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Antifouling Activity towards Mussel by Small-Molecule Compounds from a Strain of *Vibrio alginolyticus* Bacterium Associated with Sea Anemone *Haliplanella* sp.

Xiang Wang[1], Yanqiu Huang[1], Yanqing Sheng[1], Pei Su[1,2], Yan Qiu[3], Caihuan Ke[1,2], and Danqing Feng[1,2*]

[1] *State-Province Joint Engineering Laboratory of Marine Bioproducts and Technology, College of Ocean & Earth Sciences, Xiamen University, Xiamen 361000, P.R. China*

[2] *Fujian Collaborative Innovation Center for Exploitation and Utilization of Marine Biological Resources (Xiamen University), Xiamen, 361000, P.R. China*

[3] *Medical College, Xiamen University, Xiamen 361000, P.R. China*

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*Corresponding author
Phone: +86-592-2880801;
Fax: +86-592-2880802;
E-mail: dqfeng@xmu.edu.cn

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Mussels are major fouling organisms causing serious technical and economic problems. In this study, antifouling activity towards mussel was found in three compounds isolated from a marine bacterium associated with the sea anemone *Haliplanella* sp. This bacterial strain, called PE2, was identified as *Vibrio alginolyticus* using morphology, biochemical tests, and phylogenetic analysis based on sequences of 16S rRNA and four housekeeping genes (*rpoD*, *gyrB*, *rctB*, and *toxR*). Three small-molecule compounds (indole, 3-formylindole, and cyclo (Pro-Leu)) were purified from the ethyl acetate extract of *V. alginolyticus* PE2 using column chromatography techniques. They all significantly inhibited byssal thread production of the green mussel *Perna viridis*, with EC_{50} values of 24.45 μg/ml for indole, 50.07 μg/ml for 3-formylindole, and 49.24 μg/ml for cyclo (Pro-Leu). Previous research on the antifouling activity of metabolites from marine bacteria towards mussels is scarce. Indole, 3-formylindole and cyclo (Pro-Leu) also exhibited antifouling activity against settlement of the barnacle *Balanus albicostatus* (EC_{50} values of 8.84, 0.43, and 11.35 μg/ml, respectively) and the marine bacterium *Pseudomonas* sp. (EC_{50} values of 42.68, 69.68, and 39.05 μg/ml, respectively). These results suggested that the three compounds are potentially useful for environmentally friendly mussel control and/or the development of new antifouling additives that are effective against several biofoulers.

Keywords: Marine bacterium, mussel, antifouling, *Vibrio alginolyticus*, compound

Introduction

The accumulation of marine fouling organisms on human-made surfaces in the marine environment poses severe technical and economic problems around the world [1, 2]. Antifouling coatings with metal-based compounds (such as tributyltin and cuprous oxide) and synthetic organic biocides (such as Igarol and diuron) have been widely used to combat marine biofouling [3]. However, these antifoulants have been found to produce toxic effects on non-target species and may pollute the marine environment, leading to bans and regulations on their use in antifouling coatings [3–5]. Thus, environmentally friendly alternatives are urgently needed.

Natural products with antifouling activity from marine organisms are suggested to be a promising source of eco-friendly antifoulants [6, 7]. So far, a large number of secondary metabolites from marine invertebrates and seaweeds have been isolated and demonstrated to have antifouling activity [8]. However, these compounds from marine macroorganisms usually are not available in sufficient quantities for commercial application, and most

of them are difficult to produce through chemical synthesis at a cost that would be feasible on an industrial scale [6–8]. Since marine microorganisms can be cultured to produce bioactive substances on a large scale, they provide potential sources of novel environmentally friendly antifoulants. Some antifouling active compounds from marine microorganisms have been isolated and identified [9–13]. Given the vast number of microorganism species in the ocean, there is great potential for discovering antifouling active microorganisms and subsequently obtaining natural antifouling products.

Mussels are one of the most problematic fouling species. They produce byssal threads to attach themselves firmly to submerged hard surfaces such as ship hulls, steel, pilings, metal grates, and concrete. Mussel fouling is particularly known for clogging cooling systems of power plants and causing serious economic problems [14–16]. For example, it was estimated that zebra mussel in the Great Lakes cost the power industry 3.1 billion US dollars from 1993 to 1999 [17]. Chlorine is commonly used to combat mussel fouling in power stations [15, 16, 18]. However, byproducts of chlorine can affect non-target organisms, and some of them are carcinogenic [15, 19]. Finding natural marine products with antifouling activity towards mussels would aid in the development of environmentally friendly mussel control methods. Although a few compounds derived from marine microorganisms have been demonstrated as effective in inhibiting the settlement of invertebrates, such as barnacles, bryozoans, and polychaetes [9, 20, 21], little information is available on the activity of metabolites from marine bacteria on byssal attachment of mussels.

In our preliminary screening of marine bacteria for antifouling activity, we found that the crude extract of a bacterial strain called PE2, which was isolated from the sea anemone *Haliplanella* sp., showed strong antifouling activity. In this study, the bacterial strain PE2 was identified as *Vibrio alginolyticus* using morphology, biochemical, and genetic analyses. Three small-molecule compounds were isolated from its crude extract using bioassay-guided fractionation. They were all found to be active in inhibiting byssal production of mussel. These compounds also showed antifouling activity against barnacle and a marine bacterium.

Materials and Methods

Strain Isolation

The bacterial strain PE2 was isolated from the sea anemone *Haliplanella* sp. collected from the coastal region of Fujian Province, which is situated in southeast China. The isolation of bacteria was performed via serial dilution of triturated *Haliplanella* sp. samples in sterile filtered seawater (0.22 μm, FSW), followed by plating on Marine Agar 2216E plates. The plates were incubated at 28°C for 3 days. Distinct bacterial colonies were then isolated and purified. During the preliminary screening of bacteria for antifouling activity, the isolated strain PE2 exhibited significant activity and was selected for further study. It was deposited in the China General Microbiological Culture Collection Center (CGMCC) under the accession number of CGMCC No. 8815.

Identification of Strain PE2

Phenotypic characterization. Strain PE2 was cultivated overnight at 28°C on thiosulfate citrate bile sucrose (TCBS) agar, and the color of the colonies formed was observed. Cell morphology was observed by using transmission electron microscopy (JEM-2100 TEM, JEOL, Japan) after negative staining with 1% (w/v) phosphotungstic acid for 15 sec. Gram staining was performed as described by Collins *et al*. [22]. Catalase and oxidase activities were assessed according to the method of Chen *et al*. [23]. Tests of starch hydrolysis and gelatin hydrolysis were performed as described by Smibert and Krieg [24]. Production of H_2S was determined using the method described by Bruns *et al*. [25]. Melanin production was tested by using tyrosine agar plates. Growth at different NaCl concentrations (0%, 1%, 3%, 5%, and 10% (w/v)) was determined in peptone water (10 g peptone per liter). Susceptibility to antibiotics was tested on 2216E agar plates using filter-paper discs impregnated with chloramphenicol (30 μg per disc) and streptomycin (30 μg per disc). The utilization of organic compounds as sole carbon sources was determined by using a basal medium (containing 2 g $(NH_4)_2SO_4$, 0.5 g Na_2HPO_4, 0.5 g K_2HPO_4, 0.2 g $MgSO_4$, 0.1 g $CaCl_2$, and 1.5 g NaCl, per liter of distilled water) supplemented with 0.5% (w/v) concentrations of the following compounds: yeast extract, D-sorbitol, D-mannitol, D-fructose, D-galactose, D-raffinose, D-xylose, D-maltose, D-sucrose, and D-lactose.

Molecular characterization. Phylogenetic analysis was used to identify strain PE2. Genomic DNA was extracted by using the method of Sambrook *et al*. [26]. The 16S rRNA gene and the housekeeping genes *rpoD* (RNA polymerase sigma factor gene), *gyrB* (DNA gyrase subunit B gene), *rctB* (replication origin-binding protein gene), and *toxR* (transmembrane regulatory protein gene) were studied. Amplification of these genes was performed according to Pascual *et al*. [27]. The amplified products were ligated into a pMD19-T vector and sequenced. Multiple alignments of the sequences of PE2 and related species were performed using ClustalX 1.81 [28]. Phylogenetic trees based on each gene and concatenated sequences of the four housekeeping genes were constructed using neighbor-joining [29] with the program MEGA 4.1 [30]. The reliability of the topologies was determined by using bootstrap values based on 1,000 replications [31]. The gene sequences used in the analysis were deposited in GenBank under the accession numbers KT036618 for the 16S rRNA gene,

KT036619 for the *gyrB* gene, KT036620 for the *rctB* gene, KT036621 for the *rpoD* gene, and KT036622 for the *toxR* gene.

Bacterial Fermentation and Extraction

The PE2 strain was cultivated in flasks with marine broth 2216E liquid medium on a rotary shaker (150 rpm) at 28°C for 3 days. In total, 107 L of culture was prepared. The cultures were centrifuged at 9,000 ×*g* at 4°C for 20 min to separate bacterial cells from the broth supernatant. The broth supernatant was extracted three times with an equal volume of ethyl acetate (EtOAc). After solvent removal under reduced pressure, 22.9 g of crude extract was obtained.

Isolation and Identification of Antifouling Compounds

The crude extract was suspended in 90% MeOH (1 L) and further extracted with an equal volume of petroleum ether. The remaining solution was dried under reduced pressure and redissolved in 10% MeOH (1 L), followed by successive partitioning with equal volumes of dichloromethane, EtOAc, and *n*-butanol. The resultant five fractions, including the residual aqueous fraction, were evaporated to dryness under reduced pressure and were tested for antifouling activity. The dichloromethane fraction with high activity was further separated by column chromatography on silica, eluted with petroleum ether-EtOAc (15:1 then 5:1) to give compound **1** (300 mg) and three fractions (Fr. A−C). Fr. B was further isolated by column chromatography on silica, eluted with hexane-acetone (7:3) to yield compound **2** (5 mg). Fr. C was subjected to column chromatography on silica and eluted with dichloromethane–MeOH (200:1) to give two subfractions, C1 and C2. Fr. C1 was further purified by column chromatography on silica, eluted with hexane-acetone (5:1 then 4:1 then 3:1 then 2:1 then 1:1) to obtain compound **3** (30 mg). NMR spectra of the purified compounds were obtained in $CDCl_3$ on a Bruker Advance-500 FT spectrometer operating at 500 and 125 MHz for ^{1}H and ^{13}C, respectively, with tetramethylsilane as the internal standard. Chemical shifts (δ) are reported in parts per million (ppm), and coupling constants (*J*) are in Hz. ESIMS spectral data were measured in the positive-ion mode on an ABI 3200 Q-Trap mass spectrometer. Mass spectra were recorded across the range *m/z* 100−1000. Structural elucidation of the compounds was based on their spectral data (NMR, ESIMS) and on comparison with published values.

Mussel Bioassay

The antifouling activity of compounds towards the green mussel *Perna viridis* was tested by following the methods of Van Winkle [32] and Rajagopal *et al.* [33]. Briefly, *P. viridis* (shell length, 15−20 mm) was collected from submerged rafts at a fish farm in Zhangzhou, Fujian Province, China, and then kept in an aquarium with aerated seawater for 2−5 days. The byssal threads of each mussel were gently cut off with sharp scissors. Compounds **1**, **2**, and **3** were dissolved in dimethylsulfoxide (DMSO). One mussel, 20 μl of test solution, and 1.98 ml of FSW were added into each well of a 24-well plate. There were 10 replicates for each treatment and the control (1% DMSO in FSW (v/v)). After 24 h, the number of byssus threads produced by each mussel was counted and expressed in threads $mussel^{-1}$ day^{-1} [32, 33]. The dead mussels were also counted. When calculating the EC_{50} for each compound (the concentration that inhibited byssal production of mussel by 50% relative to the control), the relative rate (%) of the byssal production was used, which was obtained by dividing the number of threads in the treatment group by the number of threads in the control group. The EC_{50} was estimated using the Spearman–Karber method [34−36].

Barnacle Bioassay

Adults of *Balanus albicostatus* were collected from intertidal rocks in Xiamen, Fujian Province, China. The naupliar larvae were released from the adults and reared to the cyprid stage as described by Feng *et al*. [37]. The larval settlement experiments were performed using glass Petri dishes (6 cm diameter). Compounds **1**, **2**, and **3** were dissolved in EtOAc and applied to the dishes. After complete evaporation of the EtOAc at room temperature, about 30 larvae and 10 ml of FSW were added to each dish. There were three replicates for each treatment and the FSW control. The dishes were incubated at 25°C in darkness for 48 h. After incubation, the larvae that settled or died were counted with the aid of a stereomicroscope. The rates of larval settlement were used to calculate the EC_{50} (the concentration that inhibited barnacle settlement by 50% relative to the control) using the Spearman-Karber method [34−36].

Bacterium Bioassay

The antibacterial-settlement activity of compounds towards a marine bacterium *Pseudomonas* sp. TB4 was tested, using the method of Kelly *et al*. [38] with some modifications. The target bacterium was isolated from a natural marine biofilm on an antifouling coating after 24 h of immersion in Xiamen Bay in March 2000. The TB4 strain was stored in 20% glycerol (v/v) at −80°C. The TB4 strain is a member of the genus *Pseudomonas* sp., as indicated by analysis of the 16S rRNA gene sequence. The day before the settlement assay was performed, *Pseudomonas* sp. TB4 was inoculated in 100 ml of marine broth 2216E liquid medium and grown overnight on a rotary shaker (180 rpm) at 28°C. After centrifugation at 8,000 ×*g* at 4°C for 20 min, the broth supernatant was removed and the cells were suspended in sterile FSW to reach an optical density of 1 at 600 nm. In the bacterial settlement assay, 6-well plates were used, with a glass coverslip (24 × 24 mm) placed into each well. Compounds **1**, **2**, and **3** were dissolved in DMSO. A volume of 100 μl of each compound solution, 9.8 ml of sterile FSW, and 100 μl of bacterial suspension were added into each well of the 6-well plates. The experiment was run in three replicates. The control wells contained 100 μl of DMSO, 9.8 ml of sterile FSW, and 100 μl of bacterial suspension. The plates were incubated for 3 h in darkness at 25°C. Unsettled bacteria were removed from the coverslips by washing gently with sterile FSW.

Settled bacterial cells on coverslips were stained with 10 µg/ml of DAPI for 5 min in the dark. The coverslips were observed under an Olympus BH-2 epifluorescence microscope. The number of settled bacteria was counted in nine random fields of view on each coverslip from pictures taken with a camera (Leica DFC 420C). Bacterial settlement is presented here as the relative rate: (S/C) × 100%, where S is the number of settled bacteria in the treatment group and C the number of settled bacteria in the control group. The relative rates of bacterial settlement were used to calculate the EC_{50} (the concentration that inhibited bacterial settlement by 50% relative to the control) using the Spearman-Karber method [34−36]. To assess the effects of compounds **1**, **2**, and **3** on the growth of *Pseudomonas* sp. TB4 during the trial period, the bacterial suspension was prepared and added to the 6-well plates with the DMSO control and compound solutions as described above. Three replicates were set up. After incubation of the plates at 25°C for 3 h, 200 µl of bacterial culture was taken from each well and measured for absorbance at 600 nm using a microplate reader to assess the cell growth.

Statistical Analysis

Differences in byssus thread production of mussels, barnacle larval settlement, or bacterial settlement between the treatments and control were compared using one-way analysis of variance (ANOVA) followed by a Dunnett post hoc test. The significance level was set at $p < 0.05$.

Results

Characterization of the PE2 Strain

Cells of the PE2 strain were gram-negative, short rod-shaped (0.5−0.7 µm wide and 1−2 µm long), and motile by means of a single polar flagellum (Fig. 1). Colonies were yellow and circular with regular edges after overnight incubation on TCBS agar at 28°C. The PE2 strain grew at the NaCl concentrations of 1−10% (w/v) and there was no growth without NaCl, suggesting that NaCl is required for growth. The phenotypic characteristics of the PE2 strain are listed in Table 1.

The 16S rRNA gene sequence (1,420 bp) of strain PE2 showed the highest similarities with the species *Vibrio azureus* (JN128263) (99%), *V. neocaledonicus* (KJ841877) (99%), and *V. alginolyticus* (CP006718) (99%). This indicated that strain PE2 belonged to the genus *Vibrio*. A phylogenetic tree based on 16S rRNA gene sequences is shown in Fig. 2A. Multilocus sequence analysis is a useful tool to identify

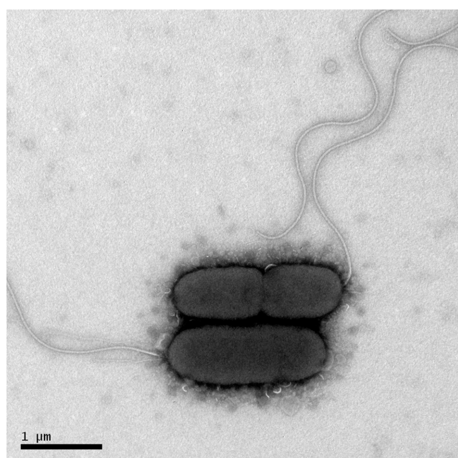

Fig. 1. Transmission electron micrograph of *Vibrio alginolyticus* PE2 (3,000× magnification).

Table 1. Phenotypic characteristics of *Vibrio alginolyticus* PE2.

Characteristic	PE2 strain
Cell shape	Short rod
Colony color on TCBS agar	Yellow
Gram stain	−
Catalase	+
Oxidase	+
Hydrolysis of	
Starch	+
Gelatin	+
Production of	
H_2S	−
Melanin	−
Growth in NaCl (% (w/v))	
0	−
1	+
3	+
5	+
10	+
Susceptibility to	
Chloramphenicol	+
Streptomycin	−
Utilization of	
Yeast extract	+
D-Sorbitol	−
D-Mannitol	+
D-Fructose	−
D-Galactose	−
D-Raffinose	−
D-Xylose	−
D-Maltose	+
D-Sucrose	+
D-Lactose	−

+, positive result/growth; −, negative result/ no growth

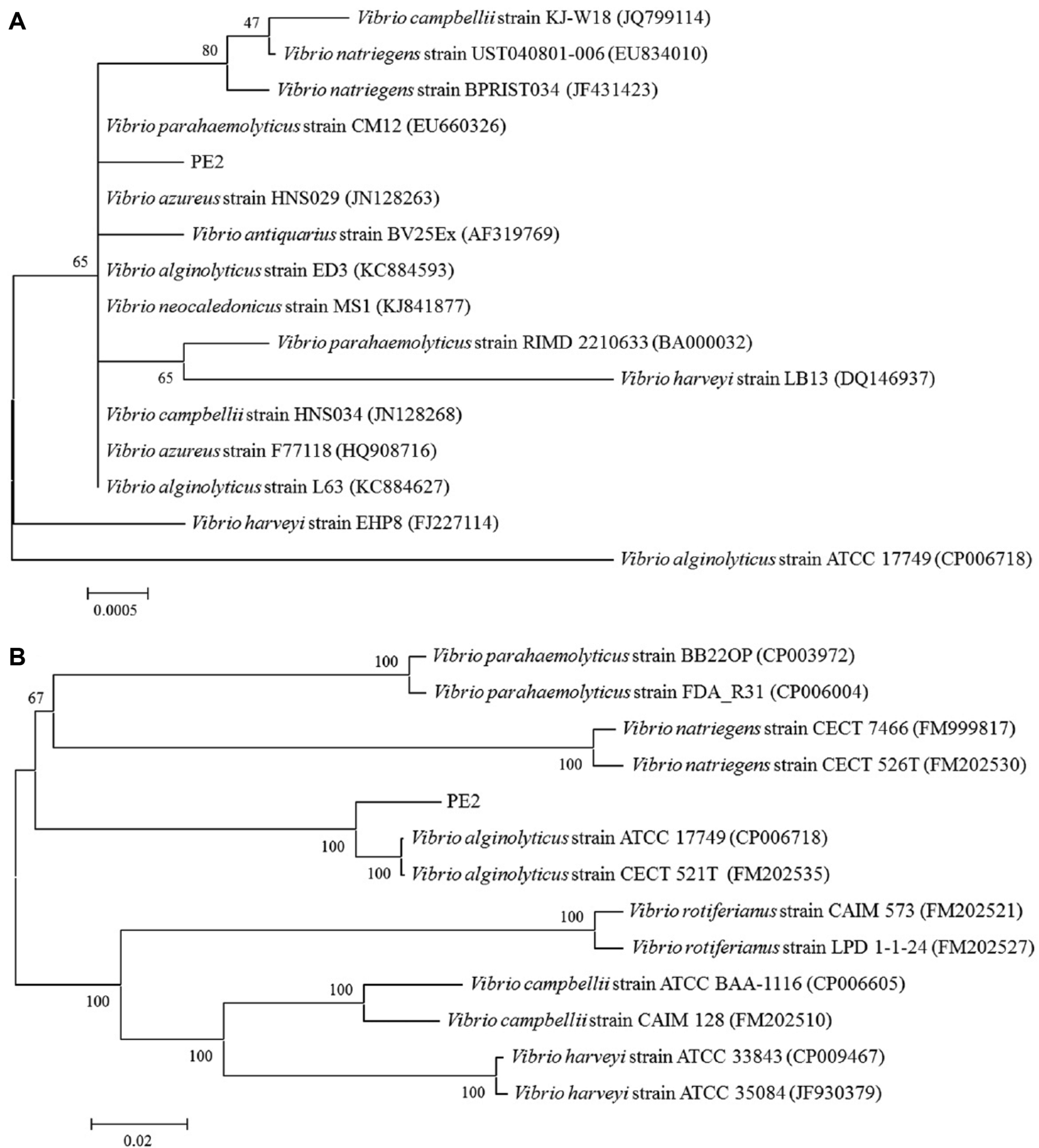

Fig. 2. Phylogenetic reconstructions based on analyses of (**A**) the 16S rRNA and (**B**) concatenated *rpoD*, *gyrB*, *rctB*, and *toxR* gene sequences both using the neighbor-joining method.

GenBank sequence accession numbers are given in parentheses. Numbers at nodes denote the level of bootstrap based on 1,000 replicates. The scale bar represents base substitutions per site.

Vibrio species [27, 39, 40]. Here, sequences of the genes *rpoD* (907 bp), *gyrB* (1261 bp), *toxR* (582 bp), and *rctB* (701 bp) were obtained and compared with those of related species. The genes *rpoD*, *gyrB*, and *toxR* all pointed to *V. alginolyticus* (CP006718) as the closest relative, with sequence similarity values of 99% (*rpoD*), 99% (*gyrB*), and 98% (*toxR*). The *rctB* gene sequence of the PE2 strain showed the highest similarity with *V. alginolyticus* (JF836251) (99%). The phylogenetic trees based on each housekeeping gene (Fig. S1), and on concatenated sequences of the four housekeeping genes (Fig. 2B) confirmed the clustering of strain PE2 and *V. alginolyticus* with high bootstrap values. Based on the genomic and phenotypic data, strain PE2 was identified as *Vibrio alginolyticus*.

Table 2. ^{1}H NMR and ^{13}C NMR data for compounds **1**, **2**, and **3** in $CDCl_3$ (δ in ppm).

Position	Compound **1**		Compound **2**		Compound **3**	
	δ_C[a]	δ_H[b]	δ_C[a]	δ_H[b]	δ_C[a]	δ_H[b]
1		8.13 (1H, brs)				5.85 (1H, brs, NH)
2	124.2	7.23 (1H, d, *J* = 2.8 Hz)	139.8	8.02 (s, 1H)	166.2	
3	102.6	6.62 (1H, d, *J* = 2.8 Hz)	120.0		59.0	4.13 (1H, dd, *J* = 8.1, 8.3 Hz)
4	127.9		123.6			
5	120.8	7.71 (1H, d, *J* = 8.2 Hz)	122.4	8.09 (d, 1H, *J* = 7.30 Hz)	170.2	
6	122.0	7.20 (1H, dd, *J* = 8.0, 7.0 Hz)	125.0	7.20 (m, 2H)	53.4	4.03 (1H, dd, *J* = 9.5, 3.5 Hz)
7	119.8	7.27 (1H, dd, *J* = 8.0, 7.0 Hz)	113.1		38.6	1.55, 2.07 (1H, each, m)
8	111.1	7.42 (1H, d, *J* = 8.0 Hz)	125.6	7.41 (d, 1H, *J* = 7.64 Hz)	23.3	1.84 (1H,m)
9	135.8		138.9		22.7	1.02 (3H, d, *J* = 6.5 Hz)
10			187.5	9.80 (s, 1H, CHO)	21.2	0.96 (3H, d, *J* = 6.5 Hz)
11					28.1	2.12, 2.35 (1H, each, m)
12					24.7	1.91, 2.05 (1H, each, m)
13					45.5	3.60, 3.55 (1H, each, m)

[a]Recorded at 125 MHz. [b]Recorded at 500 MHz.

Isolation and Identification of Compounds Produced by *Vibrio alginolyticus* PE2

Three antifouling compounds were isolated from the active dichloromethane fraction of the broth crude extract of *Vibrio alginolyticus* PE2. Compound **1** was obtained as a pale yellow crystal with ESIMS *m/z* 118.0653 $[M + H]^+$. Compound **2** was a pale yellow crystal with ESIMS *m/z* 168.0395 $[M + Na]^+$. Compound **3** was a white crystal with ESIMS *m/z* 233.1275 $[M + Na]^+$ and 249.1233 $[M + K]^+$. Their ^{1}H NMR and ^{13}C NMR data are listed in Table 2. Based on their spectral data and comparison with those reported by Devi *et al*. [41], Zan *et al*. [42], and Kumar *et al*. [43], compounds **1**, **2**, and **3** were identified as indole, 3-formylindole, and cyclo(Pro-Leu), respectively. Their chemical structures are shown in Fig. 3.

Antifouling Activity of Compounds

All of the three compounds isolated from *V. alginolyticus* PE2 in this work showed significant antifouling activity against the mussel *Perna viridis*, the barnacle *Balanus albicostatus*, and the marine bacterium *Pseudomonas* sp. TB4 (Figs. 4–6, Table 3). As shown in Table 3, among the three compounds tested, indole exhibited the highest antifouling activity against *P. viridis* with an EC_{50} of 24.45 μg/ml. All mussels survived in the DMSO controls and all the treatments of indole and 3-formylindole, indicating that these two compounds inhibited byssal production of mussels via a non-toxic mechanism. In the case of cyclo(Pro-Leu), no mussel mortality was observed in concentrations ranging from 0 to 50 μg/ml and only the concentration of 100 μg/ml exhibited toxicity, showing 80% mussel mortality. Regarding the antifouling activity against barnacles, it was noteworthy that the EC_{50} values of these compounds against *B. albicostatus* settlement were all lower than 25 μg/ml, the standard requirement established by the US Navy program as a potency criterion for natural antifoulants [44]. As shown in

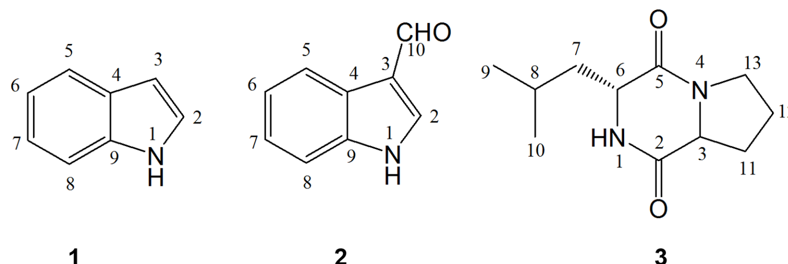

Fig. 3. Chemical structures of indole (**1**), 3-formylindole (**2**), and cyclo(Pro-Leu) (**3**) isolated from the marine bacterium *Vibrio alginolyticus* PE2.

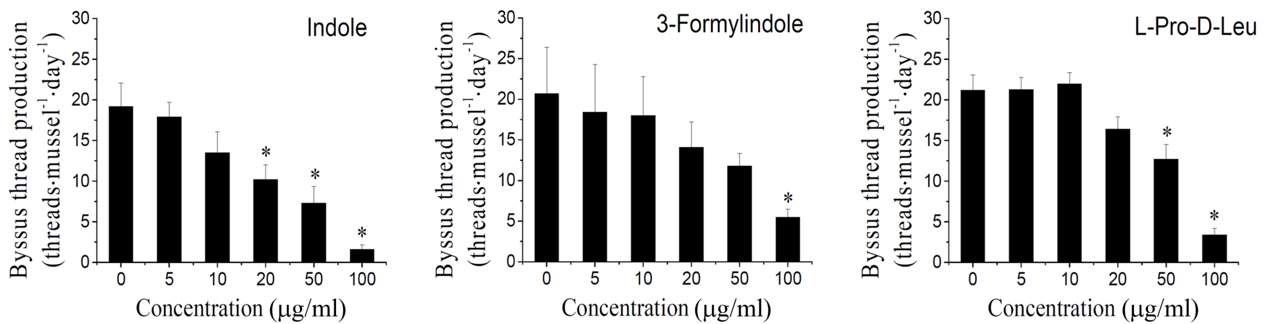

Fig. 4. Effects of indole, 3-formylindole, and cyclo(Pro-Leu) on byssal thread production of *Perna viridis*.
Data shown are the means of 10 replicates ± standard error. Data that are significantly different from the control according to a Dunnet test (ANOVA: $p < 0.05$) are indicated by an asterisk above the bars.

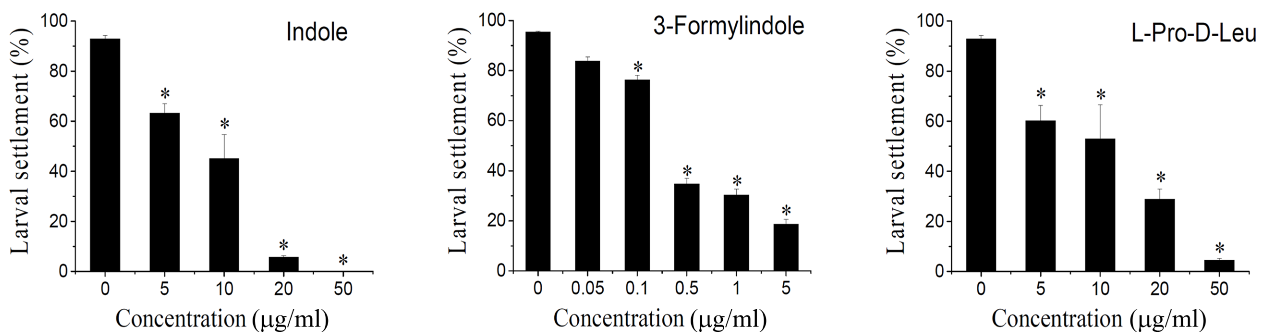

Fig. 5. Effects of indole, 3-formylindole, and cyclo(Pro-Leu) on settlement of *Balanus albicostatus* larvae.
Data shown are the means of three replicates ± standard error. Data that are significantly different from the control according to a Dunnet test (ANOVA: $p < 0.05$) are indicated by an asterisk above the bars.

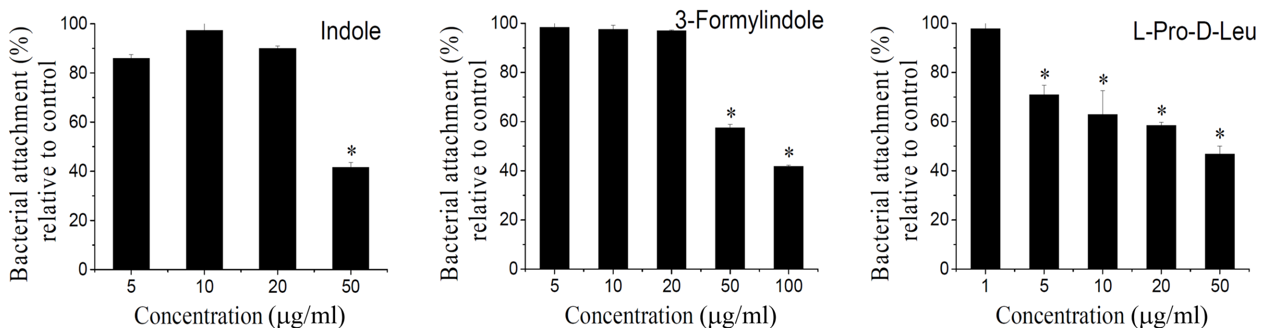

Fig. 6. Effects of indole, 3-formylindole, and cyclo(Pro-Leu) on settlement of *Pseudomonas* sp. TB4.
Data are expressed as settlement (%) relative to the control. Error bars are standard errors. Data that are significantly different from the control according to a Dunnet test (ANOVA: $p < 0.05$) are indicated by an asterisk above the bars.

Fig. 5 and Table 3, 3-formylindole had the most pronounced antilarval settlement activity against *B. albicostatus*, with an EC_{50} of 0.43 μg/ml. Furthermore, the lowest concentration of 3-formylindole capable of causing significant antifouling activity against barnacles was 0.1 μg/ml (Fig. 5), indicating the potential of this compound in the development of anti-barnacle technology. In the barnacle bioassay, barnacle cyprids showed no mortality in the control group or in any of the indole or cyclo(Pro-Leu) treatment groups. Weak toxicity to barnacle larvae was observed only in the treatments of 3-formylindole, at 1 and 5 μg/ml (with mortality rates of 16.1% and 26.1%, respectively). In the bacterium bioassay, based on the EC_{50} values, the potency rankings for inhibition of bacterial settlement among the

Table 3. Antifouling activity of indole, 3-formylindole, and cyclo(Pro-Leu) towards the mussel *Perna viridis*, the larvae of the barnacle *Balanus albicostatus*, and the marine bacterium *Pseudomonas* sp. TB4.

EC_{50} (μg/ml)	Compound		
	Indole	3-Formylindole	Cyclo(Pro-Leu)
Mussel bioassay	24.45 (24.07–24.84)	50.07 (48.98–51.19)	49.24 (48.52–49.96)
Barnacle bioassay	8.84 (8.68–9.00)	0.43 (0.41–0.44)	11.35 (11.05–11.65)
Bacterium bioassay	42.68 (42.02–43.35)	69.68 (64.47–75.32)	39.05 (36.73–41.52)

The data are expressed as EC_{50} with 95% confidence limits.

compounds, starting with the most potent, was cyclo(Pro-Leu) > indole > 3-formylindole (Table 3). No significant differences in bacterial growth were observed between the compound treatments and control after 3 h of incubation.

The only difference between the structures of indole and 3-formylindole is the presence of the formyl group in 3-formylindole (Fig. 3), but there is an obvious difference in the antifouling activity between these two compounds (Table 3). As shown in Table 3, the EC_{50} of indole against the mussel *P. viridis* was lower than that of 3-formylindole, and the same was observed in the bioassay with the bacterium *Pseudomonas* sp., indicating that the antifouling activities of indole against *P. viridis* and *Pseudomonas* sp. were more pronounced than those of 3-formylindole. However, the EC_{50} of indole against the barnacle *B. albicostatus* was greater than that of 3-formylindole, indicating that the antifouling activity of indole against *B. albicostatus* was lower than that of 3-formylindole. Analysis of the structure-activity relationship of indole and 3-formylindole suggested that the formyl group at position 3 in 3-formylindole reduces its antifouling activity towards *P. viridis* and *Pseudomonas* sp. but enhances the antifouling activity towards *B. albicostatus*.

Discussion

A large number of studies have suggested that marine microorganisms are a potential source of compounds with antimicrobial, antioxidant, antiviral, or antitumor properties [45]. In comparison, the exploration of antifouling activity of compounds from marine microorganisms is rather limited. In this study, three small-molecule antifouling compounds were isolated from a marine bacterial strain, *Vibrio alginolyticus* PE2. *V. alginolyticus* was previously reported to produce an antifouling active polysaccharide with high molecular mass (>200 kDa) [46, 47]. It is possible that *V. alginolyticus* contains a few antifouling compounds with highly different molecular masses. Another reasonable explanation for the different antifouling compounds obtained from *V. alginolyticus* in our study and the previous investigation [46] was that different strains of *V. alginolyticus* were used in these studies, and different strains of the same bacterial species may produce different active compounds [48]. The strain in our study was obtained from the sea anemone *Haliplanella* sp., whereas the strain in the previous research was obtained from the green alga *Ulva reticulata* [46].

Many bacterial species can produce indole, which is a product of tryptophan metabolism [49]. It was recently suggested that indole can act as an intercellular signal molecule and play a role in many aspects of bacterial physiology, including drug resistance, virulence induction, plasmid stability, metabolic control, and biofilm formation [49−51]. In vertebrates, indolamines, a class of biogenic amines that contain an indole ring and an amine group, have important physiological functions [52]. For example, serotonin, an indolamine, is a well-known neurotransmitter [53]. The various biological activities of indole have received increasing attention. Yang *et al*. [54] reported that indole is antibacterial and antidiatom active. Wang *et al*. [55] also found that indole inhibited larval settlement of the barnacle *Balanus amphitrite* and the bryozoan *Bugula neritina*. The activity of indole towards mussels revealed here suggests its promising potential for mussel control for the first time. Some indole derivatives with antifouling activity have been isolated from marine organisms, including a marine bryozoan [56], a bacterium [57], fungi [12], and a gorgonian [58]. Here, one simple indole derivative, 3-formylindole, was isolated from the marine bacteria *V. alginolyticus* PE2. Olguin-Uribe *et al*. [57] also isolated this compound from an *Acinetobacter* sp. bacterium associated with the ascidian *Stomozoa murrayi* and found it inhibited the settlement of *B. amphitrite* with an EC_{50} of 28 μg/ml. This study identified a new biological source of 3-formylindole and was the first to find its antifouling activity against mussel and a bacterium. Since fouling communities are made up of diverse organisms, a compound that inhibits only one fouling organism may not be effective against other foulers. The results here revealed the antifouling activity of 3-formylindole against

several different biofoulers.

Diketopiperazines, found mainly in microorganisms [59], are an important class of secondary metabolites with biological activities, including antitumor, antifungal, and antifouling activities [60−62]. Cyclo(Pro-Leu) is a diketopiperazine compound. To our best knowledge, this is the first report to isolate cyclo(Pro-Leu) from the marine bacterium *V. alginolyticus*. Cyclo(Pro-Leu) has been documented to be active in inhibiting larval settlement of the barnacle *B. amphitrite* [60]. Here, it was shown that cyclo(Pro-Leu) can also inhibit byssal production of mussel and bacterial settlement, demonstrating its antifouling efficacy against different fouling organisms.

It has been suggested that bacteria can play important roles in the development and evolution of eukaryotic hosts [63]. Furthermore, some bacteria have been shown to produce biologically active compounds that may have ecological functions for the hosts [64]. The bacterium *V. alginolyticus*, although receiving a lot of attention for being a pathogen in marine aquaculture [65, 66], has been found to be associated with many marine organisms, such as macroalgae [46, 67], sponges [68], cnidarians [69], mussels [70], and fish [71]. It is possible that in the association of *V. alginolyticus* with marine higher organisms, the biologically active substances produced by *V. alginolyticus* may be beneficial to the hosts, perhaps by acting as antifouling compounds for chemical defense of the hosts against biofoulers.

In summary, three small-molecule compounds, indole, 3-formylindole, and cyclo(Pro-Leu) that exhibited antifouling activities against both micro- and macrofouling organisms, were isolated from the marine bacterium *V. alginolyticus*. Although the anti-settlement activity of these three compounds against the barnacle *B. albicostatus* (EC_{50} 0.43–11.35 μg/ml) was far lower than that of the most potent natural product reported, bufalin (a component of the secretory substance of *Bufo vulgaris*; EC_{50} 10 ng/l) against the barnacle *B. amphitrite* [72, 73], they all meet the standard requirement by the US Navy program: For a compound to be considered a promising natural antifoulant, its EC_{50} should be lower than 25 μg/ml [44]. It was especially noteworthy that they all showed significant inhibitive effects on byssal production of mussels. The three simple compounds described herein (or their derivatives synthesized based on their simple structures) may be useful for the development of environmentally friendly mussel control and/or new antifouling agents effective against several biofoulers with commercial applicability, as indole and 3-formylindole are commercially available in large scales, and cyclo(Pro-Leu) can be produced in large amounts through fermentation technology.

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References

1. Callow JA, Callow ME. 2011. Trends in the development of environmentally friendly fouling-resistant marine coatings. *Nat. Commun*. **2:** 244-253.
2. Schultz M, Bendick J, Holm E, Hertel W. 2011. Economic impact of biofouling on a naval surface ship. *Biofouling* **27:** 87-98.
3. Yebra DM, Kiil S, Dam-Johansen K. 2004. Antifouling technology — past, present and future steps towards efficient and environmentally friendly antifouling coatings. *Prog. Org. Coat*. **50:** 75-104.
4. Guerin T, Sirot V, Volatier JL, Leblanc JC. 2007. Organotin levels in seafood and its implications for health risk in high-seafood consumers. *Sci. Total Environ*. **388:** 66-77.
5. van Wezel AP, van Wlaardingen P. 2004. Environmental risk limits for antifouling substances. *Aquat. Toxicol*. **66:** 427-444.
6. Fusetani N. 2011. Antifouling marine natural products. *Nat. Prod. Rep*. **28:** 400-410.
7. Qian PY, Xu Y, Fusetani N. 2010. Natural products as antifouling compounds: recent progress and future perspectives. *Biofouling* **26:** 223-234.
8. Qian PY, Li Z, Xu Y, Li Y, Fusetani N. 2015. Marine natural products and their synthetic analogs as antifouling compounds: 2009-2014. *Biofouling* **31:** 101-122.
9. Dash S, Jin C, Lee OO, Xu Y, Qian PY. 2009. Antibacterial and antilarval-settlement potential and metabolite profiles of novel sponge-associated marine bacteria. *J. Ind. Microbiol. Biotechnol*. **36:** 1047-1056.
10. Dobretsov S, Dahms HU, Qian PY. 2006. Inhibition of biofouling by marine microorganisms and their metabolites. *Biofouling* **22:** 43-54.
11. Dobretsov S, Xiong H, Xu Y, Levin LA, Qian PY. 2007.

Novel antifoulants: inhibition of larval attachment by proteases. *Mar. Biotechnol*. **9:** 388-397.
12. He F, Han Z, Peng J, Qian PY, Qi SH. 2013. Antifouling indole alkaloids from two marine derived fungi. *Nat. Prod. Commun*. **8:** 329-332.
13. Shao CL, Xu RF, Wang CY, Qian PY, Wang KL, Wei MY. 2015. Potent antifouling marine dihydroquinolin-2(1*H*)-one-containing alkaloids from the gorgonian coral-derived fungus *Scopulariopsis* sp. *Mar. Biotechnol*. **17:** 408-415.
14. Hosler DM. 2011. Early detection of dreissenid species: zebra/quagga mussels in water systems. *Aquat. Invasions* **6:** 217-222.
15. Jenner HA, Whitehouse JW, Taylor CJL, Khalanski M. 1998. Cooling water management in European power stations: biology and control of fouling. *Hydroécol. Appl*. **10:** 1-225.
16. Rajagopal S, Venugopalan VP, Van der Velde G, Jenner HA. 2003. Tolerance of five species of tropical marine mussels to continuous chlorination. *Mar. Environ. Res*. **55:** 277-291.
17. Idaho Invasive Species Council. 2009. Estimated potential economic impact of zebra and quagga mussel introduction into Idaho. Available from http://www.aquaticnuisance.org/wordpress/wpcontent/uploads/2009/01/Estimated-Economic-Impact-of-Mussel-Introduction-to-Idaho-Final.pdf. Accessed Oct. 28, 2014.
18. Rajagopal S, Van der Gaag M, Van der Velde G, Jenner HA. 2002. Control of brackish water fouling mussel, *Mytilopsis leucophaeata* (Conrad), with sodium hypochlorite. *Arch. Environ. Contam. Toxicol.* **43:** 296-300.
19. Allonier AS, Khalanski M, Camel V, Bermond A. 1999. Characterization of chlorination by-products in cooling effluents of coastal nuclear power stations. *Mar. Pollut. Bull*. **38:** 1232-1241.
20. Kwong TFN, Miao L, Li X, Qian PY. 2006. Novel antifouling and antimicrobial compound from a marine-derived fungus *Ampelomyces* sp. *Mar. Biotechnol*. **8:** 634-640.
21. Qi SH, Xu Y, Xiong HR, Qian PY, Zhang S. 2009. Antifouling and antibacterial compounds from a marine fungus *Cladosporium* sp. F14. *World J. Microbiol. Biotechnol.* **25:** 399-406.
22. Collins CH, Lyne PM, Grange JM. 1989. *Collins and Lyne's Microbiological Methods*. Butterworth, London & Boston.
23. Chen YG, Cui XL, Pukall R, Li HM, Yang YL, Xu LH, *et al*. 2007. *Salinicoccus kunmingensis* sp. nov., a moderately halophilic bacterium isolated from a salt mine in Yunnan, south-west China. *Int. J. Syst. Evol. Microbiol*. **57:** 2327-2332.
24. Smibert RM, Krieg NR. 1994. Phenotypic characterization, pp. 607-654. *In* Gerhardt P, Murray RGE, Wood WA, Krieg NR (eds.), *Methods for General and Molecular Bacteriology*. American Society for Microbiology, Washington, D.C. USA.
25. Bruns A, Rohde M, Berthe-Corti L. 2001. *Muricauda ruestringensis* gen. nov., sp. nov., a facultatively anaerobic, appendaged bacterium from German North Sea intertidal sediment. *Int. J. Syst. Evol. Microbiol*. **51:** 1997-2006.
26. Sambrook J, Fritsch EF, Maniatis TF. 1989. *Molecular Cloning: A Laboratory Manual*, 2nd Ed. Cold Spring Harbor Laboratory Press, New York. USA.
27. Pascual J, Macián MC, Arahal DR, Garay E, Pujalte MJ. 2010. Multilocus sequence analysis of the central clade of the genus *Vibrio* by using the 16S rRNA, *recA, pyrH, rpoD, gyrB, rctB* and *toxR* genes. *Int. J. Syst. Evol. Microbiol*. **60:** 154-165.
28. Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F, Higgins DG. 1997. The CLUSTAL_X Windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Res*. **25:** 4876-4882.
29. Saitou N, Nei M. 1987. The neighbour-joining method: a new method for reconstructing phylogenetic trees. *Mol. Biol. Evol*. **4:** 406-425.
30. Kumar S, Nei M, Dudley J, Tamura K. 2004. MEGA4: molecular evolutionary genetics analysis MEGA software version 4.0. *Mol. Biol. Evol*. **24:** 1596-1599.
31. Felsenstein J. 1985. Confidence limits on phylogenies: an approach using the bootstrap. *Evolution* **39:** 783-791.
32. Van Winkle WV. 1970. Effects of environmental factors on byssal thread formation. *Mar. Biol*. **7:** 143-148.
33. Rajagopal S, Venugopalan VP, van der Velde G, Jenner HA. 2003b. Response of fouling brown mussel, *Perna perna* (L.), to chlorine. *Arch. Environ. Contam. Toxicol*. **44:** 369-376.
34. Hamilton MA, Russo RC, Thurston RV. 1977. Trimmed Spearman–Karber method for estimating median lethal concentrations in toxicity bioassays. *Environ. Sci. Technol*. **11:** 714-719.
35. Hamilton MA, Russo RC, Thurston RV. 1978. Correction to: Hamilton MA, Russo RC, Thurston RV. 1977. Trimmed Spearman–Karber method for estimating median lethal concentrations in toxicity bioassays. *Environ. Sci. Technol*. **11:** 714-719. *Environ. Sci. Technol*. **12:** 417.
36. Reichelt-Brushett AJ, Michalek-Wagner K. 2005. Effects of copper on the fertilization success of the soft coral *Lobophytum compactum*. *Aquat. Toxicol*. **74:** 280-284.
37. Feng DQ, Ke CH, Lu CY, Li SJ. 2009. Herbal plants as a promising source of natural antifoulants: evidence from barnacle settlement inhibition. *Biofouling* **25:** 181-190.
38. Kelly SR, Jensen PR, Henkel TP, Fenical W, Pawlik JR. 2003. Effects of Caribbena sponge extracts on bacterial attachment. *Aquat. Microb. Ecol*. **31:** 175-182.
39. Cano-Gomez A, Goulden EF, Owens L, Høj L. 2010. *Vibrio owensii* sp. nov., isolated from cultured crustaceans in Australia. *FEMS Microbiol. Lett*. **302:** 175-181.
40. Thompson FL, Gevers D, Thompson CC, Dawyndt P, Naser S, Hoste B, *et al*. 2005. Phylogeny and molecular identification of vibrios on the basis of multilocus sequence analysis. *Appl. Environ. Microbiol*. **71:** 5107-5115.
41. Devi P, Wahidullah S, Rodrigues C, Souza LD. 2010. The sponge-associated bacterium *Bacillus licheniformis* SAB1: a source of antimicrobial compounds. *Mar. Drugs* **8:** 1203-1212.
42. Zan K, Chen XQ, Fu Q, Zhou SX, Xiao MT, Wen J, Tu PF. 2010. Chemical ingredients isolated from the aerial parts of

Artemisia anomala. *J. Chin. Pharm. Sci*. **19:** 95-99.

43. Kumar SN, Mohands C, Siji JV, Rajasekharan KN, Nambisan B. 2012. Identification of antimicrobial compound, diketopiperazines, from a *Bacillus* sp. N strain associated with a rhabditid entomopathogenic nematode against major plant pathogenic fungi. *J. Appl. Microbiol*. **113:** 914-924.
44. Rittschof D. 2001. Natural product antifoulants and coatings developments, pp. 543-566. *In* McClintock JB, Baker BJ (eds.), *Marine Chemical Ecology*. CRC Press, Boca Raton. USA.
45. Debbab A, Aly AH, Lin WH, Proksch P. 2010. Bioactive compounds from marine bacteria and fungi. *Microb. Biotechnol*. **3:** 544-563.
46. Harder T, Dobretsov S, Qian PY. 2004. Waterborne polar macromolecules act as algal antifoulants in the seaweed *Ulva reticulata*. *Mar. Ecol. Prog. Ser.* **274:** 133-141.
47. Wahl M, Goecke F, Labes A, Dobretsov S, Weinberger F. 2012. The second skin: ecological role of epibiotic biofilms on marine organisms. *Front. Microbiol*. **3:** 292.
48. Reichenbach H, Dworkin M. 1992. The Myxobacteria. 1992, pp. 3416-3487. *In* Balows A, Truper GH, Dworkin M, Harder W, Schleifer KH (eds.), *The Prokaryotes. A Handbook on the Biology of Bacteria: Ecophysiology, Isolation, Identijication, Applications*. Springer, New York. USA.
49. Lee JH, Lee J. 2010. Indole as an intercellular signal in microbial communities. *FEMS Microbiol. Rev*. **34:** 426-444.
50. Hu M, Zhang C, Mu Y, Shen Q, Feng Y. 2010. Indole affects biofilm formation in bacteria. *Indian J. Microbiol*. **50:** 362-368.
51. Mueller RS, Beyhan S, Saini SG, Yildiz FH, Bartlett DH. 2009. Indole acts as an extracellular cue regulating gene expression in *Vibrio cholerae*. *J. Bacteriol*. **191:** 3504-3516.
52. Catalá A. 2015. *Indoleamines: Sources, Role in Biological Processes and Health Effects*. Nova Science Publishers Inc., New York. USA.
53. Biello SM, Dafters RI. 2001. MDMA and fenfluramine alter the response of the circadian clock to a serotonin agonist in vitro. *Brain Res*. **920:** 202-209.
54. Yang C, Yu Y, Sun W, Xia C. 2014. Indole derivatives inhibited the formation of bacterial biofilm and modulated Ca^{2+} efflux in diatom. *Mar. Pollut. Bull*. **88:** 62-69.
55. Wang KL, Xu Y, Lu L, Li Y, Han Z, Zhang J, *et al*. 2015. Low-toxicity diindol-3-ylmethanes as potent antifouling compounds. *Mar. Biotechnol*. **17:** 624-632.
56. Konya K, Shimidzu N, Adachi K, Miki W. 1994. 2,5,6-Tribromo-1-methylgramine, an antifouling substance from the marine bryozoan *Zoobrotryon pellucidum*. *Fish. Sci*. **60:** 773-775.
57. Olguin-Uribe G, Abou-Mansour E, Boulander A, Débard H, Francisco C, Combaut G. 1997. 6-Bromoindole-3-carbaldehyde, from an *Acinetobacter* sp. bacterium associated with the ascidian *Stomozoa murrayi*. *J. Chem. Ecol*. **23:** 2507-2521.
58. Pénez N, Culioli G, Pérez T, Briand JF, Thomas OP, Blache Y. 2011. Antifouling properties of simple indole and purine alkaloids from the Mediterranean gorgonian *Paramuricea clavata*. *J. Nat. Prod*. **74:** 2304-2308.
59. Kelecom A. 2002. Secondary metabolites from marine microorganisms. *Ann. Brazil. Acad. Sci*. **74:** 151-170.
60. Li X, Dobretsov S, Xu Y, Xiao X, Huang OS, Qian PY. 2006. Antifouling diketopiperazines produced by a deep-sea bacterium, *Streptomyces fungicidicus*. *Biofouling* **22:** 187-194.
61. Nicholson B. 2006. NPI-2358 is a tubulin-depolymerizing agent: in-vitro evidence for activity as a tumor vascular-disrupting agent. *Anticancer Drugs* **17:** 25-31.
62. Nishanth Kumar S, Mohandas C, Nambisan B. 2013. Purification of an antifungal compound, cyclo(L-Pro-D-Leu) for cereals produced by *Bacillus cereus* subsp. *thuringiensis* associated with entomopathogenic nematode. *Microbiol. Res*. **168:** 278-288.
63. Fraune S, Bosch TCG. 2010. Why bacteria matter in animal development and evolution. *Bioessays* **32:** 571-580.
64. Holmström C, Kjelleberg S. 1999. Marine *Pseudoalteromonas* species are associated with higher organisms and produce biologically active extracellular agents. *FEMS Microbiol. Ecol*. **30:** 285-293.
65. Carmen Balebona M, Andreu MJ, Angeles Bordas M, Zorrilla I, Morinigo MA, Borrego JJ. 1998. Pathogenicity of *Vibrio alginolyticus* for cultured gilt-head sea bream (*Sparus aurata* L.). *Appl. Environ. Microbiol*. **64:** 4269-4275.
66. Lee KK. 1995. Pathogenesis studies on *Vibrio alginolyticus* in the grouper, *Epinephelus malabaricus*, Bloch et Schneider. *Microb. Pathog.* **19:** 39-48.
67. Carballeira NM, Sostre A, Stefanov K, Popov S, Kujumgiev A, Dimitrova-Konaklieva S, *et al*. 1997. The fatty acid composition of a *Vibrio alginolyticus* associated with the alga *Cladophora coelothrix*. Identification of the novel 9-methyl-10-hexadecenoic acid. *Lipids* **32:** 1271-1275.
68. Hoffmann M, Fischer M, Ottesen A, McCarthy PJ, Lopez JV, Brown EW, Monday SR. 2010. Population dynamics of *Vibrio* spp. associated with marine sponge microcosms. *ISME J*. **4:** 1608-1612.
69. Chimetto LA, Brocchi M, Gondo M, Thompson CC, Gomez-Gil B, Thompson FL. 2009. Genomic diversity of vibrios associated with the Brazilian coral *Mussismilia hispida* and its sympatric zoanthids (*Palythoa caribaeorum*, *Palythoa variabilis* and *Zoanthus solanderi*). *J. Appl. Microbiol*. **106:** 1818-1826.
70. Hariharan H, Giles JS, Heaney SB, Arsenault G, Mcnair N, Rainnie DJ. 1995. Bacteriological studies on mussels and oysters from six river systems in Prince Edward Island, Canada. *J. Shellfish Res*. **14:** 527-532.
71. Noguchi N, Hwang DF, Arakawa O, Sugita H, Deguchi Y, Shida Y, Hashimoto K. 1987. *Vibrio alginolyticus*, a tetrodotoxin-producing bacterium, in the intestines of the fish *Fugu vermicularis vermicularis*. *Mar. Biol*. **94:** 625-630.
72. Omae I. 2003. General aspects of tin-free antifouling paints. *Chem. Rev*. **103:** 3431-3448.
73. Rittschof D. 2000. Natural product antifoulants: one perspective on the challenges related to coatings development. *Biofouling* **15:** 119-127.