Antifouling activity of symbiotic bacteria from sponge Aplysina gerardogreeni

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Abstract

A key area in marine antifoulant research is the discovery of new environmentally friendly solutions that prevent biofilm formation and associated biocorrosion. Taking into consideration the natural mechanisms of marine organisms to protect against epibiosis, new biomimetic solutions can be utilised against biofouling, and marine bacteria are promising agents. Therefore, the goal of this study was to identify cultivable bacteria with antifouling (AF) activity associated with the sponge *Aplysina gerardogreeni*. A collection of 63 bacteria was isolated, and the organic extracts were assayed against various microfouler strains (16 bacteria and five microalgae). The results showed that 87% of bacterial extracts were active against the microfoulers tested. Sixteen of them can be considered to possess AF potential and belong to the genera *Bacillus, Micrococcus, Paracoccus, Pseudobacter, Pseudovibrio, Psychrobacter, Staphylocuccus* and *Terribacillus*. Bioactivity showed temporal variations; the highest activity was in February and June and the lowest in October. *Bacillus* bacteria were dominant and showed AF activities throughout the year. The results revealed those marine bacteria sponge-associated and the genus *Bacillus* in particular, are promising AF agents.

1. Introduction

Biofouling is the undesirable colonisation of man-made surfaces by microorganisms, macroalgae and invertebrates, leading to their subsequent biodeterioration. The bacterial colonisation of marine surfaces and subsequent macrofouling is a ubiquitous phenomenon (Harder, 2009) that causes enormous damage to marine structures built for commercial purposes (Dexter et al., 1993) and affects surfaces such as ship's hulls, building materials and water intake systems, due to premature corrosion (Hellio and Yebra, 2009), as well as decreased fuel efficiency and the introduction of new species into other ecosystems (Yebra et al., 2004).

The key in marine antifoulant research is to identify compounds that show antimicrobial activity and prevent formation of biofilms on ship hulls or installations such as oil rigs. Marine sponges offer a great potential in the search for antifoulant compounds, because several have shown AF activity (e.g., Hattori et al., 1998, Fusetani, 2004, Ortlepp et al., 2007, Qiu et al., 2008, Limna Mol et al., 2009, Hertiani et al., 2010, Blihoghe et al., 2011, Dobretsov et al., 2011, Wright et al., 2011, Wu et al., 2012, Xu et al., 2012 and Santos-

Acevedo et al., 2013) and others can modulate biofilm formation in a non-microbiocidal manner (Stowe et al., 2011). However, abundant microorganisms are associated with sponges and either reside on the sponge surface as epibionts or within the canal system as endobionts (Hentschel et al., 2003, Usher et al., 2004, Erwin et al., 2011, Vasundhara et al., 2012, Satheesh et al., 2012 and Papaleo et al., 2012). These organisms can constitute up to 60% of the total volume of sponge and have a large influence on the host metabolism and nutrient recycling (Wilkinson, 1980). The fact that sponge-associated bacteria produce bioactive metabolites, which were originally isolated from sponges, supports the hypothesis that some of the compounds formerly ascribed to sponges have a microbial origin (Stierle et al., 1988, Shigemori et al., 1992 and Oclarit et al., 1994).

Although marine microbes are a promising source of antifoulant compounds, only a small number of marine bacteria have been screened to date and few compounds have been successfully extracted (Qian et al., 2007). Several benefits are associated with the use of microorganisms as a source of compounds. The first advantage is that bacteria can be grown easily and can produce a large amount of compounds in a short time, compared with invertebrates and algae. In addition, strains of bacteria of the same species can produce different bioactive compounds under different culture conditions and therefore increase the number of potentially useful compounds (Armstrong et al., 2000). However, bacteria of marine origin constitute a potential resource of novel bioactive substances for other reasons; the evolution of marine life forms has involved exposure to environmental conditions different from that of their terrestrial counterparts and they have thus evolved novel molecules or compounds with increased activity (Nair and Simidu, 1987 and Barbier and Prieur, 1991).

In recent years, considerable attention has focused on the study of microorganisms, especially of marine bacteria capable of producing antifouling metabolites in association with marine invertebrates (Holmström and Kjelleberg, 1999, De Rosa et al., 2000, Egan et al., 2000 and Satheesh et al., 2012). Sponges of the genus *Aplysina* are characterised by a high abundance of associated bacteria (Friedrich et al., 1999, Friedrich et al., 2001, Hentschel et al., 2001 and Hentschel et al., 2006). For this reason, the aim was to probe the antifouling potential of bacteria isolated from the sponge, *Aplysina gerardogreeni*, from the Gulf of California in different seasons, to determinate whether bacteria possess antifouling activity and whether this activity was associated with specific seasons.

2. Materials and methods

2.1. Collection of sponges and isolation of bacteria

Aplysina gerardogreeni was collected bimonthly by scuba diving in Punta Arena, Baja California Sur, Mexico (24°03'40"N and 109°49'52"W). Sponge specimens were placed into sterile plastic bags, cooled on ice and transported immediately to the laboratory.

Under sterile conditions, 5 g sponge tissue was excised, was briefly washed in 70% ethanol and immediately immersed in sterilised and filtered seawater (SW) and ground using a mortar (Webster and Hill, 2001). The homogenate was diluted in sterile water, 100 µL of the mixture were plated onto tryptic soy agar (TSA) supplemented with 2.7% NaCl and incubated at 35 °C for 24 h. One representative of each morphotype was transferred to new plate for isolation.

Biochemical tests were performed in accordance with Barrow and Feltham (1993), using a pure strain inoculate of 24 h for each test: 1) aerobic/anaerobic growth, 2) oxidase activity, 3) catalase activity, 4) acid and/or gas production from carbohydrates, 5) oxidation of glucose, 6) methyl red, and 7) Voges-Proskauer.

2.2. Bacterial extracts

The bacterial isolates were grown in 100 mL tryptic soy broth (TSB) culture medium supplemented with 2.7% NaCl and incubated at 35 °C for 5 or 6 d to increase strain biomass. The extraction was performed at room temperature, using a 100 mL mixture of hexane and ethyl acetate (6:4 v/v) shaken constantly for 1 h. The organic phase was evaporated to dryness on a rotary evaporator at 37 °C to obtain bacterial extracts.

2.3. Antifouling assay

Stock solutions of bacterial extracts were prepared in methanol (as a carrier solvent) with final concentrations of 0.01, 0.1, 1, 10 and 50 µg mL^{-1}. An aliquot (100 µL) of these solutions was pipetted into 96-well plates. After methanol evaporation, the plates were sterilised in a UV sterilisation cabinet (Scie-plas G/E UVSC) for 30 min before being aseptically removed and inoculated with microorganisms and sealed. The qualitative assays of each concentration were run with six replicates. The bacterial extracts were considered active if four of six wells showed inhibition. Culture medium with the extract was used as positive control and wells free from extracts (only evaporated methanol) were inoculated with microorganisms suspension as a negative control (Chambers et al., 2011).

2.3.1. Antibacterial bioassay

Antibacterial assays were performed against a range of bacteria involved in biofouling (Plouguerné et al., 2008, Thabard et al., 2009, Plouguerné et al., 2010 and Chambers et al., 2011). These bacteria have wide

distribution and are reference strains from American Type Culture Collection (ATCC*): Bacillus cereus* (ATCC 14579), *Bacillus sphaericus* (ATCC 14577), *Bacillus subtilis* (ATCC 23857), *Bacillus laterosporus* (ATCC 64), *Polaribacter irgensii* (ATCC 700398), *Proteus vulgaris* (ATCC 1457), *Pseudoalteromonas elyakovii* (ATCC 700519), *Roseobacter litoralis* (ATCC 49566), *Shewanella putrefaciens* (ATCC 8071), *Vibrio aesturianus* (ATCC 35048), *Vibrio carcharie* (ATCC 35084), *Vibrio harveyi* (ATCC 14126), *Vibrio natriegens* (ATCC 33788), *Vibrio parahaemolyticus* (ATCC 17802) and *Vibrio proteolyticus* (ATCC 15338). Marine bacteria were cultivated in sterile SW enriched with 0.5% (w/v) neutralised bacteriological peptone (LP0034 Oxoid), and terrestrial bacteria were maintained in sterile deionised water enriched with 2.5% nutrient broth (CM0067 Oxoid).

The bacterial cultures were diluted according to the Amsterdam method (Amsterdam, 1996) to give a cell density of 2 × 108 cells mL^{-1}. Microplates that were coated with extracts were inoculated with 100 µL bacterial cell suspension and incubated at 20 °C for 48 h. After incubation, all the wells were compared visually with the control; a clear well indicated growth inhibition (Chambers et al., 2011).

2.3.2. Antimicroalgal bioassay

Five microphytobenthic strains provided by Algobank-Caen were studied: *Cylindrotheca closterium* AC170 (Ehrenberg) Reimann & Lewin (Bacillariophyta, Bacillariophyceae, Bacillariales, Bacillariaceae), *Lotharella globosa* AC132 Ishida & Hara (Chlorarachniophyta, Chlorarachniophyceae, Chlorarachniales, Chlorarachniaceae), *Exanthemachrysis gayraliae* AC15 Lepailleur (Haptophyta, Pavlovophyceae, Pavlovales, Pavlovaceae), *Halamphora coffeaeformis* AC713 (Agardh) Levkov (Bacillariophyta, Bacillariophyceae, Naviculales, Amphipleuraceae) and *Pleurochrysis roscoffensis* AC32 (Dangeard) Fresnel & Billard (Haptophyta, Prymnesiophyceae, Coccolithales, Pleurochrysidaceae). These strains were chosen as they are important microfoulers (Chambers et al., 2011; Bressy et al., 2010, Gastineau et al., 2012 and Jellali et al., 2013).

All strains were cultivated using f/2 media (Guillard and Ryther, 1962) and were maintained at 20 °C and constant light (incident irradiance: 140 µmol m^{-2} s^{-1}). The biomass of microalgal cultures was estimated from Chlorophyll a (Chl *a*) concentration measurements following the method described by Id-Daoud et al. (submitted). Five mL of microalgae culture was collected on a GF/F filter (Whatman) and immediately transferred to a vial containing 5 mL analytical grade methanol. The vial was kept in the dark at 4 °C for 30 min. The fluorescence of the pigment extract was measured (PolarSTAR Optima BMG Labtech, excitation: 485 nm, emission: 645 nm) and the concentration of Chl *a* determined using a calibration curve constructed using spinach Chl *a* (C5753 Sigma, extinction coefficient at 663 nm in methanol: 77 l g^{-1} cm^{-1}) solutions containing a GF/F filter that had been used to filter 5 mL sterile SW. For each species, dilutions were made to prepare aliquots of microalgal suspension with a starting concentration of 0.1 mg Chl *a* L^{-1} and 100 µL

were added to each well of previously coated microplates, under aseptic conditions. Inoculated microplates were incubated for 5 d at 20 °C in constant light (incident irradiance: 140 µmol m^{-2} s^{-1}) and all wells were compared visually with the controls; a clear well indicated growth inhibition (Chambers et al., 2011).

2.4. Bacterial identification

Bacterial strains that showed the greatest activity were identified by partial sequencing of 16S rRNA gene fragments. The DNA was obtained using a method modified from Sambrook and Russell (2001) and Ausubel et al. (2002). The PCR amplicons were purified and DNA was sequenced by MACROGEN in Korea. Searches of the Genbank database were performed using the BLAST program at the National Center for Biotechnology Information website (http://www.ncbi.nlm.nih.gov) and sequencing data were analysed by comparing the sequences of nearest relatives found by BLAST searching.

3. Results

3.1. Bacterial identification and biochemical characteristics

The DNA analysis showed that the bacteria associated with the sponge were related to *Bacillus*, *Micrococcus, Paracoccus, Pseudobacter, Pseudovibrio, Psychrobacter, Staphylocuccus* and *Terribacillus* genera and their biochemical characteristics are shown in Table 1. Of the bacteria, 50% showed growth under anaerobic conditions, 100% were positive for the catalase test and 63% for the oxidase test. The tested assimilation of carbohydrates revealed that the majority metabolised glucose (88%). An oxidative metabolism was shown by 75% bacteria, fermentation by 12.5% and the remaining 12.5% had the ability to both oxidise and ferment.

Table 1.
Biochemical characteristics of the bacterial strains isolated from the sponge *Aplysina gerardogreeni* with antifouling potential and their phylogenetically closely related species from GenBank according to 16S RNA gene sequence similarity.

ID	Close relative in GenBank	% Identity	Gram	AG	O	C	O/F	G	MR	VP
Ap06145	*Bacillus cibi*FJ607434	99	+	–	+	+	O	+	+	+
Ap0233	*Bacillus flexus*JQ818414	99	+	+	+	+	O	+	+	+
Ap0630	*Bacillus licheniformis*GU723480	99	+	+	+	+	O	+	+	+
Ap0694	*Bacillus megaterium*JN990602	99	+	+	+	+	O	+	–	+
Ap04121	*Bacillus pumilus*JX120507	98	+	–	–	+	O	+	–	+
Ap06137	*Bacillus sonorensis*JQ665284	99	+	+	+	+	O	+	+	+
Ap0475	*Bacillus* sp. AY433825	99	+	+	+	+	O	+	+	+
Ap04103	*Bacillus subtilis*JF501099	100	+	–	+	+	O	+	+	+
Ap0637	Marine bacterium A4950194	99	+	+	+	+	O	+	+	+
Ap0471	*Micrococcus luteus*JQ581526	100	+	–	–	+	O	–	–	+
Ap0624	*Paracoccus* sp. JQ691539	99	–	–	+	+	O	+	+	–
Ap04168	*Pseudovibrio* sp. JX436425	99	+	+	–	+	O/F	+	+	+
Ap0492	*Psychrobacter maritimus*HQ538762	98	–	+	+	+	O/F	–	+	–
Ap041	*Staphylococcus arlettae*FJ386956	100	+	–	–	+	F	+	+	–
Ap06116	*Staphylococcus* sp. JN615429	99	+	–	–	+	F	+	+	–
Ap0846	*Terribacillus goriensis* DQ519571.1	100	+	–	–	+	O	+	+	–

AG Anaerobic growth, O Oxidase, C Catalase, O/F Oxidation/Fermentation, G Glucose, MR Metil Red, VP Voges-Proskauer.

3.2. Antibacterial bioassay

In this study, 63 strains were isolated from *A. gerardogreeni* and the bioassay showed that 87% of sponge-associated bacteria were active, but only sixteen could be considered to have a good antifouling potential. The most active extracts against the marine bacterial strain showed activity against at least one marine bacterial strain with minimum inhibitory concentration (MIC) ranging from 0.01 to 0.1 μg mL^{-1} (Table 2). The extracts of *Bacillus licheniformis* and *B. subtilis* showed the broadest spectrum of activity, inhibiting seven out of 15 strains of bacteria at low concentrations (0.01–10 μg mL^{-1}) in the assay. Extracts from *Bacillus flexus*, *Terribacillus goriensis* and non-identified marine bacteria, were only active against marine bacteria but not against microalgae. None of the extracts was active against *Bacillus lateresporus*, *S. putrefaciens, P. irgensii, P. vulgaris, P. elyakovii, V. natriegens, V. proteolyticus* and *V. carcharie*. We observed *B. subtilis* was most sensitive test strain, as it was inhibited by 69% of the extracts, mostly at a low MIC (0.01 μg mL^{-1}) (Fig. 1A).

Table 2.
Determination of Minimum Inhibitory Concentration (MIC µg mL^{-1}) for bacteria isolated from *A. gerardogreeni* with the best antibacterial potential.

Bacteria isolated Aplysina	B1	B2	B3	B4	B5	B6	B7
Bacillus cibi	0.01		0.1	50	–	–	–
Bacillus flexus	0.01		0.01		–	–	1
Bacillus licheniformis	0.01	1	0.01	10	0.1	1	1
Bacillus megaterium	0.01	–	–	0.01	–	0.01	–
Bacillus pumilus	0.01	0.01	0.1	0.1	50	10	0.01
Bacillus sp.	0.01	50	10	–	–	–	10
Bacillus subtilis	0.01	1	0.01	10	0.1	0.1	1
Marine bacterium	0.01	–	0.01	10	–	–	–
Micrococcus luteus	50	–	0.1	–	10	10	–
Paracoccus sp.	0.01	–	–	–	–	–	–
Pseuodovibrio sp.	0.01	–	0.1	–	–	–	–
Psychrobacter maritimus	0.01	50	0.01	–	0.01	10	10
Staphylococcus arlettae	0.01	–	–	–	–	–	–
Terribacillus goriensis	–	0.01	10	–	–	–	–

B1 Bacillus subtilis, B2 B. sphaericus, B3 B. cereus, B4 Vibrio aestuarianus, B5 V. harveyi, B6 V. parahemolyticus, B7 Roseobacter litoralis, – No activity.

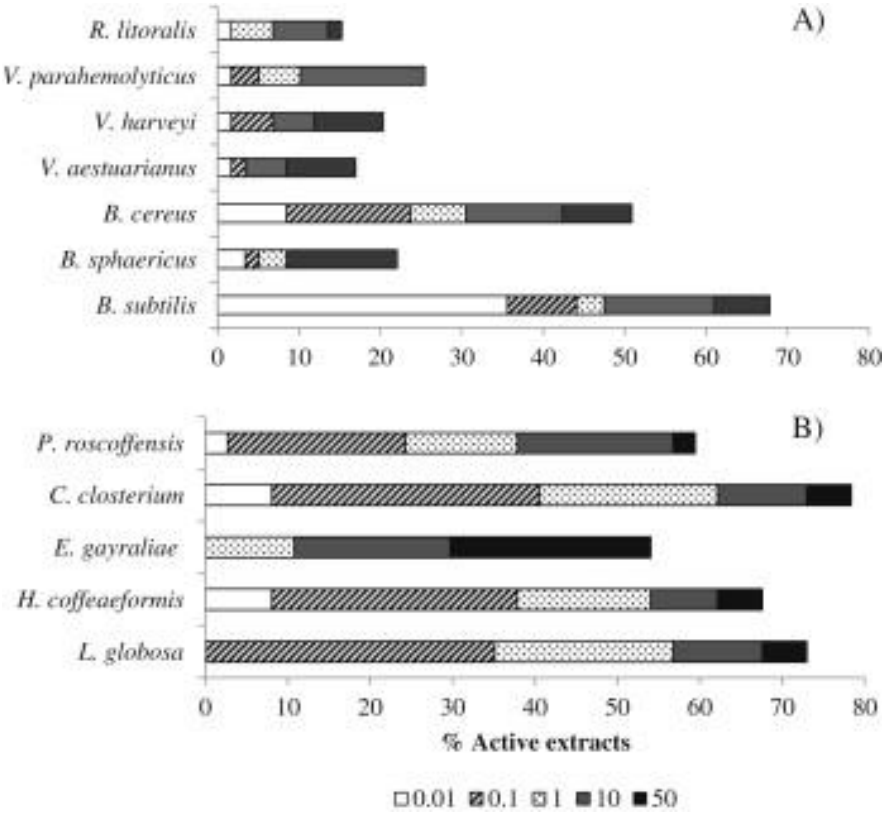

Fig. 1.
Percentage of extracts showed activity at different concentrations against: A) marine bacteria B) microalgae (□ 0.01 µg mL^{-1}, 0.1 µg mL^{-1}, 1 µg mL^{-1}, 10 µg mL^{-1}, ■ 50 µg mL^{-1}).

3.3. Antimicroalgal bioassay

In the bioassay against microalgae, the extracts of *Bacillus pumilus* and *B. subtilis* showed the best activity, with a MIC between 0.01 and 1 μg mL^{-1} and the extracts from *Bacillus sonorensis, Psychrobacter* sp. and *Staphylococcus* sp. were only active against microalgae (Table 3). The most sensitive strain was *Cylindrotheca closterium*; 80% of extracts were able to inhibit their growth, mostly at concentrations between 0.1 and 1 μg mL^{-1} (Fig. 1B).

Table 3.
Determination of Minimum Inhibitory Concentration (MIC μg mL^{-1}) for bacteria isolated from *A. gerardogreeni* with the best antimicroalgal potential.

Bacteria isolated *Aplysina*	M1	M2	M3	M4	M5
Bacillus cibi	0.1	50	50	0.01	10
Bacillus licheniformis	0.1	0.1	10	0.01	0.1
Bacillus megaterium	0.1	0.1	10	–	–
Bacillus pumilus	1	0.01	1	0.01	0.1
Bacillus sonorensis	10	0.1	50	0.1	0.1
Bacillus sp.	10	0.1	50	1	–
Bacillus subtis	0.1	0.01	1	0.1	0.01
Micrococcus luteus	0.1	0.01	–	0.1	10
Paracoccus sp.	10	–	50	1	0.1
Pseudovibrio sp.	–	–	1	0.1	0.1
Psychrobacter maritimus	1	0.1	10	0.1	1
Psychrobacter sp.	0.1	–	1	–	0.1
Staphylococcus arlettae	1	1	–	10	–
Staphylococcus sp.	1	1	–	50	1

M1 *Lotharella globosa*, M2 *Pleurochrysis roscoffensis*, M3, *Exanthemachrysis gayraliae* M4 *Cylindrotheca closterium*, M5 *Halamphora coffeaeformis*, – No activity.

3.4. Temporal variation in antifouling activity

The screening for antifouling activity over one year demonstrated that active extracts showed temporal variations. June and February provided the largest percentages of extracts active against marine bacteria (23 and 22% respectively), and October the smallest (5%) (Fig. 2A). The temporal variation of activity against microalgae was very similar to that marine bacteria; the largest number of active extracts from microalgae was obtained in February and June (35 and 32% respectively), whereas in October and December, no extracts were found active (Fig. 2B).

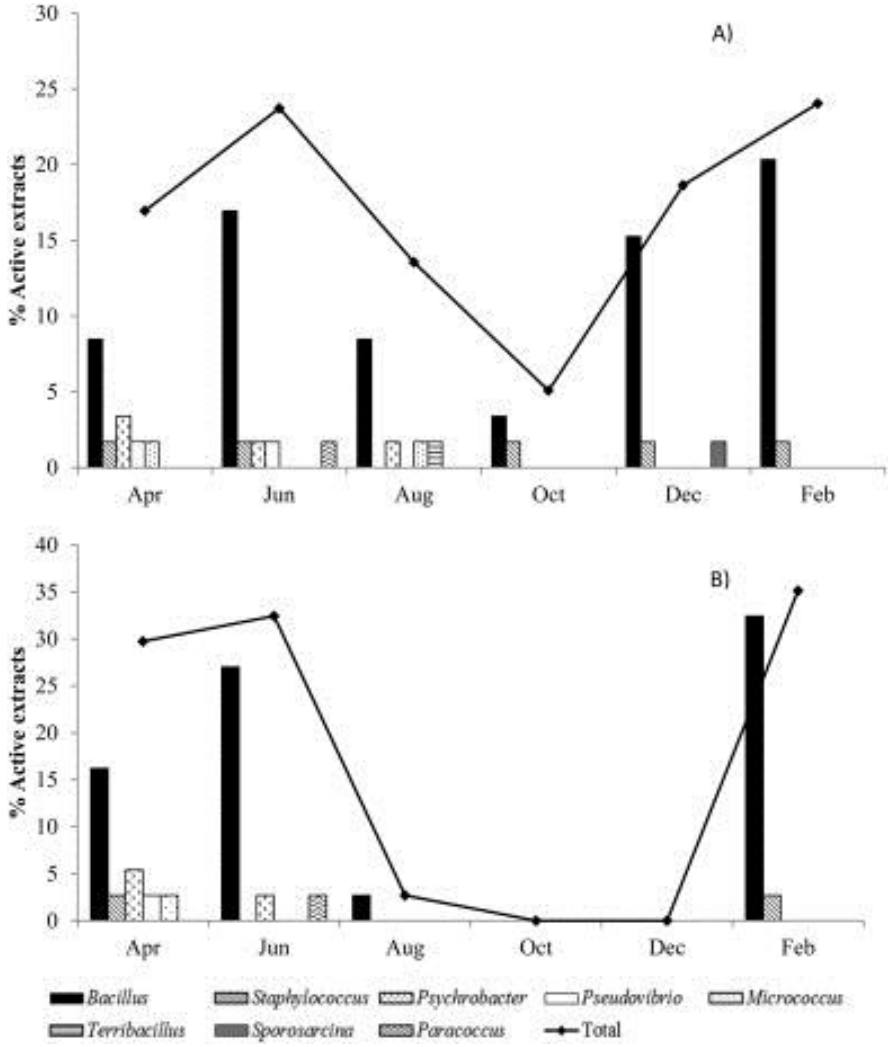

Fig. 2.
Temporal variation of the percentage of active extract (line: total, bars: by genus) in marine antifouling tests against: A) bacteria and B) microalgae.

Antifouling Bacillus strains were dominant and their extracts were active against bacteria throughout the year and against microalgae only in four months. In both cases the highest activity was in February (Fig. 2A and B). *Staphyloccocus* extracts showed antibacterial activity in all months except for August, whereas *Pseudovibrio* and *Micrococcus* strains were detected and produced active extracts in two months. *Sporosarcina, Paracoccus* and *Terribacillus* were isolated in only a single month and their extracts were active (Fig. 2A). With the exception of Bacillus, the bacteria that produced extracts active against microalgae were very specific and only showed activity at a particular time. *Staphylococcus* was active only in April and February, *Psychrobacter* in April and June. And the other extracts were active only in one month (Fig. 2B).

4. Discussion

The present study indicates that marine bacteria associated with the sponge *A. gerardogreeni*, exhibit inhibitory activity against two important groups of organisms involved in microfouling (bacteria and microalgae). The major number of these culturable bacteria identified by partial sequencing of the 16S rDNA gene, were phyllogenetically closer to the order Firmicutes.

Biochemical test showed that the microbial isolates were versatile chemo-heterotrophs, aerobes or facultative anaerobes, with a fermentative or respiratory metabolism. They were capable of fermenting carbohydrates, and most were catalase positive. These metabolic capabilities reflected their colonisation of the diverse habitats offered by the host (Gunn and Colwell, 1983, Logan and Berkeley, 1984 and Holt et al., 2000).

There is growing evidence that microorganisms associated with sponges are a source of bioactive metabolites (Fenical, 1993, Unson et al., 1994, Hentschel et al., 2001 and Selvin et al., 2010) and display inhibitory effects on the growth and attachment of microbes competing for the same niche (Holmström et al., 1999; Boyd et al., 1999). In terms of chemical ecology, some epibiotic microorganisms might play a role in protecting the host indirectly by releasing chemicals that prevent colonisation by other organisms (Yang et al., 2006).

Some marine bacteria belonging to the family Vibrionaceae and genus *Pseudoalteromonas* and *Roseobacter*, produce compounds that can reduce bacterial biofilm formation and settlement by other microorganisms (Dobretsov and Qian, 2004, Rao et al., 2006 and Bowman, 2007). However, to date, only a limited number of marine bacteria have been used to assess antifouling activity, such as the bacteria, *Pseudoalteromonas luteoviolacea, Pseudoalteromonas tunicata* and *Pseudoalteromonas aurantia* isolated from sponges and algae, which inhibited the growth of other bacteria in the marine environment (Holmström et al., 2000). In this study, the most active isolates were closely related to *Bacillus* species and moreover, showed a broader spectrum of activity, which agrees with the findings of Baam et al. (1966). Similarly, Burgess et al. (2003) found that *B. pumilus, B. licheniformis* and *B. subtilis* isolated from macroalgae and a nudibranch, produced antibiotic compounds that inhibited bacterial adherence. Furthermore, species of Bacillus showed inhibitory activity against bacteria and biofilm-forming microalgae (Satheesh et al., 2012).

Gram-positive bacilli have a higher antifouling activity than Gram-negative species against the studied microorganisms. In the terrestrial and marine environment, Gram-positive bacteria are well known as producers of biologically active secondary metabolites. Genome studies have revealed that more than 3% of bacteria contain genes that encode components of metabolite biosynthesis, such as non-ribosomal

peptide synthetase required for the biosynthesis of active compounds (Donadio et al., 2007). The *Bacillus* genus is one of the groups of microorganisms with a high potential biomedical use (Fenical and Jensen, 1993). They have been isolated with regularity from various invertebrates and have been extensively studied for the production of metabolites with antibacterial, non-stick properties, algicide activity and several pharmacologically useful compounds (Ivanova et al., 1999, Jeong et al., 2003, Pabel et al., 2003 and Zhang et al., 2004), which have a high potential in the search for novel antimicrobial substances (Muscholl-Silberhorn et al., 2008). Marine strains of *B. cereus* have been widely implicated in the biofouling of marine installations, together with microalgae and compounds that inhibit these organisms and might have potential application in AF technologies. Thakur and Anil (2000), while studying the sponge *Ircinia ramosa*, also concluded that species of the genus Bacillus were dominant and demonstrated good antibacterial activity in the two seasons that were studied. It has been reported that *B. brevis, B. cereus, B. circulans, B. laterosporus, B. licheniformis, B. polymyxa, B. pumilus*, and *B. subtilis* isolated from different substrates produce antibiotics (Madigan et al., 2005). Recent studies have shown the potential of sponge-associated Bacilli to produce antimicrobial compounds, such as the SAB1 strain of *B. licheniformis* isolated by Devi et al. (2010), and wider screening of these bacteria might aid the development of novel drugs.

The most important secondary metabolites isolated from Bacillus are peptides synthesised on the ribosome, commonly lethal to bacteria closely related to the producer (Riley and Wertz, 2002). This might explain why bioassays of antifouling agent activity were found to produce good results with extracts obtained from Bacillus isolated from *Aplysina* against other Bacillus strains (Desriac et al., 2010). It has been recently demonstrated that sponge isolates with antimicrobial activity from this genus are very abundant. This might account for the susceptibility of the test species *Bacillus* to the extracts of *Bacillus* species found in symbiosis with sponges and provides evidence that bacterial secondary metabolites play a role in inhibiting the colonisation of competing bacteria across species, as shown by the inhibition of *B. subtilis* (more sensitive strain tested) by extracts from other *Bacillus* species.

Species of *Pseudovibrio* genus have been obtained worldwide from a variety of sponges and it is believed that they are restricted to members of the phylum Porifera and have never been isolated from surrounding water samples (Enticknap et al., 2006). In this study, the strain identified as *Pseudovibrio* sp. showed high activity (0.01 μg mL^{-1}) against bacteria and microalgae. Same activity was observed for strains of *Staphylococcus, Micrococcus* and *Psychrobacter*. Recent research has found that same species isolated from sponges exhibit antimicrobial activity (Ivanova et al., 1999, Hentschel et al., 2001, Jeong et al., 2003, Ortega-Morales et al., 2008, Berrue et al., 2009, Santos et al., 2010 and Musthafa et al., 2011).

The screening for bioactive bacteria over a one year period, demonstrated that the activities of extracts obtained from the strain isolated from A. *gerardogreeni* varied between months. This indicates that

temporal screening is important if different bioactive bacteria are to be isolated. A clear trend in antifouling activity was observed against marine bacteria, with extracts of bacteria isolated from February and June being the most active, with a minimum of activity in October. The extracts from February were also the most active against microalgae. These results agree with previous research demonstrating that antifouling activity varied over a year (Hellio et al., 2004). Bernbom et al. (2011) observed a significant difference in the number of antifouling bacteria isolated in Denmark in different months, with the largest numbers in August and November. Maréchal et al. (2004) reported that the antifouling activity of *Bifurcaria bifurcata* (brown alga), was highest during spring and summer, when the fouling pressure was most intense and when values for water temperature and light intensity were high. In our case, August was the month with the maximum water temperature, however, a relationship to increased antifouling activity was not observed.

In the *A. gerardogreeni* sponge, the production of antimicrobial substance might confer several functions to a microorganism inhabiting this particular niche (Hentschel et al., 2001). In this case, the high density as well as the taxonomic diversity of bacteria within the sponge mesohyl might create an environment conducive to the production of antimicrobials and other defence compounds (Hentschel et al., 2001). Marine sponges are a potential source of less toxic and more specific natural antifoulants, but obtaining a sufficient supply of antifouling compounds from them is always difficult (Hill, 2004). So far, only a limited number of marine bacteria have been tested with regard to the production of antifouling compounds (Hill, 2004 and Kwong et al., 2006).

The advantages of microorganism as source of antifouling compounds is that 1) they can be cultivated to produce large quantities of metabolites, 2) and they have been the focus of bio-inspired applications research (Ralston and Swain, 2009), because they can be used in coatings or via microencapsulation to prevent biofouling (Holmström et al., 2000 and Yee et al., 2007). The results in our study indicate that bacteria associated with *A. gerardogreeni* such as *Bacillus, Micrococcus, Paracoccus, Pseudobacter, Pseudovibrio, Psychrobacter, Staphylocuccus* and *Terribacillus* have the potential to be used as antifoulant agents.

5. Conclusion

This study evaluated the bioactivity of some sponge-associated bacteria and identified at least 50% of these marine bacteria as having good antifouling potential. It also confirmed that bacteria associated with *A. gerardogreeni*, which were isolated from the tryptic soy agar (TSA) culture medium, are a good source of active compounds for use as antifouling agents.

Bacterial extracts showed temporal variation, with the best MIC against marine bacteria and microalgae during February and June. Extracts of bacteria from the genus *Bacillus* were most active, and therefore most promising.

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References

D. Amsterdam, Susceptibility testing of antimicrobials in liquid media, V. Lorian (Ed.), Antibiotics in Laboratory Medicine, Williams & Wilkins, Baltimore (1996), pp. 52–111

E. Armstrong, K.G. Boyd, J.G. Burgess, Prevention of marine biofouling using natural compounds from marine organisms, Biotechnol. Annu. Rev., 6 (2000), pp. 221–241

F.M. Ausubel, R. Brent, R.E. Kingston, D.D. Moore, J.G. Seidman, J.A. Smith, K. Struhl (Eds.), Short Protocols in Molecular Biology: a Compendium of Methods from Current Protocols in Molecular Biology (fifth ed), John Wiley & Sons, Inc., U.S.A (2002)

B.R. Baam, N.M. Gandhi, Y.M. Freitas, Antibiotic activity of marine microorganisms, Helgoländer Wiss. Meeresunters., 13 (1966), pp. 181–185

G.D. Barbier, D. Prieur Une nouvelle filifare: Les microorganismes marins, Biofutur, 106 (1991), pp. 41–46

G.I. Barrow, R.K.A. Feltham Cowan & Steel's Manual for the Identification of Medical Bacteria (third ed)Cambridge University Press (1993), p. 331

N. Bernbom, Y.Y. Ng, S. Kjelleberg, T. Harder, L. Gram, Marine bacteria from Danish coastal waters show antifouling activity against the marine fouling bacterium *Pseudoalteromonas* sp. strain S91 and zoospores of the green alga *Ulva australis* independent of bacteriocidal activity, Appl. Environ. Microbiol., 77 (2011), pp. 8557–8567

F. Berrue, A. Ibrahim, P. Boland, R.G. Kerr, Newly isolated marine *Bacillus pumilus* (SP21): a source of novel lipoamides and other antimicrobial agents, Pure Appl. Chem., 81 (2009), pp. 1027–1031

D. Blihoghe, E. Manzo, A. Villela, A. Cutignano, G. Picariello, M. Faimali, A. Fontana, Evaluation of the antifouling properties of 3-alyklpyridine compounds, Biofouling, 27 (2011), pp. 99–109

J.P. Bowman, Bioactive compounds synthetic capacity and ecological significance of marine bacterial genus *Pseudoalteromonas*, Mar. Drugs, 5 (2007), pp. 220–241

K.G. Boyd, D.R. Adams, J.D. Burgess, Antibacterial and repellent activities of marine bacteria associated with algal surfaces, Biofouling, 14 (1999), pp. 227–236

C. Bressy, C. Hellio, J.P. Marèchal, B. Tanguy, A. Margaillan, Bioassays and field immersion tests: a comparison of the antifouling activity of copper-free poly (methacrylic)-based coatings containing tertiary amines and ammonium salt groups, Biofouling, 26 (2010), pp. 769–777

J.G. Burgess, K.G. Boyd, E. Armstrong, Z. Jiang, L. Yan, M. Berggren, U. May, T. Piscane, A. Granmo, D.R. Adams, The development of a marine natural product-based antifouling paint, Biofouling, 19 (2003), pp. 197–205

L. Chambers, C. Hellio, K. Stokes, S. Dennington, L. Goodes, R. Wood, F. Walsh, Investigation of *Chondus crispus* as a potential source of new antifouling agentes, Int. Biodeterior. Biodegrad., 65 (2011), pp. 939–946

S. De Rosa, A. Milone, A. Kujumgiev, K. Stefanov, I. Nechev, S. Popov, Metabolites from a marine bacterium *Pseudomonas/Alteromonas*, associated with the sponge *Dysidea fragilis*, Comp. Biochem. Physiol., 126 (2000), pp. 391–396

F. Desriac, D. Defer, N. Bourgougnon, B. Brillet, P. Le Chevalier, Y. Fleury, Bacteriocin as weapons in the marine animal-associated bacteria warfare: inventory and potential applications as an aquaculture probiotic, Mar. Drugs, 8 (2010), pp. 1153–1177

P. Devi, S. Wahidullah, C. Rodrigues, L.D. Souza, The sponge-associated bacterium *Bacillus licheniformis* SAB1: a source of antimicrobial compounds, Mar. Drugs, 8 (2010), pp. 1203–1212

A.F. Dexter, M.J. Garson, M.E. Hemling, Isolation of a novel bastadin from the temperate marine sponge *Ianthella* sp, J. Nat. Prod., 56 (1993), pp. 782–786

S. Dobretsov, P.Y. Qian, The role of epibiotic bacteria from the surface of soft coral *Dendronephthya* sp. in the inhibition of larval settlement, J. Exp. Mar. Biol. Ecol., 299 (35) (2004), p. 50

S. Dobretsov, M. Teplitski, M. Bayer, S. Gunasekera, P. Proksch, V.J. Paul, Inhibition of marine biofouling by bacterial quorum sensing inhibitors, Biofouling, 27 (2011), pp. 893–905

S. Donadio, P. Monciardini, M. Sosio, Polyketide synthases and nonribosomal peptide synthetases: the emerging view from bacterial genomics, Nat. Prod. Rep., 24 (2007), pp. 1073–1109

S. Egan, T. Thomas, C. Holmström, S. Kjelleberg, Phylogenetic relationship and antifouling activity of bacterial epiphytes from the marine alga *Ulva lactuca*, Environ. Microbiol., 2 (2000), pp. 343–347

J.J. Enticknap, M. Kelly, O. Peraud, R.T. Hill, Characterization of a culturable alphaproteobacterial symbiont common to many marine sponges and evidence for vertical transmission via sponge larvae, Appl. Environ. Microbiol., 72 (2006), pp. 3724–3732

P.M. Erwin, J.B. Olson, R.W. Thacker, Phylogenetic diversity, host-specificity and community profiling of sponge-associated bacteria in the Northern Gulf of Mexico, PLOS ONE, 6 (2011), p. e26806

W. Fenical, Chemical studies of marine bacteria: developing a new resource, Chem. Rev., 93 (1993), pp. 1673–1683

W. Fenical, P.R. Jensen, Marine microorganisms: a new bio-medical resource, D.H. Attaway, O.R. Zabrosky (Eds.), Marine biotechnology: Pharmaceutical and Bioactive Natural Products, Plenum Press, New York (1993), pp. 419–457

A.B. Friedrich, H. Merkert, T. Fendert, J. Hacker, P. Proksch, U. Hentschel, Microbial diversity in the marine sponge *Aplysina cavernicola* (formerly *Verongia cavernicola*) analyzed by fluorescence *in situ* hybridization (FISH), Mar. Biol., 134 (1999), pp. 61–470

A.B. Friedrich, I. Fischer, P. Proksch, J. Hacker, U. Hentschel, Temporal variation of the microbial community associated with the Mediterranean sponge *Aplysina aerophoba*, FEMS Microbiol. Ecol., 38 (2001), pp. 105–113

N. Fusetani, Biofouling and antifouling, Nat. Prod. Rep., 21 (2004), pp. 94–104

R. Gastineau, J.B. Pouvreau, C. Hellio, M. Morançais, J. Fleurence, P. Gaudin, N. Bourgougnon, J.L. Mouget, Biological activities of purified marennine, the blue pigment responsible for the greening of oysters, J. Agric. Food Chem., 60 (2012), pp. 3599–3605

R.R.L. Guillard, J.H. Ryther, Studies of marine planktonic diatoms. I. *Cyclotella nana* Hustedt and *Detonula confervacea* Cleve, Can. J. Microbiol., 8 (1962), pp. 229–239

B.A. Gunn, R.R. Colwell, Numerical taxonomy of *Staphylococci* isolated from the marine environment, Int. J. Syst. Bacteriol., 33 (1983), pp. 751–759

T. Harder, Marine epibiosis: concepts, ecological, consequences and host defence, H.C. Flemming, P.S. Murthy, R. Venkatesan, K. Cooksey (Eds.), Marine and Industrial Biofouling, Springer-Verlag, Berlin, Germany (2009), pp. 219–231

T. Hattori, K. Adachi, Y. Shizuri, New ceramide from marine sponge *Haliclona koremella* and related compounds as antifouling substances against macroalgae, J. Nat. Prod., 61 (1998), pp. 823–826

C. Hellio, J.P. Marèchal, B. Veron, G. Bremer, A.S. Clare, Y. Le Gal, Seasonal variation of antifouling activities of marine algae from Brittany Coast (France), Mar. Biotechnol., 6 (2004), pp. 67–82

C. Hellio, D.M.Y. Yebra (Eds.), Advances in Marine Antifouling Coatings and Technologies, Woodshead Publishing, Cambridge, UK (2009)

U. Hentschel, M. Schmid, M. Wagner, L. Fieseler, C. Gernert, J. Hacker, Isolation and phylogenetic analysis of bacteria with antimicrobial activities from the Mediterranean sponges *Aplysina aerophoba* and *Aplysina cavernicola*, FEMS Microbiol. Ecol., 35 (2001), pp. 305–312

U. Hentschel, L. Fieseler, A. Wehrl, C. Gernert, M. Steinert, J. Hacker, M. Horn, Microbial diversity of marine sponges, W.E.G. Müller (Ed.), Sponges (Porifera), Springer, Berlin Heidelberg, Nueva York (2003), pp. 59–88

U. Hentschel, K. Usher, M. Taylor, Marine sponges as microbial fermenters, FEMS Microbiol. Ecol., 55 (2006), pp. 167–177

T. Hertiani, R. Edrada-Ebel, S. Ortlepp, R.W.M. van Soest, N.J. de Voogd, V. Wray, U. Hentschel, P. Proksch, From anti-fouling to biofilm inhibition: new cytotoxic secondary metabolites from two Indonesian *Agelas* sponges, Bioorganic Med. Chem., 18 (2010), pp. 1297–1311

R.T. Hill, Microbes from marine sponges: a treasure trove of biodiversity for natural products discovery, A.T. Bull (Ed.), Microbial Diversity and Bioprospecting, ASM Press, Washington (2004), pp. 177–190

J.G. Holt, N.R. Krieg, P.H.A. Sneath, J.T. Staley, S.T. Williams, Bergey's Manual of Determinative Bacteriology (ninth ed) The Williams and Wilkins Company, Baltimore, MD (2000)

C. Holmström, S. Kjelleberg, Marine *Pseudoalteromonas* species are associated with higher organisms and produce biologically active extracellular agents, FEMS Microbiol. Ecol., 30 (1999), pp. 285–293

C.P. Holmström, P. Steinburg, V. Christov, G. Christie, S. Kjellburg, Bacteria immobilized in gels: improved methodologies for antifouling and biocontrol applications, Biofouling, 15 (2000), pp. 109–117

E.P. Ivanova, M.V. Vysotskii, V.I. Svetashev, O.I. Nedashkovskaya, N.M. Gorshkova, V.V. Mijhailov, N. Yumoto, Y. Shigeri, T. Taguchi, S. Yoshikawa, Characterization of *Bacillus* strains of marine origin, Int. Microbiol., 2 (1999), pp. 267–271

R. Jellali, I. Campistron, P. Pasetto, A. Laguerre, F. Gohier, C. Hellio, J.F. Pilard, J.L. Mouget, Antifouling activity of novel polyisoprene-based coatings made from photocurable natural rubber derived oligomers, Prog. Org. Coatings, 76 (2013), pp. 1203–1214

S.Y. Jeong, K. Ishida, Y. Ito, S. Okada, M. Murakami, Bacillamide, a novel algicide from the marine bacterium, Bacillus sp. SY-1, against the harmful dinoflagellate, *Cochlodinium polykrikoides*, Tetrahedron Lett., 44 (2003), pp. 8005–8007

T.F.N. Kwong, L. Miao, X. Li, P.Y. Qian, Novel antifouling and antimicrobial compound from a marine-derived fungus *Ampelomyces* sp, Mar. Biotechnol., 8 (2006), pp. 634–640

V.P. Limna Mol, T.V. Raveendran, P.S. Parameswaran, Antifouling activity exhibited by secondary metabolites of the marine sponge, *Haliclona exigua* (Kirkpatrick), Int. Biodeterior. Biodegrad., 63 (2009), pp. 67–72

N.A. Logan, R.C.W. Berkeley, Identification of *Bacillus* strains using the API system, J. General. Microbiol., 130 (1984), pp. 1871–1882

M. Madigan, J. Martinko, J. Parker, Brock, Biology of Microorganisms, Prentice Hall, New York (2005)

J.P. Marèchal, G. Culioli, C. Hellio, H. Thomas-Guyon, M.E. Callow, A.S. Clare, A. Ortalo-Magne, Seasonal variation in antifouling activity of crude extracts of the brown alga *Bifurcaria bifurcata* (Cystoseiraceae) against cyprids of *Balanus amphitrite* and the marine bacteria *Cobetia marina* and *Pseudoalteromonas haloplanktis*, J. Exp. Mar. Biol. Ecol., 313 (2004), pp. 47–62

A. Muscholl-Silberhorn, V. Thiel, J. Imhoff, Abundance and bioactivity of cultured sponge-associated bacteria from the Mediterranean Sea, Microbiol. Ecol., 55 (2008), pp. 94–106

K.S. Musthafa, V. Saroja, S.K. Pandian, A.V. Ravi, Antipathogenic potential of marine *Bacillus* sp. SS4 on N-acyl-homoserine-lactone-mediated virulence factors production in *Pseudomonas aeruginosa* (PAO1), J. Biosci., 36 (2011), pp. 55–67

S. Nair, U. Simidu, Distribution and significance of heterotrophic marine bacteria with antibacterial activity Appl. Environ. Microbiol., 53 (1987), pp. 2957–2962

J.M. Oclarit, H. Okada, S. Ohta, K. Kaminura, Y. Yamaoka, T. Iizuka, S. Miyashiro, S. Ikegami, Anti-bacillus substance in the marine sponge, *Hyatella* species, produced by an associated *Vibrio* species bacterium Microbios, 78 (1994), pp. 7–16

B.O. Ortega-Morales, M.J. Chan-Bacab, E. Miranda-Tello, M.L. Fardeau, J.C. Carrero, T. Stein, Antifouling activity of sessile bacilli derived from marine surfaces, J. Ind. Microbiol. Biotechnol., 35 (2008), pp. 9–15

S. Ortlepp, M. Sjögren, M. Dahlström, H. Weber, R. Ebel, R. Edrada, C. Thoms, P. Proksch, Antifouling activity of bromotyrosine-derived sponge metabolites and synthetic analogues, Mar. Biotechnol., 9 (2007), pp. 776–785

C.T. Pabel, J. Vater, C. Wilde, P. Franke, J. Hofemeister, Adler, G. Bringmann, J. Hacker, U. Hentschel, Antimicrobial activities and matrix-assisted laser desorption/ionization mass spectrometry of *Bacillus* isolates from the marine sponge *Aplysina aerophoba*, Mar. Biotechnol., 5 (2003), pp. 424–434

M.C. Papaleo, M. Fondi, I. Maida, E. Perrin, A. LoGiudice, L. Michaud, S. Mangano, G. Bartolucci, R. Romoli, R. Fani, Sponge-associated microbial Antarctic communities exhibiting antimicrobial activity against *Burkholderia cepacia* complex bacteria, Biotechnol. Adv., 30 (2012), pp. 272–293

E. Plouguerné, C. Hellio, E. Deslandes, B. Véron, V. Stiger-Pouvreau, Anti-microfouling activities in extracts of two invasive algae: *Grateloupia turuturu* and *Sargassum muticuma*, Bot. Mar., 51 (2008), pp. 202–208

E. Plouguerné, C. Hellio, C. Cesconetto, M. Thabard, K. Mason, B. Véron, R.C. Pereira, B.A.P. da Gama, Antifouling activity as a function of population variation in *Sargassum vulgare* from the littoral of Rio de Janeiro (Brazil), J. Appl. Phycol., 22 (2010), pp. 717–724

P.Y. Qian, S.C.K. Lau, H.U. Dahms, S. Dobretsov, T. Harder, Marine biofilms as mediators of colonization by marine macroorganisms: implications for antifouling and aquaculture, Mar. Biotechnol., 9 (2007), pp. 399–410

Y. Qiu, Z.W. Deng, M. Xu, Q. Li, W.H. Lin, New A-nor steroids and their antifouling activity from the Chinese marine sponge Acanthella cavernosa, Steroids, 73 (2008), pp. 1500–1504

E. Ralston, G. Swain, Bioinspiration-the solution for biofouling control? Bioinspiration Biomimetics, 4 (2009), pp. 1–9

D. Rao, J.S. Webb, S. Kjelleberg, Microbial colonization and competition on the marine alga *Ulva australis*, Appl. Environ. Microbiol., 72 (2006), pp. 5547–5555

M.A. Riley, J.E. Wertz, Bacteriocins: evolution, ecology, and application, Annu. Rev. Microbiol., 56 (2002), pp. 117–137

J. Sambrook, D.W. Russell, Molecular Cloning: a Laboratory Manual, (third ed)Cold Spring Harbor Laboratory, Cold Spring Harbor, New York (2001)

M. Santos-Acevedo, C. Puentes, K. Carreño, J. Gómez-Leóna, M. Stupak, M. García, M. Pérez, G. Blustein, Antifouling paints based on marine natural products from Colombian Caribbean, Int. Biodeterior. Biodegrad., 83 (2013), pp. 97–104

O.C. Santos, P.V. Pontes, J.F. Santos, G. Muricy, M. Giambiagi-deMarval, Isolation, characterization and phylogeny of sponge associated bacteria with antimicrobial activities from Brazil, Res. Microbiol., 161 (2010), pp. 604–612

S. Satheesh, A.R. Soniamby, C.V. Sunjaiy-Shankar, S.M. Punitha, Antifouling activities of marine bacteria associated with sponge (*Sigmadocia* sp.), J. Ocean. Univ. China, 11 (2012), pp. 354–360

J. Selvin, A.S. Ninawe, G. Seghal, A.P. Lipton, Sponge-microbial interactions: ecological implications and bioprospecting avenues, Critical Rev. Microbiol., 36 (2010), pp. 82–90

H. Shigemori, M.A. Bae, K. Yazawa, T. Sasaki, J. Kobayashi, Alteramide A a new tetracyclic alkaloid from a bacterium *Alteromonas* sp. associated with the marine sponge *Halichondria okadai*, J. Org. Chem., 57 (1992), pp. 4317–4320

A.C. Stierle, J.H. Cardellina, F.L. Singleton, A marine *Micrococcus* produces metabolites ascribed to the sponge *Tedania ignis*, Experentia, 44 (1988), p. 1021

S.D. Stowe, J.J. Richards, A.T. Tucker, R. Thompson, C. Melander, J. Cavanagh, Anti-biofilm compounds derived from marine sponges, Mar. Drugs, 9 (2011), pp. 2010–2035

M. Thabard, G. Id Daoud, B. Véron, R.L. Fletcher, C. Hellio, Screening of biological activities of extracts of *Ralfsia verrucosa, Petalonia fascia* and *Scytosiphon lomentaria* (Phaeophyceae, Scytosiphonales) for potential antifouling application, Electron. J. Nat. Subst., 4 (2009), pp. 1–10

N.L. Thakur, A.C. Anil, Antibacterial activity of the sponge, *Ircinia ramose*: importance of its surface-associated bacteria, J. Chem. Ecol., 26 (2000), pp. 57–71

M.D. Unson, N.D. Holland, D.J. Faulkner, A brominated secondary metabolite synthesized by the cyanobacterial symbiont of marine sponge and accumulation of the crystalline metabolite in the sponge tissue, Mar. Biol., 119 (1994), pp. 1–11

K.M. Usher, J. Fromont, D.C. Sutton, S. Toze, The biogeography and phylogeny of unicellular cyanobacterial symbionts in sponges from Australia and the Mediterranean, Microbiol. Ecol., 48 (2004), pp. 167–177

K. Vasundhara, I. Khan, S. Prasad, Antimicrobial screening of marine bacterium G54 (Bacillus sp.) associated with the sponge Mycale mannarensis, J. Pure Appl. Microbiol., 6 (2012), pp. 1991–1996

N.S. Webster, R.T. Hill, The culturable microbial community of the Great Barrier Reef sponge *Rhopaloeides odorabile* is dominated by an alpha-Proteobacterium, Mar. Biol., 138 (2001), pp. 843–851

C.R. Wilkinson, Cyanobacteria symbiotic in marine sponges, W. Schwemmler, H.E.A. Schenck (Eds.), Endocytobiology, Endosymbiosis and Cell Biology, De Gruyter, Berlin (1980), pp. 993–1002

A.D. Wright, A. McCluskey, M.J. Robertson, K.A. MacGregor, C.P. Gordon, J. Guenther, Anti-malarial, anti-algal, anti-tubercular, anti-bacterial, anti-photosynthetic, and anti-fouling activity of diterpene and diterpene isonitriles from the tropical marine sponge Cymbastela hooperi, Org. Biomol. Chem., 9 (2011), pp. 400–407

Z. Wu, C. Shao, C. Wang, Review on the secondary metabolites from *Phyllospongia* sponges and their bioactivities, Chin. J. New. Drugs, 21 (2012), pp. 52–58

Y. Xu, N. Li, W. Jiao, R. Wang, Y. Peng, S. Qi, S. Song, W. Chen, Antifouling and cytotoxic constituents from the South China Sea sponge *Acanthella cavernosa*, Tetrahedron, 68 (2012), pp. 2876–2883

L.H. Yang, O.O. Lee, T. Jin, X.C. Li, P.Y. Qian, Antifouling properties of 10-β- formamidokalihinol-A and kalihinol A isolated from the marine sponge *Acanthella cavernosa*, Biofouling, 22 (2006), pp. 23–32

D.M. Yebra, S. Kiil, K. Dam-Johansen, Antifouling technology -past, present and future steps towards efficient and environmentally friendly antifouling coatings, Prog. Org. Coatings, 50 (2004), pp. 75–104

L.H. Yee, C. Holmström, E.T. Fuary, N.C. Lewin, S. Kjelleberg, P.D. Steinberg, Inhibition of fouling by marine bacteria immobilised in K-carrageenan beads, Biofouling, 23 (2007), pp. 287–294

H.L. Zhang, H.M. Hua, Y.H. Pei, X.S. Yao, Three new cytotoxic cyclic acylpeptides from marine *Bacillus sp*, Chem. Pharm. Bull., 52 (2004), pp. 1029–1030