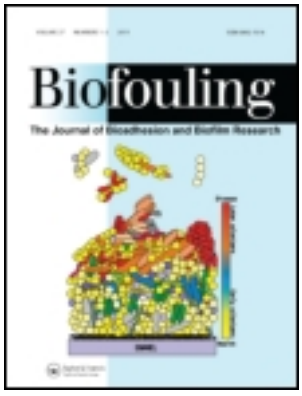

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Inhibition of marine biofouling by bacterial quorum sensing inhibitors

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Seventy eight natural products from chemical libraries containing compounds from marine organisms (sponges, algae, fungi, tunicates and cyanobacteria) and terrestrial plants, were screened for the inhibition of bacterial quorum sensing (QS) using a reporter strain *Chromobacterium violaceum* CV017. About half of the natural products did not show any QS inhibition. Twenty four percent of the tested compounds inhibited QS of the reporter without causing toxicity. The QS inhibitory activities of the most potent and abundant compounds were further investigated using the LuxR-based reporter *E. coli* pSB401 and the LasR-based reporter *E. coli* pSB1075. Midpacamide and tenuazonic acid were toxic to the tested reporters. QS-dependent luminescence of the LasR-based reporter, which is normally induced by N-3-oxo-dodecanoyl-L-homoserine lactone, was reduced by demethoxy encecalin and hymenialdisin at concentrations >6.6 μM and 15 μM, respectively. Hymenialdisin, demethoxy encecalin, microcolins A and B and kojic acid inhibited responses of the LuxR-based reporter induced by N-3-oxo-hexanoyl-L-homoserine lactone at concentrations >0.2 μM, 2.2 μM, 1.5 μM, 15 μM and 36 μM, respectively. The ability to prevent microfouling by one of the compounds screened in this study (kojic acid; final concentrations 330 μM and 1 mM) was tested in a controlled mesocosm experiment. Kojic acid inhibited formation of microbial communities on glass slides, decreasing the densities of bacteria and diatoms in comparison with the control lacking kojic acid. The study suggests that natural products with QS inhibitory properties can be used for controlling biofouling communities.

Keywords: quorum sensing; inhibitors; antifouling; natural products; biofilms

Introduction

In the marine environment, all natural and artificial substrata are quickly colonized by micro- and macroorganisms in a process that is known as biofouling. Micro- and macrofoulers can cause severe industrial problems by increasing drag, promoting metal corrosion and reducing heat transfer efficiency of heat exchangers (Yebra et al. 2006; Schultz et al. 2010). Biocides that are used to control biofouling are not effective against some bacterial and diatom species (Cassé and Swain 2006; Molino et al. 2009a, 2009b; Fay et al. 2010), which colonize entire surfaces coated with antifouling (AF) paints and affect their performance. Therefore, development of new ways to regulate the density of microbes on AF coatings is urgently required.

Interference with bacterial quorum sensing (QS) has been proposed as one potential approach for controlling biofouling (Dobretsov et al. 2009; Choudhary and Schmidt-Dannert 2010; Qian et al. 2010; Xiong and Liu 2010). QS is a population density-dependent gene regulatory mechanism, which relies on the production and perception of threshold concentrations of low molecular weight signal molecules that activate transcriptional regulators (Antunes et al. 2010). In Gram-negative bacteria, QS is affected by N-acyl homoserine lactones (AHLs). These QS molecules are typically produced by the AHL synthethases (homologues of the LuxI) and actively or passively redistributed in the environment (Waters and Bassler 2005; Dickschat 2010). When the intracellular concentration of AHLs reaches a certain threshold level, the AHL molecules bind to the LuxR-type receptor, and this leads to the formation of active dimers allowing interactions with QS-dependent promoter sequences (Boyer and Wisniewski-Dye 2009). Once active complexes within promoter sequences are established, transcription of QS genes responsible for luminescence, biofilm formation, virulence and other relevant processes is effected (Waters and Bassler 2005; Irie and Parsek 2008).

Because bacterial QS is central to the interactions of bacteria with their eukaryotic hosts, it is not surprising that many multicellular organisms evolved

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different mechanisms to interfere with bacterial QS (reviewed by Dobretsov et al. 2009; Goecke et al. 2010). One of the well-studied examples of organisms producing QS inhibitors is the red alga *Delisea pulchra* (Manefield et al. 1999), which produces a suite of halogenated furanones that reduce bacterial adhesion to algal surfaces and inhibit bacterial swarming (Maximilien et al. 1998). Several recent studies have demonstrated that extracts of Great Barrier Reef marine invertebrates (Skindersoe et al. 2008a) and cyanobacteria from Florida waters (Dobretsov et al. 2010) are similarly capable of inhibiting bacterial QS.

It has been proposed that QS inhibitors can be used for antimicrobial protection in aquaculture (Defoirdt et al. 2004; Dobretsov et al. 2009). In the laboratory, it has been shown that synthetic furanones inhibited development of microbial biofilms (Dobretsov et al. 2007). In short-term field and laboratory experiments, furanones produced by *D. pulchra* strongly inhibited attachment of marine bacteria on rocks and seaweeds (Maximilien et al. 1998).

In the present study, 78 natural products from marine invertebrates (mostly sponges), terrestrial plants, fungi and cyanobacteria were screened for the inhibition of bacterial QS reporters. The activities of the most potent and abundant QS inhibitors, such as demethoxy encecalin, midpacamide, tenuazonic acid, hymenialdisin, microcolins A and B and kojic acid, were further investigated using different reporter strains. The AF performance of kojic acid was tested in a mesocosm experiment. The main aims of the study were to investigate: (1) the effects of natural products on QS pigment production in *Chromobacterium violaceum* CV017; (2) the activity of demethoxy encecalin, midpacamide, tenuazonic acid, hymenialdisin, microcolins A and B and kojic acid using the LuxR-based and the LasR-based reporters; and (3) the AF performance of kojic acid in a mesocosm experiment.

Material and methods

Compounds used in this study

All natural products analyzed in this study were previously isolated by the research groups of P. Proksch and V. Paul (Table 1). The compounds were isolated from sponges, tunicates, fungi, plants and cyanobacteria and are representative major groups of natural products. All isolated compounds were fully characterized structurally by mass spectrometry as well as by one and two dimensional NMR spectroscopy (^{1}H, ^{13}C, COSY, HMBC). All compounds were dissolved in methanol (Fisher Scientific, USA) yielding a stock solution (0.2 mg ml^{-1}).

QS inhibition bioassays

A reporter strain *Chromobacterium violaceum* CV017 was used for screening for QS inhibitors. This biosensor strain produces N-hexanoyl homoserine lactone, which induces production of the purple pigment violacein *via* the AHL receptor CviR (Chernin et al. 1998). Methanol solutions of the compounds were added into wells of microtiter plates (Nunc, Denmark), solvents were evaporated and extracts were re-dissolved in 3 μl of dimethyl sulfoxide (DMSO). DMSO in empty wells were used as controls. Experiments were conducted according to Dobretsov et al. (2010). Briefly, bacterial cells from an overnight culture of CV017 were centrifuged and washed with sterile distilled water. Five ml of soft LB agar (Difco) were mixed with 500 μl of washed culture of CV017, and 100 μl of this mixture were applied to each well. The plates were incubated overnight at 30°C. A reduction in violacein production was compared to the control treatments visually. The bioassays were repeated three times and the mean minimum inhibitory concentration (MIC) in μM was calculated.

A toxicity assay was performed according to Dobretsov et al. (2010) in order to test the effect of compounds on the growth of the reporter strain *C. violaceum* CV017. Briefly, solutions of compounds in DMSO were applied onto glass fiber disks (1 cm diameter) and these disks were placed onto LB agar (Difco, USA) inoculated with *C. violaceum* CV017. Growth inhibition around the disk corresponds to antibacterial activity of the compound against the reporter strains. This experiment was repeated three times. The results are expressed as the mean minimal amount of compound in moles that inhibit growth of the reporter. DMSO was used as a control.

In order to further investigate QS inhibitory properties, the most active QS inhibitors were selected. Since some of these inhibitors were isolated in low quantity, only demethoxy encecalin, tenuazonic acid, midpacamide, hymenialdisin and microcolins A and B (Figure 1, Table 1) were used in this study. Additionally, the QS inhibitory properties of kojic acid, a compound that was used for the mesocosm experiment (see below) were evaluated. Several bioassays were performed using *E. coli*-based reporters. Before the bioassays, all compounds were re-dissolved in DMSO. Midpacamide was tested at 0.4–46 μM, tenuazonic acid was assayed at 0.1–102 μM, demethoxy encecalin at 0.04–20 μM, hymenialdisin at 0.007–150 μM, microcolins A and B at 0.015–150 μM and kojic acid at 4–330 μM. Possible toxic effects of compounds on metabolic activity or luminescence of the reporters were tested using a

control construct containing a pTIM2442 plasmid in *E. coli* DH5α (Alagely et al. 2011), in which the *lux* cassette is controlled by a constitutive phage lambda promoter. Compounds were tested at maximal inhibitory concentrations. Midpacamide was tested at 46 μM, tenuazonic acid was tested at 102 μM, demethoxy encecalin at 20 μM, hymenialdisin at 150 μM, microcolins A and B at 150 μM and kojic acid at 330 μM. This experiment was performed with eight replicates. For the LasR-based bioassay (*Pseudomonas aeruginosa* LasR/LasI QS system) the LasR-based reporter (pSB1075) was used (Winson et al. 1998), which emits light in response to AHLs with long (>C10) acyl side chains. Both direct and indirect bioassays with non-toxic compounds were preformed according to Alagely et al. (2011). In the direct bioassays, the reporter *E. coli* pSB1075 was exposed to the compounds dissolved in DMSO; in indirect bioassays, N-3-oxo-dodecanoyl-L-homoserine lactone (3-oxo-C12-HSL) (final concentration of 2 μM) was also added in order to stimulate QS for this reporter. This experiment was conducted twice with eight replicates each time; only the data for the second experiment are included. Additionally, the effect of non-toxic compounds on QS was tested in a LuxR-based reporter *E. coli* pSB401 (Winson et al. 1998) that contained the *luxR* P_{luxI}-*luxCDABE* transcriptional fusion which emits light in response to AHLs with medium (C6–10) acyl side chains. In the direct bioassays, the reporter *E. coli* pSB401 was exposed to the compounds of interest dissolved in DMSO. In the indirect bioassay, N-3-oxo-hexanoyl-L-homoserine lactone (3-oxo-C6-HSL) (final concentration of 10 μM) was also added in order to stimulate QS of pSB401. The experiment was performed twice with eight replicates each time, and the data for the second experiment are reported. Since the reporters pSB1075 and pSB401 could give variable counts per second (CPS) for a number of technical and biological reasons (see Alagely et al. 2011), two DMSO controls viz. positive (reporters with AHLs) and negative (reporters without AHLs) were included. All dilutions of tested compounds were assayed together with the same positive and negative controls and replicated eight times. For all bioassays, compounds were added to the wells of a black microtiter plate and serially diluted. Reporter suspensions (in LB broth) were thoroughly mixed with 3% DMSO solutions of compounds. Luminescence and optical density of the reporter suspensions (OD_{595}) were measured every hour using a multimode microtiter plate reader Victor–3 (Perkin Elmer). The data obtained in direct and indirect bioassays are presented as 'relative bioluminescence' in order to take into account the population density of the reporters. To calculate relative bioluminescence (RB), the following formula was used:

$$RB = (B_s)/OD_{595}$$

where B_s is bioluminescence of each sample measured in CPS, and OD_{595} is optical density of the reporter culture measured at 595 nm. The differences between the treatments and the positive control were compared by ANOVA followed by a Dunnet test (Zar 1996).

Mesocosm experiment

Since several grams of QS inhibitor were required for the mesocosm experiment, kojic acid, a commercially available QS inhibitor discovered within this study was selected. Kojic acid was dissolved in unfiltered seawater from the Marina Bandar Rawdha (Muscat, Oman) to give final concentrations of 330 μM and 1 mM. Three 3 l sterile plastic containers were filled with 1 l of the Kojic acid solutions. One sterile microscope slide (size 25 × 75 mm) was immersed horizontally in each container. Sterile glass slides placed into 1 l of unfiltered seawater from the Marina Bandar Rawdha were used as a control. Each treatment was replicated three times and the experiment was conducted twice. Each experiment was analyzed separately. Containers with slides were kept under continuous illumination (light intensity 2500 lux) in controlled conditions (temperature = 25°C) for 7 days. At the end of experiment, slides were taken out and fixed with 3% formaldehyde solution in seawater. The slides were stained with the DNA-binding fluorochrome 4,6-diamidino-2-phenylindole (DAPI, Fluka Chemie AG, Switzerland) solution (0.5 mg ml^{-1}). The number of bacteria in 10 randomly selected fields of view was counted under an epifluorescence microscope (Axiophot, Zeiss, Germany; magnification 1000×; λ_{Ex} = 359 nm, λ_{Em} 441 nm). The number of diatoms in 10 randomly selected fields of view was counted under a microscope (Nikon Eclipse, USA; magnification 400×). For counting bacteria and diatoms, tables of random x and y coordinates were generated using MS Excel and these were used to select random field of views. Treatments were coded; codes were masked prior to the scoring of the treatments. Densities of bacteria and diatoms were log-transformed to normalize the data. The normality assumption was verified with the Shapiro-Wilk test (Zar 1996). Differences in densities of microorganisms between the treatments and the control were compared by ANOVA followed by a Dunnet test (Zar 1996).

Table 1. Origin of the natural products tested and their effect on quorum sensing and growth of *C. violaceum* CV017.

Abbreviation	Name	Origin		Groups of compounds	Reference	QS (μM)	Growth (mole $\times$ 10^{-6})
		Species	Group				
			First group of compounds				
1	Demethoxy encecalin	*Baccharis cassinaefolia*	Plant	Benzopyran	Proksch and Rodriguez (1982)	3.92 ± 1.11	–
2	Orientin	*Polygonum orientale*	Plant	Flavonoid	Weber (2007)	4.46 ± 1.3	–
3	Kuanoniamin D	*Cystodytes* sp.	Tunicate	Alkaloid	Eder et al. (1998)	5.55 ± 3.11	–
4	Malyngamide A	*Lyngbya majuscula*	Cyanobacterium	Amide	Cardellina et al. (1979)	7.46 ± 2.11	–
5	Ageliferin	*Agelas conifera*	Sponge	Alkaloid	Hertiani et al. 2010	11.29 ± 1.93	–
6	Microcolin A	*Lyngbya* sp.	Cyanobacterium	Peptide	Koehn et al. (1992)	15.23 ± 2.75	–
7	Mauritamide B	*Agelas nakamurai*	Sponge	Alkaloid	Hertiani et al. (20100	36.76 ± 4.47	–
8	Pinoresinol	*Pinus* ssp.	Plant	Lignan	Weber (2007)	41.85 ± 6.13	–
9	Microcolin B	*Lyngbya* sp.	Cyanobacterium	Peptide	Koehn et al. (1992)	43.21 ± 3.14	–
10	Gallic acid	*Plants*	Plant	Phenol	Bayer (2009)	64.66 ± 12.27	–
11	Glucobrassicin	*Brassica napus* var. Napus	Plant	Alkaloid	Weber (2007)	133.71 ± 16.39	–
12	Meleagrin	*Penicillium chrysogenum*	Fungus	Alkaloid	Rusman (2006)	138.42 ± 22.18	–
13	Alterporriol E	*Alternaria porri*	Fungus	Polyketide	Aly et al. (2008)	298.26 ± 57.30	–
14	Kojic acid	*Aspergillus* spp.	Fungus	Pyranone	Indriani (2008)	239.25 ± 8.92	–
15	4-(4,5-dibromo–1-methyl-1H-pyrrole-2-carboxamido) butanoic acid	*Agelas* sp.	Sponge	Alkaloid	Hertiani et al. (2010)	271.73 ± 9.13	–
16	Hymenialdisin	*Hymeniacidon aldis*	Sponge	Alkaloid	Supriyono et al. (1995)	308.52 ± 8.19	–
17	Dulcitol	*Spatoglossum sp.*	Brown alga	Sugar	Queiroz et al. (2008)	380.02 ± 56.23	–
18	Midpacamide	*Agelas mauritiana*	Sponge	Alkaloid	Hertiani et al. (2010)	458.61 ± 34.11	–
19	Tenuazonic acid	*Alternaria tenuis*	Fungus	Alkaloid	Hassan (2007)	517.03 ± 39.71	–
			Second group of compounds				
20	Malyngamide B	*Lyngbya majuscula*	Cyanobacterium	Amide	Cardellina et al. (1978)	5.89 ± 1.77	17.27 ± 1.26
21	(+)Avarol	*Dysidea avara*	Sponge	Terpene	Putz (2009)	6.36 ± 2.78	0.40 ± 0.04
22	Alternariol monomethyl ether	*Alternaria* sp.	Fungus	Polyketide	Aly et al. (2008)	7.32 ± 1.25	7.35 ± 1.16
23	Aaptamin	*Aaptos aaptos*	Sponge	Alkaloid	Supriyono (1997)	8.76 ± 2.38	0.61 ± 0.02
24	8-OH-manzamien A	*Acanthostrongylophore ingens*	Sponge	Alkaloid	Edrada (1998)	12.41 ± 1.34	0.50 ± 0.03
25	Lyngbyastatin 3	*Lyngbya majuscula*	Cyanobacterium	Peptide	Williams et al. (2003)	12.00 ± 3.41	16.19 ± 1.67
26	Aeroplysinin	*Aeroplysisnin* sp.	Sponge	Alkaloid	Ebel (1997)	16.55 ± 5.51	0.31 ± 0.02
27	(−) Dibromophakelline	*Pseudaxinyssa cantharella*	Sponge	Alkaloid	Hertiani (2007)	17.99 ± 5.10	0.46 ± 0.05
28	Alterlactone	*Alternaria* sp.	Fungus	Polyketide	Aly et al. (2008)	24.31 ± 5.29	1.74 ± 0.13
29	Emodin	*Rhamnus purshiana*	Plant	Polyketide	Debbab (2007)	25.90 ± 5.14	0.93 ± 0.04
30	Encecalin	*Eupatorium californica*	Plant	Benzopyran	Proksch and Rodriguez (1982)	30.12 ± 6.39	1.29 ± 0.09
31	Agelanesin C	*Agelas* sp.	Sponge	Alkaloid	Hertiani et al. (2010)	36.30 ± 8.94	0.91 ± 0.01
32	Cyclo Colorenon (1)	*Porella vernicosa*	Plant	Terpene	Handayani (1998)	91.60 ± 16.22	1.38 ± 0.04
33	Aerothionin	*Aplysina aerophoba*	Sponge	Alkaloid	Ebel (1997)	244.47 ± 19.71	0.27 ± 0.02
34	(−) Agelasidine D	*Agelas clathrodes*	Sponge	Alkaloid	Hertiani et al. (2010)	454.90 ± 14.22	0.46 ± 0.06
35	Altersolanol A	*Alternaria solani*	Fungus	Polyketide	Aly et al. (2008)	594.71 ± 48.39	0.41 ± 0.04
			Third group of compounds				
36	Curacin D	*Lyngbya majuscula*	Cyanobacterium	Lipid	Marquez et al. (1998)	–	69.88 ± 3.54
37	Alternariol sulphate	*Alternaria* sp.	Fungus	Polyketide	Aly et al. (2008)	–	0.59 ± 0.06
			Fourth group of compounds				
38	Alteric acid	*Alternaria* sp.	Fungus	Polyketide	Aly et al. (2008)	–	–
39	Alternariol	*Alternaria tenuis*	Fungus	Polyketide	Aly et al. (2008)	–	–
40	Altenuene 4′-Epialtenuene	*Alternaria* sp.	Fungus	Polyketide	Aly et al. (2008)	–	–
41	Altenusin	*Alternaria tenuis*	Fungus	Polyketide	Aly et al. (2008)	–	–

(*continued*)

Table 1. (*Continued*).

Abbreviation	Name	Origin		Groups of compounds	Reference	QS (μM)	Growth (mole $\times$ 10^{-6})
		Species	Group				
42	Alterporriol D	*Alternaria porri*	Fungus	Polyketide	Aly et al. (2008)	–	–
43	Ampelanol	*Ampelomyces* sp.	Fungus	Polyketide	Hassan (2007)	–	–
44	Aposhaerin A	*Aposphaeria* sp.	Fungus	Polyketide	Hassan (2007)	–	–
45	Atromentine	*Aglaia odorata*	Fungus	Phenol	Duong (2006)	–	–
46	Chaetomin	*Chaetomium cochliodes*	Fungus	Alkaloid	Aly et al. (2008)	–	–
47	Cochliodinol	*Chaetomium globosum*	Fungus	Alkaloid	Aly et al. (2008)	–	–
48	Citrinin	*Penicillium citrinum*	Fungus	Phenol	Hiort et al. (2004)	–	–
49	Cyclo(L-Pro-L-Val)	*Aspergillus flavipes*	Fungus	Peptide	Indriani (2008)	–	–
50	Cyclo(L-Tyr-L-Pro)	*Alternaria alternata*	Fungus	Peptide	Indriani (2008)	–	–
51	Cytochalasin E	*Rosellinia necatrix*	Fungus	Alkaloid	Indriani (2008)	–	–
52	Equisetin	*Fusarium equiseti*	Fungus	Alkaloid	Kjer (2010)	–	–
53	Ageraton	*Ageratum houstonianum*	Plant	Benzofuran	Kunze (1995)	–	–
54	Aglaiol	*Aglaia odorata*	Plant	Terpene	Duong (2006)	–	–
55	(+) Curcudiol	*Didiscus flavus*	Sponge	Terpene	Hertiani (2007)	–	–
56	Piscidinol A	*Phellodendron chinense*	Plant	Terpene	Duong (2006)	–	–
57	Septicine	*Tylophora asthmatica*	Plant	Alkaloid	Moustafa (2009)	–	–
58	Ellagic acid	Plants	Plant	Phenol	Bayer (2009)	–	–
59	Agelasine I	*Agelas* sp.	Sponge	Alkaloid	Hertiani et al. (2010)	–	–
60	Agelanesin A	*Agelas* sp.	Sponge	Alkaloid	Hertiani et al. (2010)	–	–
61	Agelanesin B	*Agelas* sp.	Sponge	Alkaloid	Hertiani et al. (2010)	–	–
62	Agelanin A	*Agelas* sp.	Sponge	Alkaloid	Hertiani et al. (2010)	–	–
63	Agelanin B	*Agelas* sp.	Sponge	Alkaloid	Hertiani et al. (2010)	–	–
64	(+) Agelasidine-C	*Agelas nakamurai*	Sponge	Alkaloid	Hertiani et al. (2010)	–	–
65	Agelongine	*Agelas longissima*	Sponge	Alkaloid	Hertiani et al. (2010)	–	–
66	(−)-Ageloxime D	*Agelas nakamurai*	Sponge	Alkaloid	Hertiani et al. (2010)	–	–
67	Aldisine	*Hymeniacidon aldis*	Sponge	Alkaloid	Hertiani (2007)	–	–
68	Aplysamine-2	*Psammaplysilla purpurea*	Sponge	Alkaloid	Hertiani (2007)	–	–
69	E/Z-aplysinopsin	*Verongia spengelii*	Sponge	Alkaloid	Hertiani (2007)	–	–
70	(+)Avarone	*Dysidea avara*	Sponge	Terpene	Putz (2009)		–
71	Bastadin- 4	*Ianthella basta*	Sponge	Alkaloid	Ortlepp et al. (2007)	–	–
72	2 Bromoaldisine	*Hymeniacidon aldis*	Sponge	Alkaloid	Hertiani (2007)	–	–
73	4-(4-Bromo-1H-pyrrole-2-carboxamido) butanoic acid	*Agelas nakamurai*	Sponge	Alkaloid	Hertiani (2007)	–	–
74	4-Bromopyrrole-2-carboxamide	*Agelas nakamurai*	Sponge	Alkaloid	Hertiani (2007)	–	–
75	Dienone dimethoxyketal	*Pseudoceratina purpura*	Sponge	Alkaloid	Fendert (2000)	–	–
76	Hymenidin	*Agelas clathrodes*	Sponge	Alkaloid	Supriyono (1997)	–	–
77	Mauritamide C	*Agelas nakamurai*	Sponge	Alkaloid	Hertiani et al. (2010)	–	–
78	Dragonamide C	*Lyngbya* cf. polychroa	Cyanobacterium	Peptide	Gunasekera et al. (2008)	–	–

Note: Quorum sensing inhibition is reported as the mean of three replicates $\pm$ SD (MIC) (μM). The toxicity effect of compounds is presented as a minimal amount of natural product necessary to inhibit growth of the reporter strain. Compounds are sorted according to their bioactivity. The first group contains compounds that only inhibited QS of *C. violaceum* CV017; the second group includes compounds that inhibited QS of *C. violaceum* CV017, but had some toxicity; the third group represents compounds that inhibited growth of CV017; the fourth group includes compounds that did not have any bioactive properties.

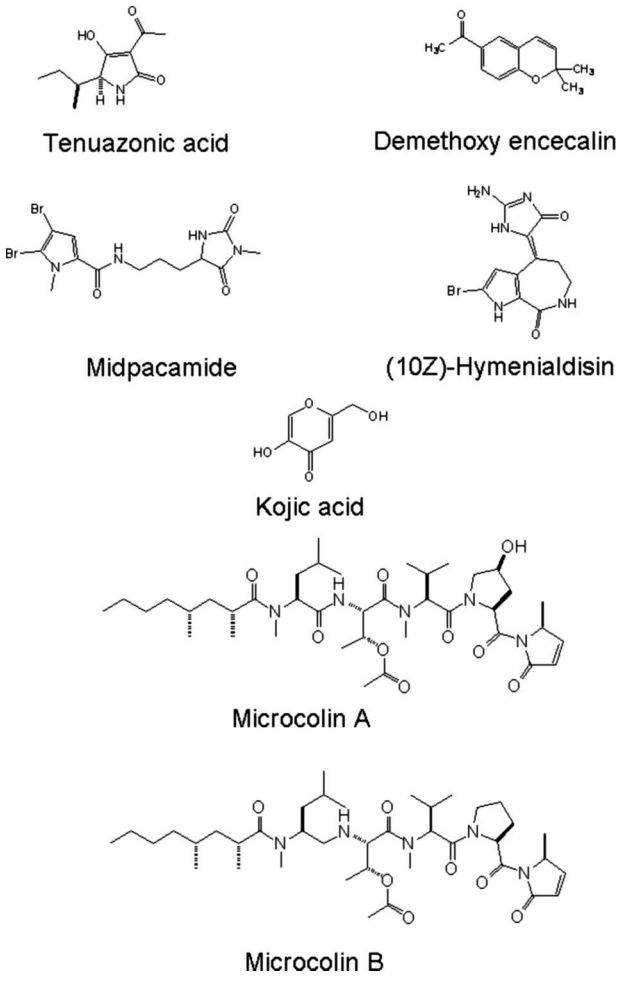

Figure 1. Structures of tenuazonic acid, demethoxy encecalin, midpacamide, (10Z)-hymenialdisin, microcolins A and B and kojic acid.

Results

QS reporter inhibition bioassays

All tested compounds (Table 1) can be separated into four groups according to their bioactivity. The first group contains compounds that only inhibited QS of *C. violaceum* CV017. The second group includes compounds that inhibited QS of *C. violaceum* CV017 and had some antibiotic properties. The third group represents compounds that only inhibited growth of CV017. The fourth group includes compounds that did not have any bioactive properties in the bioassays. A high proportion (51%) of the natural products did not show any activity. The proportion of compounds that only inhibited QS of *C. violaceum* CV017 without toxicity was the second highest (24%). Twenty percent of compounds inhibited QS of CV017, but had some antibiotic properties. Only a few compounds had only antibacterial activity. Compounds from all tested groups of organisms exhibited some QS inhibitory

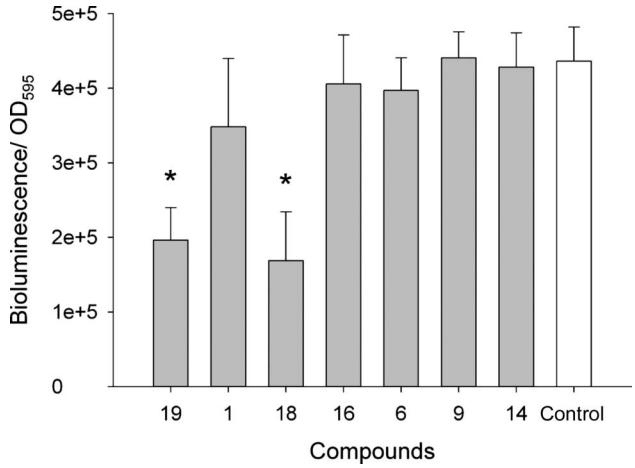

Figure 2. The effect of tenuazonic acid (#19), demethoxy encecalin (#1), midpacamide (#18), hymenialdisin (#16), microcolins A (#6) and B (#9), and kojic acid (#14) on the bioluminescence of *E. coli* DH5α containing a pTIM2442 plasmid. The data are shown as means + SD of the relative bioluminescence (bioluminescence/OD_{595}) of the reporter with added compounds in dimethyl sulfoxide (DMSO) ($n = 8$) or without (only DMSO, control, $n = 8$). Toxic compounds highlighted by asterisks had significantly (Dunnet, $p < 0.05$) lower relative bioluminescence compared to the control. Midpacamide was tested at 46 μM, tenuazonic acid was tested at 102 μM, demethoxy encecalin was tested at 20 μM, hymenialdisin was tested at 150 μM, microcolins A and B at 150 μM and kojic acid at 330 μM. Measurements were taken every hour but the results obtained at 4 h are shown.

activity. All major groups of investigated natural products demonstrated some QS inhibitory properties (Table 1).

In the *C. violaceum* CV017 bioassay, the QS inhibitory concentrations of tested compounds varied from 3.92 μM to 517 μM (Table 1). Many of the tested natural products had MICs below 100 μM. Demethoxy encecalin from the plant *Baccharis cassinaefolia* was the most effective QS inhibitor in this investigation.

The QS inhibitory properties of selected compounds (demethoxy encecalin, tenuazonic acid, midpacamide, hymenialdisin, microcolins A and B and kojic acid) (Figure 1) were further investigated in LasR based and LuxR-based bioassays. Prior to the tests, the possible toxic effects of compounds on metabolic activity or luminescence of the reporters were evaluated with the *E. coli* pTIM2442 reporter. This constitutively luminescent reporter demonstrated that both midpacamide and tenuazonic acid significantly (ANOVA, Dunnet test, $p < 0.05$) inhibited luminescence of the reporter in the absence of AHLs at the maximal inhibitory concentrations (Figure 2), suggesting that they were either toxic or inhibited luminescence either directly (ie by affecting the luciferase enzyme) or indirectly (by affecting metabolism). This

constitutive reporter, when used in conjunction with the toxicity assays, allowed the elimination of compounds that were both generally toxic and those that inhibited the bioassay. Therefore, compounds that were either toxic or otherwise negatively affected the pTIM2442 reporter were not used for further studies. Demethoxy encecalin, hymenialdisin, microcolins A and B and kojic acid were not toxic and did not interfere with luminescence of *E. coli* pTIM2442. Bioactivity of these compounds was further studied. In the direct assays, none of these compounds at any tested concentrations stimulated QS responses in the reporter pSB1075 based on the LasR system (Figure 3). Both demethoxy encecalin and hymenialdisin at concentrations above 6.6 μM and above 15 μM, correspondingly, significantly reduced the QS dependent luminescence of the reporter *E. coli* pSB1075 induced by 3-oxo-C12-HSL (Figure 3A, B). Microcolin A, microcolin B and kojic acid did not significantly (ANOVA, Dunnet test, $p < 0.05$) inhibit QS dependent luminescence of the reporter *E. coli* pSB1075 at the tested concentrations (data not shown). Background bioluminescence of pSB1075 without 3-oxo-C12-HSL (negative control) was 89 $\pm$ 36 CPS, and bioluminescence of this reporter with the cognate AHL (positive control) was 3760 $\pm$ 369 CPS. None of the compounds at the tested concentrations induced luminescence of the reporter *E. coli* pSB401 in the direct assays, suggesting that the compounds were not capable of stimulating QS responses in this reporter based on the LuxR system of *Vibrio fischeri* (Figure 4). Both hymenialdisin and demethoxy encecalin at concentrations above 0.2 μM and 2.2 μM significantly (ANOVA, Dunnet test, $p < 0.05$) reduced the QS dependent luminescence of the reporter *E. coli* pSB401 induced by 3-oxo-C6-HSL compared to the control (Figure 4A, B). Kojic acid inhibited QS dependent luminescence of the reporter induced by 3-oxo-C6-HSL only at concentrations above 36 μM (Figure 4C). Both microcolin A and microcolin B significantly (ANOVA, Dunnet test, $p < 0.05$) reduced QS dependent luminescence of the reporter induced by 3-oxo-C6-HSL at concentrations above 1.5 μM and above 15 μM, correspondingly (Figure 4D, E). Background bioluminescence of pSB401 without 3-oxo-C6-HSL (negative control) was 46 $\pm$ 17 CPS, and bioluminescence of this reporter with HSL was 31739 $\pm$ 5493 CPS.

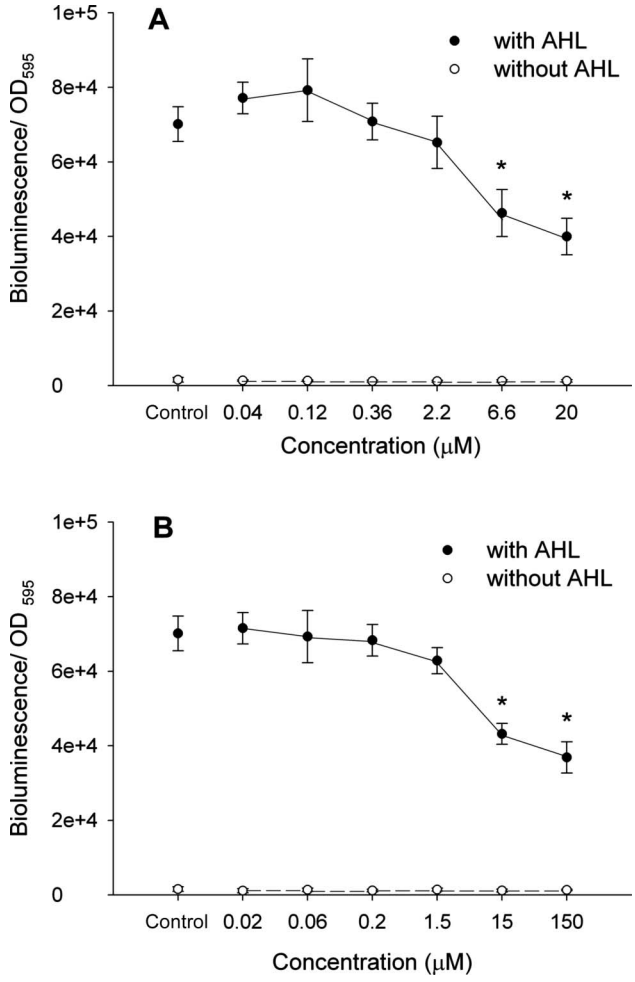

Figure 3. The effect of **A**: demethoxy encecalin and **B**: hymenialdisin on QS dependent bioluminescence of the LasR-based reporter *E. coli* pSB1075. Data ($n = 8$) show means $\pm$ SD of the relative bioluminescence (bioluminescence/OD_{595}) of the reporter with added compounds or without them (control). ● (with AHL) show the relative bioluminescence of the reporter *E. coli* pSB1075 induced by 3-oxo-C12-HSL (final concentration of 2 μM); ○ (without AHL) show relative bioluminescence of the reporter without 3-oxo-C12-HSL. Compound concentrations that significantly (Dunnet, $p < 0.05$) inhibited QS of the reporter compared to the control are marked with asterisks. All treatments and controls contained dimethyl sulfoxide (DMSO). Measurements were taken every hour, but only the results obtained at 4 h are shown.

Mesocosm experiment

Kojic acid reduced the formation of microbial communities on glass slides (Figure 5A, B). In both experiments, the bacterial densities in the presence of 330 μM and 1 mM of kojic acid were significantly reduced (ANOVA, Dunnet test, $p < 0.05$) in comparison with those on the control slides (Figure 5A). Similarly, significantly lower (ANOVA, Dunnet test, $p < 0.05$) densities of diatoms were observed in biofilms developed with kojic acid solutions in two repeated experiments (Figure 5B).

Discussion

In this study, 78 different natural products from marine organisms (sponges, algae, fungi, tunicates

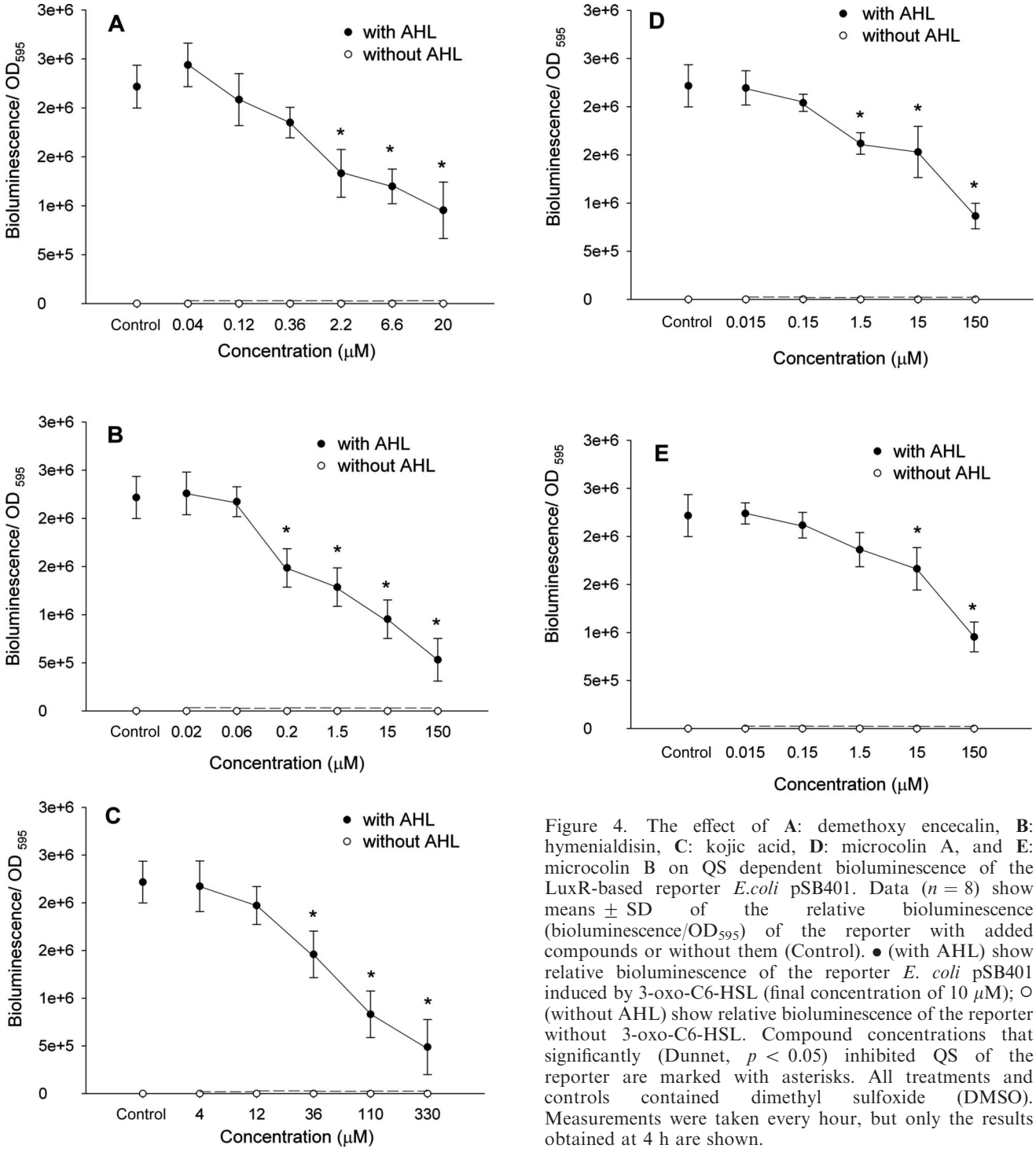

Figure 4. The effect of **A**: demethoxy encecalin, **B**: hymenialdisin, **C**: kojic acid, **D**: microcolin A, and **E**: microcolin B on QS dependent bioluminescence of the LuxR-based reporter *E.coli* pSB401. Data ($n = 8$) show means $\pm$ SD of the relative bioluminescence (bioluminescence/OD_{595}) of the reporter with added compounds or without them (Control). ● (with AHL) show relative bioluminescence of the reporter *E. coli* pSB401 induced by 3-oxo-C6-HSL (final concentration of 10 μM); ○ (without AHL) show relative bioluminescence of the reporter without 3-oxo-C6-HSL. Compound concentrations that significantly (Dunnet, $p < 0.05$) inhibited QS of the reporter are marked with asterisks. All treatments and controls contained dimethyl sulfoxide (DMSO). Measurements were taken every hour, but only the results obtained at 4 h are shown.

and cyanobacteria) and terrestrial plants were screened for their ability to inhibit bacterial QS. Marine natural products have rarely been screened for QS inhibitory compounds (reviewed by Dobretsov et al. 2009; Ni et al. 2009) in comparison with synthetic compounds (Muh et al. 2006; Soulere et al. 2010). The results demonstrate that a large proportion of tested compounds (51%) did not interfere with bacterial QS and only 24% of compounds inhibited the QS of *C. violaceum* CV017. Inhibition of the *C. violaceum* reporter could be due to the compound's ability to inhibit QS in the reporter (either directly by blocking AHL perception, or indirectly by affecting, for example, the transcription of the AHL receptor gene or the stability of the QS transcript). Inhibition of the *C. violaceum* reporter could also be due to the direct or

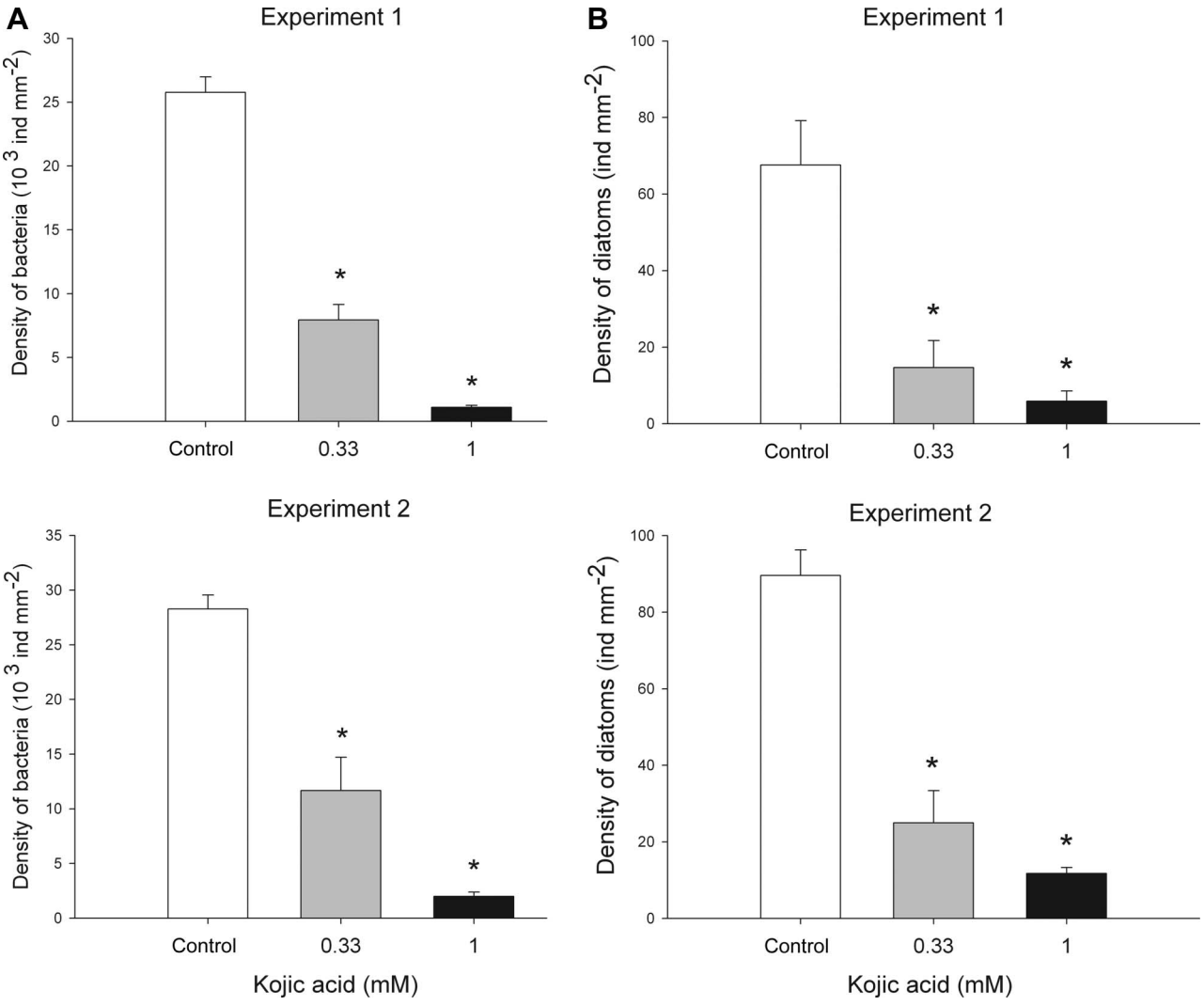

Figure 5. Mean densities of **A:** bacteria (ind mm^2) and **B:** diatoms (ind mm^2) on glass slides exposed to 330 μM and 1 mM kojic acid solutions prepared with unfiltered seawater from the Marina Bandar Rawdha (Muscat, Oman). Three glass slides were incubated in containers filled with unfiltered seawater for 7 days to allow development of biofouling. Sterile glass slides placed into unfiltered seawater without addition of kojic acid were used as a control. Bars are the means of three replicates + SD. Data that are significantly different from the control (ANOVA, Dunnet test: $p < 0.05$) are indicated by an asterisk above the bars. Data are from two independent experiments.

indirect inhibition of the synthesis of the tryptophan derivative violacein, a purple pigment that serves as a read-out for this bioassay. Some of the tested natural products inhibited the *C. violaceum* reporter at concentrations >100 μM. These concentrations were extremely high, and are unlikely to occur in the marine environment (Hmelo and Van Mooy 2009). Therefore, it is likely that these compounds are not truly functioning as QS inhibitors in nature. This fact may explain the relatively high rate of finding QS inhibitors in this study and in other investigations (Skindersoe et al. 2008a).

Some of the *C. violaceum* CV017 QS inhibitors found in this study have antibiotic properties. Due to the different application method, QS inhibitory concentrations of compounds cannot be directly compared to the amount of compounds used for the toxicity bioassay. Further, toxicity of some QS inhibitors was determined by the pTIM2442 reporter, which suggested that indeed some QS inhibitors demonstrate some antibiotic activity. This fact is not novel (Skindersoe et al. 2008b). Previously, 12 antibiotics at sub-lethal concentrations were screened for their QS inhibitory activity (Skindersoe et al. 2008b). The antibiotics azithromycin, ceftazidime, and ciprofloxacin at concentrations of 0.1–11 μM inhibited QS of a LuxR based reporter based on the QS circuit of *Vibrio fischeri*. In a previous study, the cyanobacterial

antibiotic malyngolide produced by the cyanobacterium, *Lyngbya majuscula*, inhibited QS at concentrations ranging from 3.57 μM to 57 μM (Dobretsov et al. 2010). The mechanism of this QS inhibition remains unknown but it was suggested that antibiotics can change bacterial membrane permeability, thus affecting the flux of QS signals (Skindersoe et al. 2008b).

The QS activities of compounds similar to those tested in this study have been investigated earlier. It has been shown that malyngamide C and 8-epi-malyngamide C inhibited luminescence of the LasR-based *E. coli* reporter induced with 3-oxo-C12-HSL at concentrations of 10 μM–1 mM (Kwan et al. 2010), likely by inhibiting transcription of the *lasR* gene. In the present study, malyngamides A and B, which are structurally different from malyngamide C, inhibited QS-dependent violacein production by *C. violaceum* CV017 at similar concentrations. Extracts of the plants *Moringa oleifera* and *Acacia nilotica* that contained gallic and ellagic acids had anti-QS potential (Singh et al. 2009a, 2009b). Epigallocatechin gallate (salt of gallic acid) and ellagic acid inhibited LasR-based and Lux-R based QS at concentrations of 15–30 μM (Huber et al. 2003). In the present study, only gallic but not ellagic acid inhibited QS of *C. violaceum* CV017 at 64.7 μM. Different reporters used in both studies likely explain such differences in the results.

The activity of the compounds identified as QS inhibitors of the *C. violaceum* reporter was tested using semi-synthetic LuxR and LasR reporters based on *E. coli*. These reporters contain AHL receptor genes on a multi-copy plasmid (Winson et al. 1998, Alagely et al. 2011). It was not possible to investigate the effect of all promising QS inhibitors in this experiment because most of the natural products tested in this study were only available in low quantities. Midpacamide and tenuazonic acid were toxic to the reporters at the tested concentrations. Demethoxy encecalin and hymenialdisin interfered with induced luminescence of LasR and LuxR reporters, while kojic acid and microcolin A and B only interfered with LuxR reporters. The results of the experiments were consistent and reproducible. The inhibitory concentrations of hymenialdisin, demethoxy encecalin, kojic acid and microcolins A and B were comparable with ones of natural furanones (Maximilien et al. 1998; Martinelli et al. 2004), ellagic acid (Huber et al. 2003), malyngolide (Dobretsov et al. 2010) and manoalide (Skindersoe et al. 2008a).

Usually, QS inhibitors have been tested in the laboratory against monocultures of pathogens (see review by Dobretsov et al. 2009) or environmental isolates (Maximilien et al. 1998). Only a few studies have investigated the effect of quorum sensing inhibitors, such as furanones, on multispecies of bacteria in the laboratory (Dobretsov et al. 2007) and for over 2 h in the field (Maximilien et al. 1998). In the latter study, crude extracts from the red alga *Delisea pulchra* and pure furanone–1 and -2 at a concentration of 1 μg cm^{-2} applied to Perspex disks or glass Petri dishes inhibited attachment of bacteria to $<20\%$ of control numbers. In this study, the AF performance of the QS inhibitor, kojic acid, was tested against environmental microbes. This compound was selected because of its QS inhibitory activity in the experiments and its commercial availability that ensured sufficient quantities of the compound. In a preliminary field experiment (data not shown) kojic acid incorporated into a non-toxic paint matrix at a concentration of 0.5% significantly reduced the densities of bacteria and diatoms growing on the paint and decreased macrofouling over 1 month. Interpretation of these results poses unique technical and scientific challenges that make it difficult to attribute inhibition of micro- and macrofouling solely to the QS inhibitory activity of kojic acid. Therefore, a controlled mesocosm experiment with kojic acid at non-toxic (330 μM) and a 3-fold higher concentration was conducted. In this experiment, which was repeated twice, kojic acid at non-toxic concentrations inhibited bacterial density 2.5–3.2 fold and diatom density 4.7–3.6 fold in biofilms on glass slides.

How did kojic acid affect microfouling in the present experiment? It is possible that kojic acid inhibited QS of bacteria and this led to low bacterial attachment/recruitment and biofilm formation. This was supported by the data showing that kojic acid at tested concentrations inhibited QS of the reporters and was not toxic to the reporters or the diatom *Amphora coffeaeformis* (data not presented). Kojic acid is widely used as a food additive to prevent enzymatic browning, and in cosmetic preparations as a skin-lightening or bleaching agent because of its tyrosinase inhibitory action (Cabanes et al. 1994; Burdock et al. 2001). Unfortunately, direct measurements of AHLs in the biofilms treated and not treated with kojic acid are technically challenging and would not help prove this hypothesis. Previous studies suggested that QS inhibitors, such as furanones, affect microbial composition and the densities of certain groups of bacteria (Dobretsov et al. 2007), shifting the composition of microbial communities from being dominated by Gram-negative bacteria to those dominated by Gram-positive species (Maximilien et al. 1998; Kjelleberg et al. 2001). In this case, a decrease in AHL concentrations might reflect changes in microbial composition. Changes in bacterial species composition and chemical compound production could possibly result in changes in diatom communities, as presence of particular bacterium may affect recruitment of diatoms (Gawne et al. 1998; Wigglesworth-Cooksey

and Cooksey 2005). Alternatively, there is a possibility that kojic acid reduced the formation of microbial communities by means other than QS inhibition. For example, kojic acid could have a toxic effect on some marine bacteria and diatoms more sensitive than the tested reporters. It is possible that kojic acid could inhibit other regulatory cascades that affect biofilm formation. Brominated furanones are known to inhibit multiple regulatory pathways leading to biofilm formation even without interference with QS (Janssens et al. 2008). Overall, the results of this experiment demonstrate the high AF potential of kojic acid.

In conclusion, the results of this study suggest that screening of natural products is a promising way to find novel QS inhibitors. Natural products with QS inhibitory properties can control the formation of microbial communities and may potentially be used in the future for AF applications.

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