–	–	–	0.12	UI
14.12	0.6	–	–	–	Octadecane
14.21	0.6	–	–	–	UI
14.42	–	1.16	–	–	UI
14.74	–	–	1.5	–	UI
15.05	–	–	1.93	–	UI
15.12	–	0.17	–	–	UI
15.38	1.32	0.43	1.06	–	Nonadecane
15.46	–	0.54	–	0.55	*n*-Hexadecanoic acid
16.06	0.64	0.77	–	0.31	2-Pentadecanone, 6,10,14-trimethyl
16.24	–	0.21	0.44	0.31	Neophtadiene
16.78	2.37	–	–	–	Eicosane
16.89	0.95	2.77	–	1.34	Trans-2-nonadecene
17.3	–	–	2.94	–	UI
17.97	–	1.12	5.02	1.01	9-Hexadecanoic acid, methyl ester, (Z)
18.09	2.96	1.35	–	–	Dibutyl phthalate,1,2-Benzenedicarboxylic acid, dibutyl ester
18.22	–	–	–	4.43	UI
18.4	6.21	17.25	–	–	UI
18.5	–	1.88	–	–	Bicyclo[3.1.1]heptan-3-one, 2,6,6-trimethyl-, (1à,2á,5à)-,.3-Pinanone
18.84	7.76	14	–	2.46	Heptadecanoic acid
18.93	–	–	6.31	–	UI
19.31	–	0.91	0.99	1.64	UI
19.53	–	–	18.47	–	UI
19.82	–	–	1.87	–	UI
20.13	1.72	–	–	1.98	UI
21.56	–	–	–	2.02	Heneicosane
21.59	0.92	–	2.09	–	UI
21.65	–	–	0.78	–	9,12,15-Octadecatrienoic acid, methyl ester, (Z,Z,Z)-

Table 3 (continued)

Peak retention time (min)	Extracts of microbial mats				Close match
	Hammam	Bowshar	Nakhl	Rustaq	
21.88	0.56	–	2.69	–	9-Octadecenoic acid (Z)-*methyl* ester
21.92	–	–	0.85	–	UI
22.33	10.24	8.05	13.75	39.03	Phytol
22.54	–	7.95	–	–	9,12-Octadecadienoic acid (Z,Z)-*methyl* ester
22.78	–	10.28	–	–	UI
22.9	35.87	4.13	–	–	Oleic Acid(9-Octadecenoic acid (Z)-)
23.08	–	–	4.54	–	Linoleic acid ethyl ester
23.15	–	–	1.67	–	UI
23.28	–	1.24	–	-	Octadecanoic acid
23.42	–	–	6.05	–	UI
23.84	–	0.32	–	–	Hexadecanoic acid, butyl ester
23.94	–	–	1.38	–	Octadecanoic acid ethyl ester
24.33	–	0.41	1.22	–	UI
24.53	1.88	1.18	–	5.75	Docosane
26.57	3.59	–	–	5.1	Tricosane
27.83	–	–	0.26	–	Hexanedioic acid, bis(2-ethylhexyl) ester
28.28	–	–	0.56	–	9-Hexacosene
28.52	–	2.6	0.43	2.33	Tetracosane
28.56	3.07	–	–	–	UI
28.24	0.78	0.33	–	–	UI
29.03	0.49	0.43	–	–	UI
30.24	0.52	0.23	–	–	Palmitic acid, *N*-octyl ester
30.38	–	–	17.17	–	Bis(2-ethylhexyl) phthalate
30.47	5.42	4.88	–	5.45	Pentacosane
32.31	2.65	2.88	–	2.13	Hexacosane
34.16	1.88	2.11	–	1.57	UI
36.48	1.27	0.02	–	1.09	Octacosane
39.35	1.02	1.19	–	0.78	Nonacosane
Total	30	42	29	28	

The data presented the relative area of the peaks in %. Peaks were identified by the NIST Mass Spectral library. Compounds that have a probability lower than 75% to the closest match with the library are marked as unidentified (UI). Compounds that are absent in the extracts are marked as (–)

mats' cyanobacteria and aerobic heterotophs raise the question of why would these microbes produce such compounds in mixed microbial communities? Previous studies demonstrated that microorganisms within biofilms produce quorum-sensing compounds to coordinate their behaviour (Huang et al. 2007; Decho et al. 2009). These compounds were shown to vary during the day and in the night (Decho et al. 2009) and within different biofilms (Huang et al. 2007). It was also described that bacterial isolates produce compounds that inhibit the growth of other bacteria (Volk and Furkert 2006, Biondi et al. 2008; Dobretsov et al. 2010), fungi (Piccardi et al. 2000; Bottone and Peluso 2003) and microalgae (Smith and Thanh Doan 1999; Blom et al. 2006; Volk 2006; Gantar et al. 2008). Similarly, cyanobacteria and other microorganisms in our microbial mats produced allelopathic compounds, like quorum-sensing inhibitors, antibacterial and anti-diatom compounds, in order to regulate densities of other micro-organisms and form stable and cooperative associations. The highest quorum-sensing inhibition, anti-diatom and

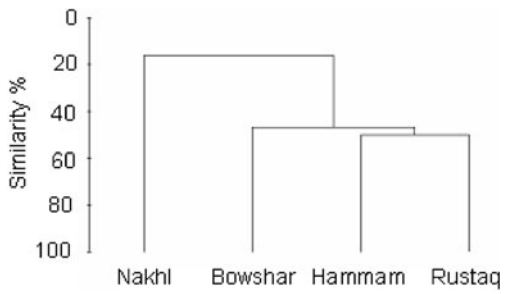

Fig. 1 Cluster analysis of similarity between GC-MS chromatograms of non-polar extracts from the four studied cyanobacterial mats

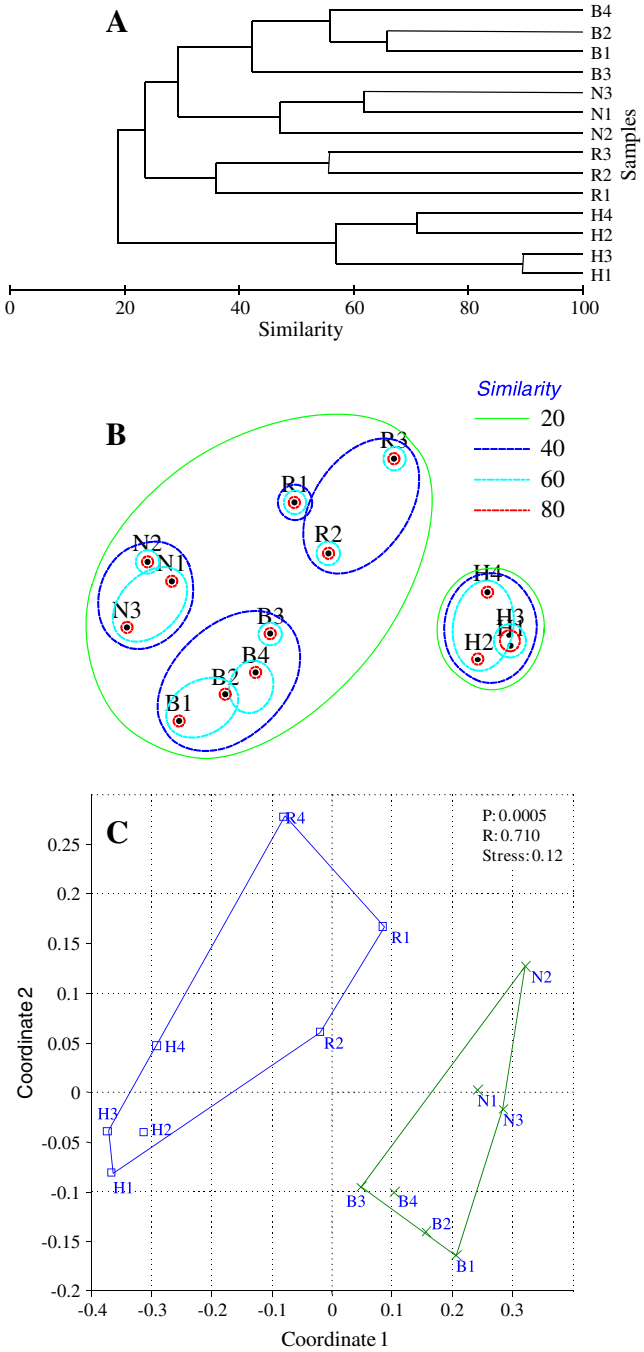

Fig. 2 Cluster analysis (**a**) and MDS plot (stress=0.12) (**b**) showing similarities among the four studied microbial communities based on ARISA profiles. (**c**) MDS plot showing comparison between antibiotic producing (N and B) and not producing (R and H) microbial communities. *H* Hammam, *B* Bowshar, *N* Nakhl, *R* Rustaq. *Digits next to letters* represent different replicates

antibacterial activities were detected in Nakhl cyanobacterial mats, which had the lowest number of OTUs (as shown by ARISA profiles) and the highest number of unidentified compounds (as shown by GC-MS analysis). It is conceivable that the microbes from this cyanobacterial mat produce bioactive compounds to suppress growth and attachment of other microorganisms, such as bacteria and diatoms, thus resulting in a low microbial diversity at this site.

Non-polar extracts of the studied cyanobacterial communities were more active than the polar ones in inhibiting growth of diatoms and bacterial QS. In opposite, polar extracts of Nakhl mats had strongly antibacterial properties. In a previous study performed on cyanobacterial isolates from microbial mats ethyl acetate extracts exhibited the highest antibacterial, antifungal and cytotoxic activities (Biondi et al. 2008). Like in other hot spring mats (Robinson and Eglinton 1990; van der Meer et al. 2008; Jungblut et al. 2009), the GC-MS analysis of the non-polar extracts of our mats revealed different abundance of saturated and unsaturated fatty acids and polyalcohols. The detection of the known environmental pollutant caprolactam in the extracts of three out of four cyanobacterial mats could be explained by the fact that the detected compounds represent not only those extracellular compounds released by microbes but also those trapped in the mat matrix. While GC-MS is a powerful technique, it can only detect low molecular weight compounds (<400) of low and medium polarity. This limits our analysis and does not allow us to compare all (polar and non-polar) metabolites in the communities. Results of this study do not allow us to assign a certain bioactivity to particular compounds. Furthermore, quorum-sensing inhibitory activity of Rustaq mats was probably due to the presence of benzofuranone. Furanones and their synthetic analogues are specific and potent inhibitors of quorum-sensing-regulated behaviours in Gram-negative bacteria, including expression of virulence genes, production of antibiotics, bacterial motility, swarming and biofilm formation (Kjelleberg et al. 1997; Defoirdt et al. 2004; Dobretsov et al. 2009).

The difference in the chemical environment and bioactivities of our cyanobacterial mat extracts could be mainly attributed to differences in microbial community composition and/or physical-chemical conditions of the springs differences. ARISA technique suggested that each spring developed its own unique microbial community with more than 40% differences in OTUs composition, as inferred from pairwise OTUs comparison. Additionally, microbial composition of antibiotic producing and non-producing cyanobacterial mats were significantly different, suggesting that different microorganisms of the studied mats might have produced different types of chemical compounds. Indeed, mat-building cyanobacteria and heterotrophic microorganisms were shown to produce different types of mat lipids (Shiea et al. 1991; Zeng et al. 1992; Zhang et al. 2004; Jungblut et al. 2009). Furthermore, differences in microbial communities in the top and bottom layers of a cyanobacterial mat were shown to result in a vertical distribution of non-polar compounds (Zeng et al. 1992). While hydrocarbons and polar lipid fatty acids dominated the top layer, wax esters, long-chain diols,

alkylglycerols and octadecanol were abundant in deeper layers of the cyanobacterial mats. Clear variations in pH, flow velocity, conductivity and temperature among the investigated springs could also affect the physiology of microorganisms and their secondary metabolites. In a particular study, in situ changes in pH from 6.8 to 9.4 during a diurnal cycle were shown to result in differential degradation of short and long-chain AHLs in a microbial mat (Decho et al. 2009). It has been shown that changes in temperature and nutrients affect production of enzymes, antibacterial, antifungal, anticancer and antifouling compounds in monocultures of cyanobacteria under laboratory conditions (Biondi et al. 2008; Selvin et al. 2009).

In conclusion, this study showed the ability of cyanobacteria and aerobic heterotrophic bacterial communities in hot spring mats to produce antibacterial, antidiatom and quorum-sensing inhibitory compounds under in situ conditions. Future research should focus on screening such communities for more bioactivities and identifying the microganisms and the conditions needed for the production of these compounds.

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