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Antibacterial and anti-diatom activity of Hong Kong sponges

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ABSTRACT: In this study, we link laboratory results with field evidence of microbial settlement responses to crude extracts of several sponge species from Hong Kong waters, including *Callyspongia* sp. 1, *Callyspongia* sp. 2, *C. pulvinata*, *Mycale adhaerens*, *Haliclona cymaeformis* var. 1, *H. cymaeformis* var. 2 and *Halichondria* sp. (Porifera: Demospongiae). Laboratory experiments showed that 6 out of 7 sponge extracts inhibited growth and caused mortality of the pennate diatom *Nitzschia paleacea* at tissue-level concentration. In disk-diffusion bioassays, extracts of the sponge *Halichondria* sp. inhibited the growth of 4 bacterial strains out of 11 isolated from inanimate substrata; other sponge extracts inhibited growth of only 1 or 2 bacterial strains. For field experiments, sponge metabolites were immobilized in a Phytagel® matrix and exposed to natural microbial communities. After 2 d of exposure, bacterial densities on the gels with sponge extracts were lower than on control gels. Terminal restriction fragment length polymorphism (TRFLP) analysis of PCR-amplified bacterial community 16S rRNA genes obtained from these gels demonstrated that the communities were different between the sponge extracts and control gels. After 7 d of exposure, 6 (86%) extracts suppressed the recruitment of pennate diatoms and 3 (43%) sponge extracts suppressed the recruitment of bacteria. These results suggest that sponge extracts can modify the structure of bacterial communities and decrease the density of bacteria and diatoms in microfouling communities.

KEY WORDS: Sponge allelochemistry · Secondary metabolites · Bacteria · Diatoms · Microbial community · TRFLP · South China Sea

INTRODUCTION

Inanimate and animate surfaces in the marine environment are continuously exposed to, and colonized by, microorganisms and propagules of metacellular organisms. Bacteria and diatoms are among the first organisms that attach to surfaces (ZoBell 1939, Characklis & Cooksey 1983) and form natural biofilms that are highly variable over time and heterogeneous in composition. Development of a biofilm on the surface of living organisms can provide advantages to the host and/or cause damage, inhibit growth, or even lead to necrosis and death of these organisms (Mitchell & Chet 1975, Dixon et al. 1981, Walls et al. 1993). In invertebrates, such as sponges, the extent of microbial colonization is possibly influenced by chemical effects of bioactive metabolites produced either by the host itself or by symbiotic organisms (Wahl 1997, Kelly et al. 2003).

Sponges are a rich source of unique and diverse bioactive metabolites (Sera et al. 1999, Faulkner 2000, Blunt et al. 2003). Antibiotics, such as plakortin from *Plakortis halichondroides* (Higgs & Faulkner 1978), manoalide from *Luffariella variabilis* (De Silva & Scheuer 1980), furospongin-1 from *Spongia officinalis* (Anderson et al. 1994) and aerothionin from *Aplysina gerardogreeni* (Encarnacion-Dimayuga et al. 2003) have been isolated from marine sponges. These and other antibiotics isolated from marine sponges are active against human pathogens and other bacteria that are not found in the living environment of sponges. Only a few investigations, however, have

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examined the antibacterial activity of secondary metabolites of sponges against bacteria isolated from marine waters (Kelman et al. 2001, Kelly et al. 2003). Until now, there has been no experimental field evidence regarding the specific physiological effects of sponge-derived metabolic compounds on microorganisms occurring on external and internal surfaces or in the tissues of sponge.

In the present study, we collected 7 species of sponges from the Class Demospongiae that are commonly found in Hong Kong coastal waters, and studied their antibacterial and anti-diatom activity in laboratory and field experiments. Bacterial communities developing on gel surfaces with sponge crude extracts were compared by the culture-independent, polymerase chain reaction (PCR)-based molecular fingerprinting technique of terminal restriction fragment length polymorphism (TRFLP) (Liu et al. 1997, Harder et al. 2004). The rationale for this approach was to overcome the disadvantages associated with culture-dependent techniques and to reveal the effects of sponge metabolites on the formation of bacterial communities under natural conditions. This approach combines restriction fragment analysis of a phylogenetic marker with automated sequencing gel technology and permits the rapid profiling of a bacterial community. Briefly, 16S rRNA genes are PCR amplified from community DNA being directly extracted from the bacterial communities developed in the presence of sponge extracts. One primer is labeled at the 5' terminus with a fluorescent dye that allows the investigator to track only the terminal fragment of a restriction digest. As a general rule, a single species will contribute a uniquely sized terminal fragment although several species may have terminal fragments of identical size. The TRFLP method has been used successfully for the characterization of bacterial communities in marine samples (Moesender et al. 1999, Harder et al. 2003, Qian et al. 2003, Lee & Qian 2004) and has certain advantages over denaturing gradient gel electrophoresis (DGGE) and 16S rRNA (DNA) gene cloning in terms of its effectiveness, sensitivity and consistency in differentiating microbial communities (Moesender et al. 1999, Brodie et al. 2002, Fuhrman et al. 2002).

In this study, we proposed to determine whether (1) the sponge extracts affected the growth of bacteria and diatoms isolated from the natural environment in laboratory experiments; (2) the sponge extracts affected the density of bacteria and diatom communities in the field experiments; (3) the sponge extracts affected the structure of bacterial communities and the species composition in the field experiments; (4) the results of the laboratory experiments correspond with findings of the field experiments.

MATERIALS AND METHODS

Collection of sponges. Specimens of the sponges *Callyspongia* (*Cladochalina*) sp. 1 (ZMAPOR 17596), *Callyspongia* (*Cladochalina*) sp. 2 (ZMAPOR 17598), *Mycale adhaerens* (ZMAPOR 17597), *Haliclona* (*Gellius*) *cymaeformis* var. 1 (ZMAPOR 17599), *H. cymaeformis* var. 2 (ZMAPOR 17602), *Halichondria* sp. (ZMAPOR 17603) and *C. pulvinata* (ZMAPOR 17601) were collected from Hong Kong coastal waters at depths of 1 to 3 m, either near the fish farm next to the Wong Shek Pier (22° 25′ N, 114° 20′ E), or at the fish farm in Port Shelter (22° 22′ N, 114° 16′ E). The sponges were carefully retrieved to the water surface, transferred to large buckets, and brought to the laboratory. The wet weight was measured at the fish farm and the tissue volume was determined by water displacement. Reference samples were stored in 96% ethanol for subsequent taxonomic identification. Samples are held in the type collection in the Zoological Museum, University of Amsterdam and have the collection acronyms used above, i.e. ZMAPOR.

Chemical extraction of sponges. In the laboratory, sponge tissues were extracted twice in 1:1 dichloromethane:methanol for 8 h with gentle agitation. The combined extracts were filtered through Whatman No. 1 paper filter and reduced by rotary evaporation to a concentration that was volumetrically equivalent to 10% of the original sample tissue. Tissue-level concentrations were adjusted prior to experiments.

Laboratory experiments. ***Diatom culture:*** The diatom *Nitzschia paleacea* was isolated from a natural biofilm developed on microscopic slides exposed to fouling for 5 d at the fish farm in Port Shelter. The isolation was performed according to Harder et al. (2002). The diatoms were cultivated in aerated 400 ml Erlenmeyer flasks at 24°C with overhead fluorescent illumination (0.3×10^{16} quanta s^{-1} cm^{-1}). When a visible film developed in the culture flask, diatom suspensions were prepared by brushing the culture flask with a sterile paint brush. This algal suspension was then used in the following experiments.

Anti-diatom bioassay: Anti-diatom activity of the sponge extracts was tested according to a protocol developed by Dobretsov & Qian (2002). Prior to the experiment, the sponge extracts were dried by rotary evaporation and re-dissolved in dimethylsulfoxide (DMSO) to a concentration that was volumetrically equivalent to 10% of the original sample tissue. Extracts were tested at tissue-level concentrations. Three hundred µl of the extract solution were added to Petri dishes (in 8 replicates) containing 2.7 ml of algal suspension of the diatom *Nitzschia paleacea* (about 12×10^4 cells ml^{-1}). After an incubation period of 2 d at 25 ± 1°C at a light:dark cycle of 15:9 h, the

numbers of dead and living diatoms were counted on 5 replicates of each plate under a light microscope (Axiophot, Zeiss; magnification 400×). Empty diatom shells were considered as dead diatoms and those with distinct chloroplasts were considered as live ones. A 10% (v/v) solution of DMSO in filtered (pore size 0.22 µm) seawater (FSW) was used as a control. Three more Petri dishes were used for chlorophyll *a* (chl *a*) measurements. The amount of chl *a* was determined by a fluorometer technique (Lorenzen 1967).

Anti-bacterial assays: To investigate the antibacterial effects of the sponge extracts, the extracts were pipetted onto circular paper discs (Whatman No. 1; disc volume = 20 µl) to obtain tissue-level concentrations. An additional set of discs loaded with 10 µg ml^{-1} of streptomycin were used as a control. Eleven strains of bacteria were tested: *Vibrio hollisae* (HKUST strain destination/GenBank accession no) (UST991130-052/X74707), *V. harveyi* (UST020129-010/AJ391203), *V. halioticoli* (UST010723-002/AJ391203), *V. furnissii* (UST010723-010/AJ4211445), *V. proteolyticus* (UST991130-038/X74723), *V. alginolyticus* (UST981130-062/X56576), *Vibrio* sp. (UST950701-007/AF064637), *Pseudoalteromonas* sp. (UST010723-006/U80834), *Rodovulum* sp. (UST950701-12/D32245), *Staphylococcus aureus* (UST950701-005/AF343958), *S. haemolyticus* (UST950701-004/AF343957). These strains were obtained from the culture collection of the Hong Kong University of Science and Technology (HKUST) and originated from 7 d old marine natural biofilms in the vicinity of the Port Shelter fish farm. Each strain was inoculated onto agar plates, then the dried discs with either the sponge extracts or with the control solution were placed onto the agar plates. The agar plates were incubated for 24 h at 30°C until bacteria developed a film. The observed zones of growth inhibition between the disc and the bacterial film were measured to the nearest 0.2 mm.

Field experiments. ***Gel-immobilization of sponge tissue extracts:*** The method for the immobilization of organic tissue extracts into gels was adopted from Hendrikson & Pawlik (1995) and Harder et al. (2004) with slight modification. Briefly, about 500 ml of the sponge extract (for the preparation of extracts see 'Chemical extraction of sponges' above) were dried by rotary evaporation. These extracts were dissolved in above DMSO at a concentration 10× times that of the tissue level. Gels were prepared by adding Phytagel (Sigma Chemical) to a stirred beaker of boiling double distilled water to yield a 4% (w/v) gel concentration. After the gel solution cooled down to 70°C, the DMSO crude extracts were diluted to tissue-level concentration with the liquid gel solution and vigorously mixed for even distribution of the extract in the viscous gel. In preliminary experiments, inhibition zones of heated 70°C DMSO crude extracts did not differ from inhibition zones of non-heated extracts. A control was prepared accordingly with pure DMSO. Then, 5 ml of the gel solution with (treatment) and without (control) extract were transferred to Falcon® Petri dishes and were allowed to solidify for 24 h. These dishes were used in the bioassays (see below).

Development of microbial communities on gel-coated Petri dishes: Gel-coated Petri dishes were used for microbial colonization in the 2 bioassays. In the first bioassay, each Petri dish with sponge extract or control was placed in a 10 l aquarium (1 Petri dish per treatment per aquarium), which was continuously supplied with natural seawater pumped from the HKUST pier (from a depth of 5 m) at a flow rate of 12 l h^{-1}. The separation of experimental and DMSO-control gels in individual aquaria allowed us to avoid potential cross contamination of leaching extracts and to exclude pseudoreplication. Six replicate aquaria per treatment were used. After 48 h of exposure to natural seawater, the discs were retrieved. Three of the 6 treatment replicates were fixed in 4% formaldehyde solution in seawater for subsequent bacterial and diatom counts; the other 3 were immediately used for the extraction of total bacterial community DNA, as described below.

A second bioassay with sponge extracts loaded on gels was performed at the HKUST pier for 7 d at a depth of 0.5 m below the low water mark. There were 4 replicates in each treatment. At the end of the experiments all Petri dishes were fixed in 4% formaldehyde solution in seawater for subsequent bacterial and diatom counts.

Counts of bacteria and diatoms: The attached bacteria were visualized using DNA-binding fluorochrome 4,6-diamidino-2-phenylindole (DAPI, Fluka Chemie) at 0.5 mg ml^{-1}. Formalin-fixed dishes were rinsed with autoclaved (120°C for 30 min) filtered (0.22 µm) seawater (AFSW) and stained with DAPI for 15 min. The number of bacteria in 5 haphazardly selected fields of view was estimated under an epifluorescence microscope (Axiophot, Zeiss; magnification 1000×; λ_{Ex} = 359 nm, λ_{Em} = 441 nm). The attached diatoms in 5 haphazardly selected fields of view were counted under the light microscope (Axiophot, Zeiss; magnification 400×). The 7 common diatom species on the plates were identified according to taxonomic keys (Jin et al. 1985).

Total bacterial community DNA extraction and purification: The entire area (19.625 cm^2) of the experimental or control Petri dishes with gel-loaded extracts was completely swabbed with sterile cotton buds for the collection of bacteria following the method described by Lee & Qian (2004). Swabs from each gel were individually suspended in 1 ml extraction buffer (100 mM Tris-HCl, 100 mM EDTA, 100 mM sodium phosphate, 1.5 M sodium chloride, 1% CTAB; at pH 8) in 2 ml micro-centrifuge tubes.

For lysing, the samples were subjected to 3 cycles of freezing and thawing followed by 2 h incubation in 20% sodium dodecylsulfate (SDS) at 65°C. The cotton buds were removed and, after centrifugation (10 000 × *g* for 5 min), the total DNA in the supernatant was extracted and purified in a volume of 24:1 chloroform:isoamyl-alcohol, followed by precipitation in isopropanol at room temperature for 15 min. The precipitated DNA was washed with cold (–20°C) 70% ethanol and resuspended in 50 µl of autoclaved double-distilled water and frozen until use.

Polymerase chain reactions: PCR of 16S rRNA genes (rDNA) of bacterial community DNA was performed in a total volume of 25 µl containing 1 ml of DNA template, 250 µM each of desoxyribonucleotide triphosphate (dATP, dCTP, dGTP, dTTP; Pharmacia Biotechnology), 1 U of DNA taq polymerase (Amersham Biosciences) and 0.8 µM each of universal primers: 341F forward (5′-CCTACGGGAGGCAGCAG-3′) and 926R reverse (5′-CCGTCAATTCCTTTRAGTTT-3′) (Amann et al. 1990, Lee et al. 1993). The 926R primer was labeled at the 5′ end with 6-carboxy fluorescein (FAM) dye.

The thermocycling conditions were as follows: a hot start at 95°C for 2 min (1 cycle); 95°C for 30 s (15 cycles), 56°C for 3 min and 72°C for 3 min. The annealing temperature started at 56°C and was reduced to 40°C in increments of 1°C cycle^{-1}; 10 cycles of 95°C for 30 s, 40°C for 3 min and 72°C for 3 min; and 72°C for 10 min. Amplified DNA (4 µl of PCR mixtures) was visualized by gel electrophoresis on a 1.5% agarose gel in TAE buffer.

Terminal restriction fragment length polymorphism analysis: Fluorescently labeled PCR products were purified with the Wizard® PCR preps DNA purification system (Promega) according to the manufacturer's protocol. Purified amplicons were digested with 20 U *Msp*I (Boehringer Mannheim Biochemicals) at 37°C for 6 h. Aliquots of digested products (10 µl) were mixed with 0.5 µl of internal size standard (ET550-R, Amersham Biosciences). This mixture was denatured for 2 min at 95°C and immediately chilled on ice prior to capillary electrophoresis on a MegaBACE genetic analyzer (Amersham Biosciences) operated in the genotyping mode. After electrophoresis, the length of the fluorescently labeled terminal restriction fragments (TRFs) was determined by comparison with internal size standards using the 'Fragment Profiler' software (Amersham Biosciences). TRFs that differed by less than 0.5 bp were considered identical.

Fragment lengths that were present in 3 samples were used to produce a representative TRF profile of each bacterial community. To make the comparisons of microbial communities reliable, a modification of the analytical procedure proposed by Dunbar et al. (2001) was utilized. The present approach reduces the variation being caused mainly by small irreproducible peaks of the electropherogram and uses only a reproducible subset of data from replicate TRF profiles. The peak heights of individual TRFs were calculated as a percentage of their abundance within a sample in order to account for DNA quantity differences between replicate profiles (Brodie et al. 2002). Phylogenetic ribotypes were assigned to the same terminal restriction fragments by the TAP-TRFLP Java Applet program (retrievable from the RPD II Website [http://35.8.164.52/html/TAP-trflp.html#program]), which performs a simulated restriction digestion of the submitted sequences in the prokaryotic 16S rRNA database.

Statistical analysis. Widths of inhibition zones produced by different extracts were square root transformed prior to analysis using a Dunnet test (Zar 1996) to compare each treatment with the control. In cases where no inhibition zone was produced, a value of ½ was given to improve the transformation. Densities of bacteria and diatoms on the Petri dishes with gels were log transformed to ensure normality and homogeneity of variance (Zar 1996). In the case of the absence of diatoms in a field of view, a value of log (y+1) was assigned to improve the transformation. In all cases, the normality assumption was verified by the Shapiro-Wilk test (Shapiro & Wilk 1965). The differences between the experimental and control treatments were determined by 1-way ANOVA followed by an LSD post-hoc test (Zar 1996). In all cases, the threshold for significance was 5%. TRF patterns of different bacterial community DNA samples were subjected to cluster analysis. Bray-Curtis similarities were used to produce a similarity matrix based on the total number of TRFs observed in all samples and the presence or absence of these TRFs in individual samples. For the construction of a dendrogram demarcating the similarity of microbial communities on the gels, group average linkage in the hierarchical, agglomerative clustering algorithm was performed using the PRIMER program (Plymouth Marine Laboratory).

RESULTS

Laboratory experiments

Anti-diatom bioassay

There were significant differences in the densities of the benthic diatom *Nitzschia paleacea* exposed to extracts from Hong Kong sponges (Fig. 1A, ANOVA: $F = 7.98$, $p < 0.0001$). All sponge extracts except *Cally-*

spongia sp. 1 significantly (LSD test, $p > 0.05$) suppressed the growth of *N. paleacea*. The chemical extract of *Callyspongia* sp. 1 showed no effect on the mortality of the diatom while other extracts were toxic to *N. paleacea* (Fig. 1B). More than 98% of diatom cells died in the presence of the extracts of *Mycale adhaerens*, *Haliclona cymaeformis* var. 1, *H. cymaeformis* var. 2, and *Halichondria* sp. The mean amount of chl *a* in the diatom biofilm was different among extract treatments from the 7 Hong Kong sponges (Fig. 1C, ANOVA: $F = 14.09$, $p < 0.000001$). The extracts of *Mycale adhaerens*, *H. cymaeformis* var. 1, *H. cymaeformis* var. 2, and *Halichondria* sp. caused the lowest value of chl *a* in the diatoms, while in the treatments with the extracts of the sponges *Callyspongia* sp. 2 and *C. pulvinata*, a moderate value of chl *a* was found. The highest value of chl *a* in the *Callyspongia* sp. 1 treatment was not significantly different from the control (Fig. 1C; LSD test, $p > 0.05$).

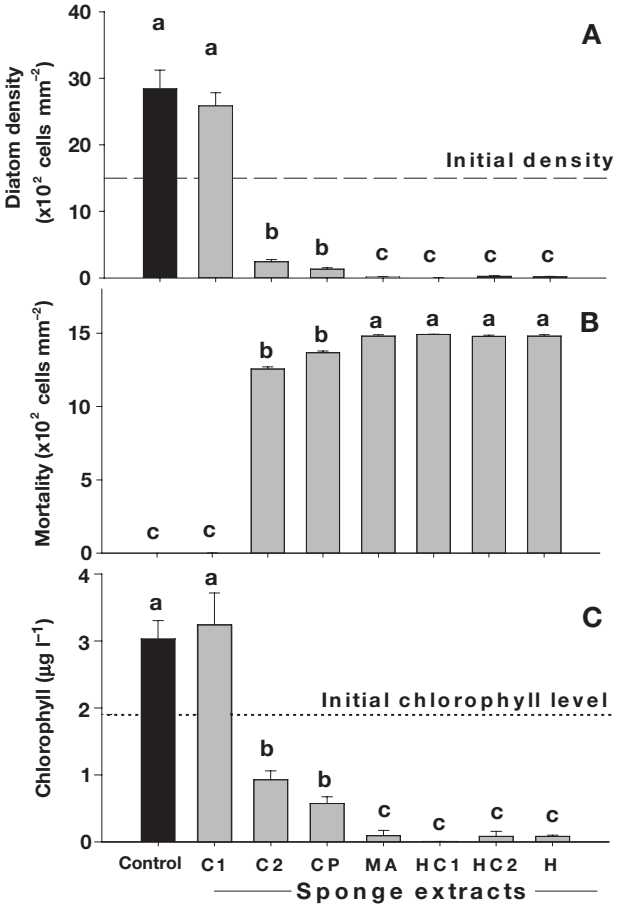

Fig. 1. Mean density (cells mm^{-2}) of live cells (A), dead diatoms (B) and the mean amount of chlorophyll *a* ($\mu g\ l^{-1}$) (C) of the benthic diatom *Nitzschia paleacea* after 2 d experiments with extracts of Hong Kong sponges. Extracts of sponges *Callyspongia* sp. 1 (C1), *Callyspongia* sp. 2 (C2), *Callyspongia pulvinata* (CP), *Mycale adhaerens* (MA), *Haliclona cymaeformis* var. 1 (HC1), *Haliclona cymaeformis* var. 2 (HC2), and *Halichondria* sp. (H) were tested at tissue-level concentrations. A 4% (v/v) solution of dimethylsulfoxide (DMSO) in seawater was used as a control (Control). Data are means ± SE of 5 replicates. Different letters above the bars indicate data that are significantly different in the LSD test ($p < 0.05$, 1-way ANOVA)

Antibacterial assay

All sponge extracts inhibited the growth of the bacterium *Vibrio harveyi* (inhibition zones from 0.8 to 1.0 mm). Out of 8 extracts only the extract of *Halichondria* sp. inhibited the growth of 4 species of bacteria, such as *V. harveyi*, *V. proteolyticus*, *V. alginolyticus*, *Pseudoalteromonas* sp. (inhibition zones from 0.9 to 4.6 mm), while the extract of *Haliclona cymaeformis* var. 2 inhibited the growth of 2 species of bacteria, such as *V. harveyi* and *V. proteolyticus* (inhibition zones from 0.8 to 1.0 mm). Sponge extracts had smaller antibiotic effects than the streptomycin control (Dunnett test, $p > 0.05$), except the growth inhibition of the bacterium *Pseudoalteromonas* sp. caused by the extract of *Halichondria* sp.

Field experiments

Experiments with gel-immobilized sponge tissue extracts

Epifluorescence microscope counts revealed that densities of bacteria on gel surfaces were significantly different among the treatments (Fig. 2, ANOVA: $F = 4.43$, $p < 0.0005$). All sponge extracts significantly decreased (LSD, $p < 0.05$) the density of bacteria on the gels. The recruitment of bacteria on the gels with extracts of *Mycale adhaerens*, *Callyspongia pulvinata*, *Haliclona cymaeformis* var. 2 and *Halichondria* sp. was more than 2 times less intensive than on the control (Fig. 2).

Bacterial community profile

TRFLP revealed that the extracts of *Haliclona cymaeformis* var. 1 had significantly lower (ANOVA: LSD, $p < 0.05$) numbers of TRFs (14) compared to the control (TRFs = 31). Communities developed on other gels had bacterial ribotype numbers similar to the control (TRFs = 29 to 37).

According to the Bray-Curtis similarity matrices based on the presence (indicated by 1) or absence (indicated by 0) of a given TRF in a pattern, the bacterial

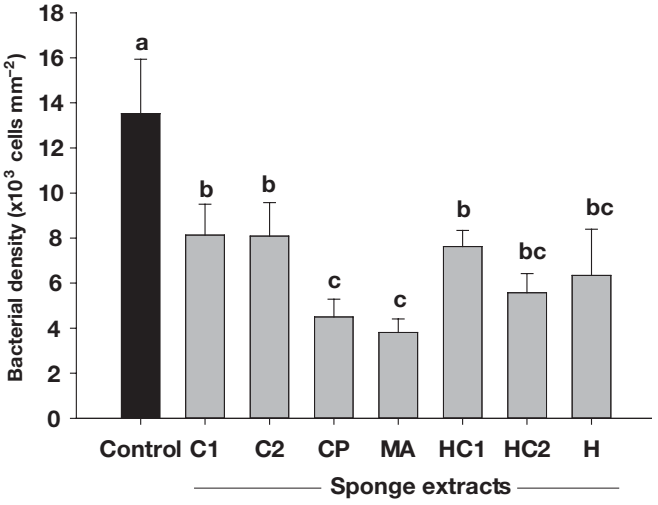

Fig. 2. Densities of bacteria developed on gel-immobilized sponge extracts at tissue-level concentration. Petri dishes were exposed to fouling for 48 h in 10 l aquaria continuously supplied with natural seawater from the University pier (depth 5 m) at a flow rate of 12 l h^{-1}. For the control (Control), a 4% (v/v) solution of DMSO in seawater was used. Data are means ± SE of 3 replicates. Different letters above the bars indicate data that are significantly different in the LSD test ($p < 0.05$, 1-way ANOVA). See Fig. 1 for definition of sponge extracts

communities were divided into several groups (Fig. 3). Bacterial communities developed on gels with sponge extracts were different from those developed on controls. Communities from the gels with the extract of *Haliclona cymaeformis* var. 1 had a low similarity (<40%) with the other bacterial communities, forming an independent cluster. Communities found on gels with the extracts of *Callyspongia* sp. 2 and *Halichondria* sp. were highly similar (for some replicates > 80% similarity), while extracts of the other sponges formed distantly related clusters in the dendrogram (Fig. 3).

Bacterial communities developed on control and experimental gels were all characterized by the absence of certain TRFs (i.e. ribotypes) and the presence of other TRFs (Fig. 4). For example, the presence of TRFs at 206 bp in the control and experimental bacterial communities indicated the presence of one or several bacterial species that were not affected by sponge metabolites. Bacterial communities on gels with the extracts of the sponges *Callyspongia* sp. 2 and *Haliclona cymaeformis* var. 1 were characterized by the absence of certain ribotypes found in the control (Fig. 4). At the same time, bacterial communities developed on gels with the extracts of *Callyspongia* sp. 1, *C. pulvinata*, *Haliclona cymaeformis* var. 2, *Halichondria* sp. and *Mycale adhaerens* had additional TRFs, which suggests that the growth of particular types of bacteria were effectively enhanced by sponge metabolites.

The TRFLP technique also provides preliminary information about relative abundances and identities (according to the TAP-TRFLP Java Applet program from the RPD II Website) of ribotypes within a bacterial community. In all treatments, the most abundant ribotype (TRFs of 206 bp length) were possibly represented by actinomycetes (mostly *Rhodococcus*, *Mycobacterium* and *Streptosporangium* spp.) as well as δ-Proteobacteria (mostly *Cystobacter* spp.). Extracts of the sponges *Mycale adhaerens*, *Haliclona cymaeformis* var. 1 and *H. cymaeformis* var. 2 allowed the growth of the second most common ribotype with a TRF length of 422 bp, which was probably *Pseudomonas* spp. (γ-Proteobacteria). In the gel with the *Callyspongia* sp. 2 extract the ribotype with a TRF length of 76 bp was probably *Fibrobacter* spp. (*Fibrobacter/Acidobacteria* group). At the same time, we did not find close matches with the RPD II Website database of TRFs of lengths of 37, 40 and 101 bp, which occur in microbial communities developed on gels with other sponge extracts. This suggests that ribotypes of bacteria developed on gels with sponge extracts have not yet been identified.

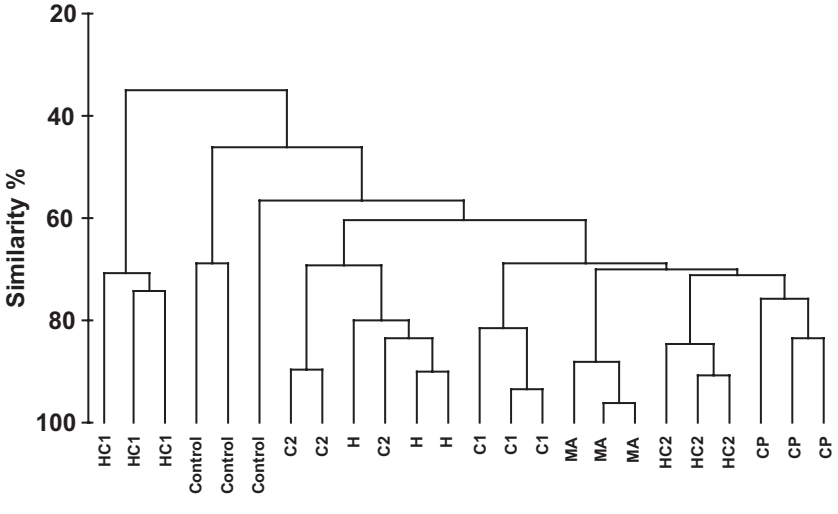

Fig. 3. Cluster analysis indicating similarity of bacterial communities developed on gel-immobilized sponge extracts (see Fig. 1 for definitions) and the control without extract (Control). There were 3 replicates in the experiment. The dendrogram was constructed by using similarity matrices of terminal restriction fragments of bacterial community DNA samples determined by the Bray-Curtis similarity coefficients

Density of bacteria and diatoms after 7 d

After 7 d of experiment, bacteria and diatoms, but not invertebrates or macroalgae, were found on control and treatment gels. Bacteria were predominantly rod- and cocci-shaped. The common diatoms belonged to the genera *Achnanthes*, *Nitzschia*, *Navicula*, *Licmophora*, *Synedra* and *Diploneis*.

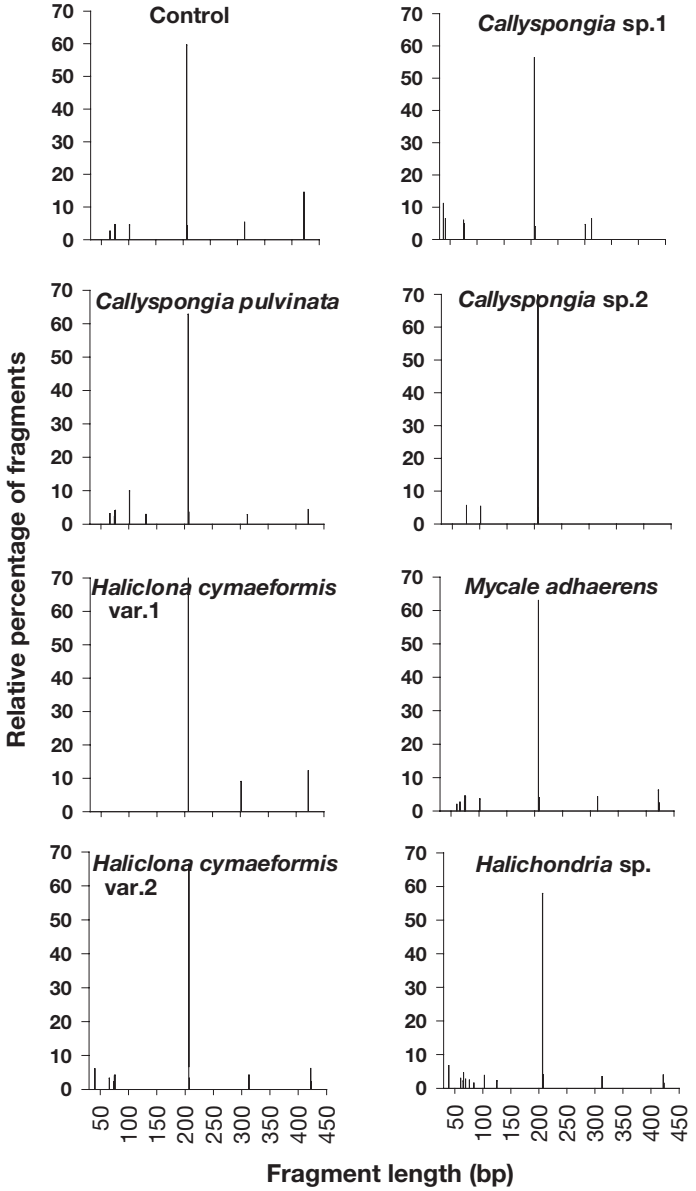

Fig. 4. Representative bacterial terminal restriction fragment (TRF) profiles derived from *Msp*I digestion of PCR-amplified bacterial community 16S rDNA obtained from gel-immobilized extracts of the 7 sponges and the control without extract (Control). The bar positions represent fragment lengths occurring consistently in each of 3 replicates, while the bar heights represent mean relative abundances of TRFs among the 3 replicates

The mean densities of bacteria differed among the control and experimental gels (Fig. 5A, ANOVA: $F = 3.325$, $p < 0.004$). The density of bacteria on gels with the extracts of *Haliclona cymaeformis* var. 1, *Mycale adhaerens* and *Callyspongia pulvinata* was lower (LSD; $p < 0.05$) than in the control. The density of bacteria on gels with the extracts of *Callyspongia* sp. 1, *Callyspongia* sp. 2, *H. cymaeformis* var. 2 and *Halichondria* sp. was not significantly different (LSD; $p > 0.05$) from the control.

The mean densities of diatoms on the gels were different (Fig. 5B, ANOVA: $F = 9.931$, $p < 0.000001$). Diatom densities on the gels with all sponge extracts except *Callyspongia* sp. 2 were significantly lower than that on the control gel (LSD test, $p < 0.05$). The extract of *Haliclona cymaeformis* var. 1 had the lowest density of diatoms.

DISCUSSION

Marine sponges contain chemical compounds that have shown antibacterial (Newbold et al. 1999) and antialgal (Faimali et al. 2003, Dobretsov et al. 2004) activity in laboratory bioassays. The present study combined laboratory and field experiments to test whether secondary metabolites from 7 sponges (*Callyspongia* sp. 1, *Callyspongia* sp. 2, *C. pulvinata*, *Mycale adhaerens*, *Haliclona cymaeformis* var. 1, *H. cymaeformis* var. 2 and *Halichondria* sp.) dominant in Hong Kong waters could suppress marine microfouling or affect the microbial species composition of biofilms.

The laboratory experiments demonstrated that all sponge extracts except *Callyspongia* sp. 1 inhibited growth and caused mortality of the pennate diatom *Nitzschia paleacea*. After 7 d in the field, all sponge extracts except for *Callyspongia* sp. 2 effectively suppressed the growth of the benthic diatoms *Achnanthes* spp., *Nitzschia* spp., *Navicula* spp., *Licmophora* spp., *Synedra* spp., and *Diploneis* spp. According to our personal observations, sponges in Hong Kong waters do not appear to have substantial diatom fouling on their surface. These findings suggest that sponges can control the growth of diatoms by chemical compounds.

One possible reason for the inhibition of diatom growth in our experiments is the toxicity of sponge compounds (Beccerro et al. 1997, Lee & Qian 2003, Dobretsov et al. 2004). It has been demonstrated that spherous cells of the encrusting sponge *Crambe crambe* produced toxic guanidine alkaloids, which seem to have multiple functions in nature, such as antifouling, antipredation, and allelopathic functions in interspecies space competition (Beccerro et al. 1997). Substances exuded by the sponge *Aplysina fistularis* were toxic to nudibranch veliger larvae and caused

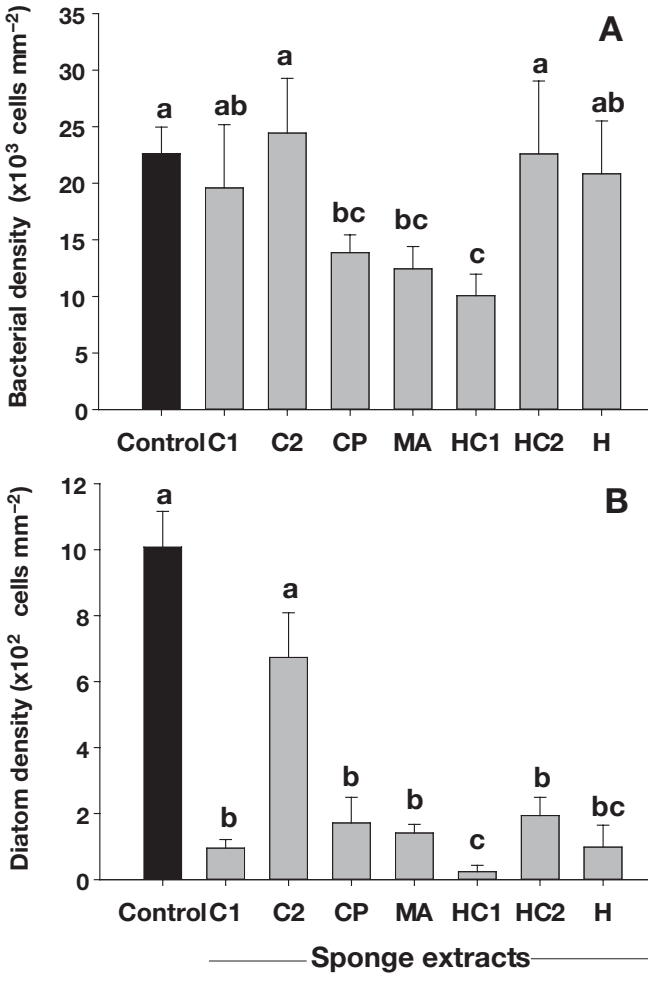

Fig. 5. Mean densities of (A) bacteria and (B) diatoms developed on gel-immobilized sponge extracts (see Fig. 1 for definitions) and the control without extract (Control) after 7 d of field exposure. Extracts were tested at tissue-level concentrations. Data are means ± SE of 4 replicates. Different letters above the bars indicate data that are significantly different according to an LSD test ($p < 0.05$, 1-way ANOVA)

abnormal behavior in several of the adult invertebrates tested by Thompson et al. (1985). The sponges *Mycale adhaerens* (Lee & Qian 2003) and *Callyspongia* (*Euplacella*) *pulvinata* (Dobretsov et al. 2004) inhibited larval settlement of the tubeworm *Hydroides elegans* by excreting toxic waterborne compounds.

In the laboratory disk-diffusion experiments, we demonstrated that most of the sponge extracts had no or only weak inhibitive effects on the growth of 11 bacterial species originally isolated from a natural biofilm. Only the growth of one pathogenic bacterium, *Vibrio harveyi* (Lui et al. 1996, Soto-Rodriguez et al. 2003), was inhibited by all sponge extracts. After the 2 d running seawater experiment, all sponge extracts inhibited the number of bacteria settling on agar matrices (Fig. 2), while after 7 d in the sea, only extracts of *Mycale adhaerens*, *Haliclona cymaeformis* var. 1 and *Callyspongia pulvinata* had low bacterial recruitment (Fig. 5A). This may suggest that the concentration of sponge-derived chemicals in the gels was low compared to what was found on the surface of these sponges or that most sponge compounds were washed out from the gels after 7 d. Results of the field experiments contradict results of the disk-diffusion bioassay which indicated that extracts of *Haliclona cymaeformis* var. 1, *M. adhaerens* and *C. pulvinata* did not inhibit the growth of most of the bacterial isolates at tissue-level concentrations. Possible reasons for this phenomenon could be that bacteria may be affected by sponge metabolites at any phase of the colonization process by a number of different mechanisms. The measurement of inhibition zones in the disk-diffusion bioassay, indicating bacteriological or bacteriostatic effects, reflects only 1 mechanism (inhibition of bacterial growth) by which bacterial abundance can be affected. In field experiments, sponge extracts may affect bacterial colonization by several mechanisms including inhibition of growth, chemotaxis (repulsion), cell motility (spreading) and production of external polymers (attachment). It has been shown that antibacterial activities of compounds are not necessarily correlated with the prevention of bacterial attachment (Wahl et al. 1994, Steinberg et al. 1997, Kelly et al. 2003). For example, Wahl et al. (1994) found that there was no correlation between bacterial abundance on Pacific ascidian surfaces and the antibiotic activity of extracts, while there was a correlation between bacterial abundance and effects of extracts on attachment. Analogously, crude extracts of the Caribbean sponges *Ailochroia crassa*, *Chondrilla nuculata*, *Ectyoplasia ferox* and *Iotrochota birotulata* inhibited the attachment of the bacterium *Vibrio harveyi* but did not inhibit bacterial growth (Newbold et al. 1999, Kelly et al. 2003).

In the present investigation, the sponges *Haliclona cymaeformis* var. 1 and var. 2 were collected from different locations in Hong Kong waters (Wong Shek Pier and Port Shelter, respectively). Laboratory and field experiments with the extracts of these species affected microbial communities differently. For example, a low number of bacterial ribotypes were found in communities formed on the gels with the metabolites from *H. cymaeformis* var. 1 compared to the communities affected by *H. cymaeformis* var. 2. Moreover, after 7 d in the sea, bacterial and diatom densities on gel surfaces with the extracts of *H. cymaeformis* var. 1 were lower than those with *H. cymaeformis* var. 2. These results could be explained by several hypothe-

ses. First, intraspecific differences between *H. cymaeformis* var. 1 and var. 2 may cause a difference in the production of secondary metabolites. Second, at different locations sponges may have a different composition of microbial symbionts, which are known to be responsible for the production of many chemical compounds isolated form sponges (cf. Faulkner 2000, Fusetani 2003). A third possibility could be that different environmental conditions may cause differences in the metabolic activity of the sponges, e.g. the concentrations of the feeding-repellent sceptrin were different in the sponge *Agelas conifera* from different locations of the Caribbean (Assmann et al. 2000). However, the effect of environmental conditions on the production of antifouling compounds by the sponge *H. cymaeformis* needs to be investigated in the future.

Bacterial communities developed on control and experimental gels were different from each other and are characterized by the absence of certain TRFs (i.e. ribotypes) and the presence of others (Fig. 4). This suggests that sponge metabolites play an important role in controlling surface bacterial populations. The recruitment of some bacterial strains may be suppressed by sponge metabolites, whereas the settlement and growth of other bacterial strains may be mediated. For example, the absence of certain ribotypes (e.g. 66 bp, 312 bp) in bacterial communities exposed to extracts of the sponges *Callyspongia* sp. 2 and *Haliclona cymaeformis* var. 1 indicated that corresponding species of bacteria were effectively inhibited. Bacterial communities developed on gels with the extracts of *Callyspongia* sp. 1, *C. pulvinata*, *H. cymaeformis* var. 2, *Halichondria* sp. and *Mycale adhaerens* had additional TRFs (e.g. 37 bp, 422 bp), which suggests that some extract components were used by certain bacteria and thus enhanced attachment/growth of these bacteria. Cross-referencing these TRF data with the ribosomal sequence database (RPD II) provided some information on the possible identity of those ribotypes found in the community profiles, although the database may not capture the full profile of the marine microbial environment.

Several previous investigations have shown that sponge extracts selectively inhibited the recruitment and growth of bacteria (Newbold et al. 1999, Kelly et al. 2003, Lee & Qian 2004). Extracts of the sponges *Pachychalina* sp., *Acanthella cavernosa* and *Xestospongia testudinaria* changed bacterial community profiles on the hydrogel surface, suggesting that the settlement of some bacteria was prevented in running seawater experiments (Harder et al. 2004). Sponge chemical and mucus production leads to the formation of sponge-specific microbiota, which is different from microbial communities associated with nearby inanimate substrata (Burja & Hill 2001, Webster & Hill 2001, Hentschel et al. 2002, Lee & Qian 2004). The sponges *Aplysina aerophoba* and *Theonella swinhoei* from the Mediterranean Sea, Red Sea and Pacific Ocean had a uniform microbial community which is phylogenetically different from that of marine plankton or marine sediments (Hentschel et al. 2002). Similarly, bacterial communities on the surface of the sponge *Mycale adhaerens* were different from the communities developed on surfaces of Petri dishes exposed to fouling in the sponge habitat (Lee & Qian 2004).

In the present study, inhibition of diatom growth in laboratory experiments corresponded in most cases with the suppression of diatom growth in the field experiments. There was no correspondence between the results of disk-diffusion bioassays and of the recruitment of bacteria on gel surfaces in the field experiments. This raises questions about the ecological relevance of results obtained through laboratory experiments. The activities of natural antibiotics and microbial repellents are conventionally investigated by the standard agar disk-diffusion assay (Acar 1980) or by the chemotaxis bioassays in laboratory experiments (Wahl et al. 1994, Boyd et al. 1999). In these assays, secondary metabolites are affecting selected bacterial strains grown on nutrient-rich media and exposed to certain concentrations of metabolites, while in the field, sponge-derived metabolites affect both culturable and unculturable bacteria at densities at which they occur in the natural environment. The choice of microbial test organisms introduces biases and uncertainty to the assays applied (Eilers et al. 2000). In the present investigation we exposed sponge compounds to the pool of naturally occurring bacterial and diatom communities under flow conditions and studied bacterial composition by culture-independent methods. However, the conceptual design of the immobilization of extracts at tissue level concentration rests on the assumption that host metabolites are evenly distributed throughout the host tissue (Jensen et al. 1996). Since this assumption will only in rare cases correspond with the real situation, our attempts do not realistically reflect ecologically relevant concentrations on experimental substrata at this moment.

In conclusion, our experiments demonstrated that chemical compounds of sponges can control diatom and bacterial fouling in the sea, probably by the suppression of growth, attachment and/or spreading of some bacteria and diatom species and enhancing the growth of others. The results of the present study offers a useful survey of the capacity of Hong Kong sponge extracts to suppress bacterial and diatom fouling in laboratory and field experiments. Such a survey will be used as a basis for subsequent bioassay-guided isolation of active compounds in our future experiments.

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