

# Allelochemical defense against epibiosis in the macroalga *Caulerpa racemosa* var. *turbinata*

Sergey Dobretsov<sup>1</sup>, Hans-Uwe Dahms<sup>1</sup>, Tilmann Harder<sup>2</sup>, Pei-Yuan Qian<sup>1,\*</sup>

<sup>1</sup>Department of Biology & Coastal Marine Laboratory, Hong Kong University of Science and Technology, Clear Water Bay, Kowloon, Hong Kong SAR, PR China

<sup>2</sup>Institute of Chemistry and Biology of the Marine Environment (ICBM), University of Oldenburg, Carl von Ossietzkystr. 9–11, 26111 Oldenburg, Germany

**ABSTRACT:** The abundance and diversity of microorganisms on the surface of the tropical green macroalga *Caulerpa racemosa* var. *turbinata* and the effect of algal surface and waterborne compounds on fouling organisms were investigated both in laboratory and field experiments. As shown via electron microscopic enumeration, the abundance of epibiotic bacteria and diatoms on algal frond surfaces was not significantly different from the reference biofilms harvested from stones in the *C. racemosa* habitat. The analysis of Terminal Restriction Fragment Length Polymorphism of DNA from algal surface-associated bacterial communities revealed that despite a similar abundance of these bacteria, the community profile on algal frond surfaces differed significantly from that of inanimate, undefended substrates. These results suggest that the alga regulate the occurrence of certain bacterial ribotypes. This result was in accordance with the fact that different bacterial communities formed on the artificial substrata (i.e. Petri dishes) placed in the *C. racemosa* habitat and alga-free control sites. Neither *C. racemosa* conditioned seawater (CCW) nor hexane surface extracts affected the growth of bacterial isolates from biofilms. However, only CCW exhibited a toxic effect on the larvae of the fouling polychaete *Hydroides elegans*, and evoked abnormal larval development in a concentration-dependent fashion. At sublethal concentrations, the <1 kD fraction of CCW inhibited the larval settlement of *H. elegans* and the bryozoan *Bugula neritina*. Caulerpenyne, the prominent bioactive metabolite in the genus *Caulerpa*, was not detected in CCW by chromatographic procedures. Our data suggest that waterborne compounds other than caulerpenyne are involved in the chemical defense of the alga *C. racemosa*.

**KEY WORDS:** *Caulerpa racemosa* · Algae · Microbial communities · Larval settlement · Epibiosis · Antifouling · Chemical defense

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## INTRODUCTION

Many marine algae have evolved efficient strategies to control epibiosis either chemically by producing defensive compounds (Nylund & Pavia 2003, Bhadury & Wright 2004, Fusetani 2004) and/or physically by sloughing and secreting mucus (Steinberg et al. 1997, Nylund & Pavia 2005). It has been shown that macroalgae produce antibacterial (Devi et al. 1997, Hellio et al. 2000, 2001), antifungal (König & Wright 1997, Hellio et al. 2000), and antilarval compounds (De Nys et al. 1995, Schmitt et al. 1995, Walters et al. 1996, Harder &

Qian 2000, Hellio et al. 2004). Antifouling metabolites of red and brown algae, such as diterpene alcohols (Schmitt et al. 1995), brominated phenols (Phillips & Towers 1982), sesquiterpenoids (de Nys et al. 1998), and halogenated furanones (Steinberg et al. 1997, 1998) repel propagules or inhibit growth of microorganisms. Contrary to brown and red algae, comparative allelochemical studies of green algae are rare (Mtolera & Semesis 1996, Walters et al. 1996, Harder & Qian 2000, Harder et al. 2004). In general, antifouling compounds from marine algae cover a small molecular size range of relatively unipolar metabolites, which stay

\*Corresponding author. Email: boqianpy@ust.hk

in close association with their site of production. These physical-chemical attributes have been suggested to be critical for bioactive substances to function as natural antifoulants in the absence of strong boundary-layer effects (Steinberg et al. 1998, 2001, 2002).

Field observations in Hong Kong waters from 1999 to 2005 revealed that the fronds of the green macroalga, *Caulerpa racemosa* (J. Agardh) var. *turbinata* (Eubank, 1946) (later *Caulerpa racemosa*), were conspicuously free of epibiotic macroorganisms throughout the year (authors' obs.), indicating effective mechanisms of antifouling control. The alga *C. racemosa* is native to the tropics, and thus subject to intense herbivore and fouling activity. The natural products of at least 14 species of *Caulerpa* from around the world have been studied in detail (Paul & Fenical 1986, Faulkner 1995). Most species produce the water-soluble sesquiterpene caulerpenyne (CYN) (Paul et al. 2006), which can reach up to 1.3% of algal dry mass (Aguilar-Santos 1970, Jung et al. 2002). As CYN is the most predominant toxin, the toxicity of *Caulerpa* algae is attributed to the production of this compound (Meyer & Paul 1992, Schroeder et al. 1998). For instance, CYN exhibits antibiotic activity (Paul & Fenical 1986), and has cytotoxic effects on eggs of some marine mammals (Pesando et al. 1996, Pedrotti & Lemée 1999). However, the effect of *Caulerpa* metabolites on ecologically relevant marine organisms has only been studied in a few cases (Selvi & Selvaraj 2000, Smyrniotopoulos et al. 2003, Freile-Pelegrin & Morales 2004, Paul et al. 2006). It is also believed that CYN is present at the *Caulerpa* frond surface but is quickly degraded in the presence of seawater (Amade & Lemée 1998).

During 2 severe algal blooms in the summers of 2004 and 2005, we collected *Caulerpa racemosa* to conduct a series of experiments in order to elucidate whether algal metabolites, particularly CYN, affect both microbial and macroscopic colonizers on algal surfaces and nearby substrata. These studies consisted of a comparison of the abundance of microorganisms on the surface of *C. racemosa* fronds and stones in close proximity to the alga. Additionally, we also investigated a long range effect of waterborne compounds of *C. racemosa* on the formation of microbial communities on artificial substrata in the *Caulerpa* habitat and 50 m away from it. We used molecular fingerprinting techniques to analyze microbial community patterns; assessed the effects of both waterborne and surface-attached algal metabolites on bacterial growth, larval development and settlement of the fouling polychaete, *Hydroides elegans*, and the bryozoan, *Bugula neritina*, in laboratory bioassays. Finally, we analyzed the waterborne and surface-attached algal metabolites for the presence of CYN by flash chromatography and GC-MS.

## MATERIALS AND METHODS

**Collection of algae.** *Caulerpa racemosa* was collected from the seafloor at a depth of 2 to 3 m next to the pier of the Coastal Marine Laboratory of the Hong Kong University of Science and Technology, Port Shelter Bay (22° 19' N, 114° 16' E) in June and July 2004 (for microbial enumeration, preparation of the conditioning of seawater and bacterial community analysis) and in July 2005 (for isolation and identification of CYN, isolation of surface-associated molecules). In all cases, algal holdfasts were carefully detached without damaging the fronds. The algal tissue volume (measured by water displacement) and the algal wet weight were determined immediately after collection. The algae were carefully washed with 0.22 µm filtered and autoclaved seawater (AFSW) to flush away external debris.

**Scanning electron microscopy.** Small algal fronds (n = 5) and reference substrata (small flat stones) in the proximity (5 to 15 cm away from *Caulerpa* stands) of dense aggregations of *C. racemosa* (n = 5) were fixed in 5% buffered formalin and dehydrated in an increasing ethanol series, dried by the critical point procedure, and sputtered with gold (see Dobretsov & Qian, 2002 for details). The specimens were examined with a JEOL 6300F (70 eV) scanning electron microscope (SEM) at magnifications of 1000 and 5000×; bacteria and diatoms were counted in 10 selected fields of view (8000 and 300 µm<sup>2</sup> respectively) per replicate.

**Analysis of bacterial community patterns.** Biofilms were harvested from algal surfaces and artificial substrata (i.e. polystyrene Petri dishes) by surface swabbing according to Liu et al. (1997). Substrates used in the investigation were: (1) algal blades and stones, with surface areas of about 20 cm<sup>2</sup>, in close proximity (5 to 10 cm) to the algae (n = 5); (2) 24 h old biofilms developed on Petri dishes, with surface areas of 70 cm<sup>2</sup> (n = 10), at a depth of 2 m in the *C. racemosa* microhabitat and about 50 m away from it in an area without this alga (reference microhabitats: n = 10). The biofilm swabs were suspended in 0.8 ml of lysis buffer (1% Triton X-100, 20 mM Tris-HCl at pH 8.2). The amplification of 16S rDNA was performed with the PCR primer pair: 341F (5'-CCTACGGGAGGCAGCAG-3') and 926R-Fam (5'-CCGTCAATTCCTTTRAGTTT-3') (Amann et al. 1990, Lee et al. 1993). The 926R primer was labeled at the 5' end with 6-carboxy fluorescein (FAM) dye. The thermocycling conditions were as follows: 95°C for 2 min (1 cycle); 95°C for 30 s (15 cycles), 60°C for 3 min and 72°C for 3 min. The annealing temperature started at 60°C and was reduced to 45°C in increments of 1°C cycle<sup>-1</sup>; 10 cycles of 95°C for 30 s, 45°C for 3 min and 72°C for 3 min; and 72°C for 10 min. Amplified DNA (4 µl of PCR mixtures) was visualized by gel electrophoresis on a 1.5% agarose gel in Tris-

acetate-ethylenediaminetetraacetic acid (TAE) buffer. Fluorescently labeled PCR products were purified with the Wizard<sup>®</sup> PCR preps DNA purification system (Promega) according to the manufacturer's protocol. Purified amplicons were digested with 20U *MspI* (Boehringer Mannheim Biochemicals) at 37°C for 6 h. Ten  $\mu$ l of digested products were mixed with 0.5  $\mu$ l of internal size standard (ET550-R, Amersham Biosciences). This mixture was denatured for 2 min at 95°C and immediately chilled on ice prior to capillary electrophoresis on a MegaBACE<sup>™</sup> genetic analyzer (Amersham Biosciences) operated in the genotyping mode. After the electrophoresis, the length of the fluorescently labeled terminal restriction fragments (TRFs), was determined by comparison with internal size standards by using the 'Fragment Profiler' software (Amersham Biosciences). The lengths of TRFs obtained by the analyzer were rounded up to the nearest integral values. Peaks that were less than 1.5 bp apart from a larger peak were classified as its 'shoulders' and thus eliminated (Dunbar et al. 2001). For each sample, peaks over a threshold of 50 fluorescence units (Blackwood et al. 2003) and whose peak heights contributed for at least 1% to the integrated height (Buchan et al. 2003, Luna et al. 2006) were used for analysis. Terminal fragments <35 and >500 bp were excluded from the analysis to avoid detection of primers and uncertainties of size determination.

**Preparation of *Caulerpa*-conditioned seawater (CCW).** The green alga *C. racemosa* was incubated at 25°C for 1 h in untreated seawater from the collection site (1:1 gravimetric ratio: original concentration). Seawater from a reference site (an area 50 m away from the *Caulerpa* stand) served as the control. After incubation, CCW and the seawater control were filtered through 0.22  $\mu$ m membranes (Millipore) and used directly in disc diffusion assays, larval bioassays, or were subjected to ultrafiltration in an ice bath (YM-100, YM-10, YM-1: Millipore). This procedure yielded filter residues of 100, 10 and 1 kD molecular weight cut-off and a <1 kD filtrate. Residues were redissolved in AFSW to the original volume of CCW. The filtrates and residues were immediately assayed for the ability to inhibit the metamorphosis of *Hydroides elegans* larvae. The <1 kD fraction was stored at -20°C and used for the identification and isolation of CYN.

**Isolation of surface-attached metabolites.** Surface-associated metabolites from the alga were extracted using the technique of De Nys et al. (1998). For this 206 g (wet weight) of the alga was dip-extracted in 100 ml of hexane (Fisher Scientific) for 30 s. The effect of the solvent on epithelial cells was investigated by an epifluorescence microscope at 1000 $\times$  magnification (Zeiss Axiophot). The hexane extracts were concentrated under vacuum at 29°C, re-dissolved in dimethyl sulfoxide (DMSO) (Sigma) and stored until use at

-20°C. In order to use extracts at surface concentrations in the bioassays corresponding to natural surface concentrations of metabolites, the ratio of surface area:wet weight was calculated for small algal pieces. Based on this ratio the total area of extracted algae was calculated. Prior to the experiments, the hexane extract was diluted with AFSW in order to obtain the algal surface level concentrations (SLC) in the multi-well plates.

**Isolation and identification of CYN.** CYN was purified from algae according to Amade & Lemée (1998). The compound was detected at 254 nm and identified on the basis of its characteristic UV spectrum in a mixture of hexane and ethyl acetate 95:5, with a  $\lambda_{\max}$  of 257 nm and shoulders at 270 and 285 nm. In order to determine the presence of CYN in the <1 kD fraction of CCW, 200 ml of CCW were extracted with 5 ml of hexane:ethyl acetate (95:5%) for 24 h at 0°C. Five replicated samples of CCW were used. The extract was concentrated under vacuum and redissolved in ethanol (25  $\mu$ g ml<sup>-1</sup>). The concentrate (5 ml) was filtered through a silica cartridge (Clean SPE Si, Alltech Assoc.) with 10 ml of hexane:ethyl acetate (95:5%) followed by 10 ml of ethyl acetate (100%). Both fractions were concentrated under vacuum and subjected to high performance liquid chromatography (HPLC, Hewlett-Packard HP1100) on a silica column (LiChrospher Si 100, Merck<sup>®</sup>; particle size = 5  $\mu$ m; pore size 100 Å) and elution with hexane:ethyl acetate (95:5%) with UV detection at 254 nm. All fraction peaks were concentrated under vacuum and subsequently analyzed by a coupled GC-MS with electron impact ionization (Varian CP-3800, ion trap Varian-4000). Analogously, we determined the presence of CYN in the hexane extracts (surface-attached metabolites). The extracts were concentrated under vacuum and the presence of CYN in the extracts was analyzed by GC-MS. In order to determine the detection limit of CYN by HPLC, different concentrations of CYN in hexane:ethyl acetate (95:5%) ranging from 1 mg ml<sup>-1</sup> to 0.01  $\mu$ g ml<sup>-1</sup> were analyzed. The chromatographic conditions were the same as described above.

**Disc diffusion bioassay.** Antibacterial activity of *Caulerpa* extracts was tested against bacterial strains belonging to  $\gamma$ -Proteobacteria (*Vibrio gallicus* UST-030701-074, *V. parahaemolyticus* UST030701-224, *Marinobacter* sp. UST040317-15, *M. flavimaris* UST-040317-005, *Pseudoalteromonas flavipulchra* UST-040317-169, *Alteromonas marina* UST030701-337, *Psychrobacter pacificensis* UST040317-196, and *Pelagibacter variabilis* UST040317-109),  $\alpha$ -Proteobacteria (*Ruegeria atlantica* UST030701-046 and *Erythrobacter flavus* UST030701-080), *Cytophaga-Flexibacter-Bacteroides* (*Flexibacter* sp. UST991130-045 and *Tenacibaculum mesophilum* UST991130-047), and to the

*Firmicutes* groups (*Bacillus atrophaeus* UST040317-111, *B. pumilus* UST040317-29, *B. thuringiensis* UST-040317-179, and *Staphylococcus* sp. UST040317-166). These isolates were obtained from the Bacterial Culture Collection of the Hong Kong University of Science and Technology (numbers indicate the sample identification) and isolated from a 7 d old subtidal biofilm from the region of the investigation. Bacteria were grown in marine broth (3% yeast extract, 5% peptone in artificial seawater) at 37°C for 24 h. Aliquots of 200 µl of cell culture ( $10^6$  CFU ml<sup>-1</sup>) were plated onto Petri dishes containing bacteriological agar No. 1 (Oxoid). Sterile paper discs of 6 mm diameter (Whatman No. 1) were loaded with 20 µl of CCW disc<sup>-1</sup>, or appropriate amounts of surface attached metabolites in order to obtain 0.5×, 1× and 2× SLC concentrations. The paper discs were air dried and placed on inoculated agar plates. Bactericidal effects were determined after 24 h of incubation at 30°C by measuring the diameter of the inhibition zone. All measurements were performed in triplicates and in 3 repeats. Control discs were prepared with seawater and hexane (both at 20 µg disc<sup>-1</sup>).

**Larval culture.** Adults of the polychaete tubeworm, *Hydroides elegans*, and the bryozoan, *Bugula neritina*, were collected from submerged rafts of a fish farm in Yung Shue O, Hong Kong (114° 21' E, 22° 24' N). Larval cultures were prepared and maintained according to Pechenik & Qian (1998). Competent larvae of *H. elegans* and *B. neritina* were used for settlement bioassays (Bryan et al. 1997). Competence of *H. elegans* larvae was determined by their morphology and by a bioassay with a settlement inducer, the phosphodiesterase inhibitor 3-isobutyl-methylxanthine (IBMX) at 10<sup>-4</sup> M solution in AFSW (Pechenik & Qian 1998).

**Larval development assay.** The laboratory bioassay was performed 3 times on different larval batches with replication (n = 5) in multiwell polystyrene dishes (#3047, FALCON), each containing 20 early trochophore larvae of *Hydroides elegans* (which hatched no longer than 15 min before the onset of the experiment) in 1 ml of CCW with dilutions of *Caulerpa racemosa* in AFSW at 1×, 0.2×, 0.1×, and 0.01×. The percentage of mortality and abnormal development (indicated by asymmetries, failure of ciliary band formation, and intrusions of ciliary bands) was determined after 24 h. During the assay, larvae were not fed.

**Larval settlement assay.** The laboratory bioassay was repeated 6 times with different larval batches in polystyrene dishes each containing 20 competent larvae of *Bugula neritina* or *Hydroides elegans* and CCW (0.01×) or surface-attached metabolites (0.25×, 0.5×, 1× and 2× SLC) as well as CYN (concentration = 0.006–1.2 mg ml<sup>-1</sup>). In all bioassays, AFSW was used as a positive control. In bioassays with larvae of *H. elegans*, 10<sup>-4</sup> M IBMX (see above) in AFSW was used as an artificial

stimulator for larval settlement (Dobretsov & Qian 2002). Prior to the experiments, *H. elegans* larvae were treated with IBMX at 10<sup>-4</sup> M solution in AFSW for 1 h, washed in AFSW, and subsequently used for the assays. Larval settlement assays for *H. elegans* were run at 28°C under continuous illumination for 24 h, and for 1 h without illumination in the case of *B. neritina*. After these periods, dishes were emptied and the settled juveniles were counted under the dissecting microscope. Mortality and settlement values were transformed into percentages.

**Statistical analysis.** The densities of bacteria and diatoms on the surface of algae and artificial substrates were compared by a Student *t*-test. The percent values of larval attachment in response to experimental treatments were arcsine-transformed. To improve the arcsine-transformation, replicates with no attachment were given the value of  $(4 \times n)^{-1}$ , where n is the number of larvae in a single replicate (Zar 1999). The homogeneity and normality of the data were analyzed with Levene's and Shapiro-Wilk's *W* test, respectively, at a confidence level of 95%. Data were analyzed by 1-way ANOVA. LSD post-hoc multiple comparison tests or multiple pair-wise comparisons versus negative controls (Dunnnett's test) were employed at the 95% confidence level (Conover & Iman 1981). Statistical calculations were performed with the software package Statistica (StatSoft). TRF patterns of different bacterial community DNA samples were subjected to cluster analysis where Bray-Curtis similarities produced a similarity matrix based on the total number of TRFs observed in all samples compared to the presence or absence of these TRFs in individual samples. For the construction of a dendrogram that demarcated the similarity of microbial communities on the gels, group average linkage in a hierarchical, agglomerative clustering algorithm was performed using the PRIMER program (Plymouth Marine Laboratory).

## RESULTS

### Enumeration of microbial epibionts on *Caulerpa racemosa*

Scanning electron microphotographs proved the presence of attached bacteria and diatoms on the algal surface. The bacterial abundances on the algal surface were slightly but not significantly lower (Student *t*-test, df = 8; *t* = 0.69, *p* > 0.05) than those on the surface of reference substrata (i.e. stones of the ambient environment) (Fig. 1). The diatom abundance was not significantly different (Student *t*-test, df = 8; *t* = 0.31, *p* > 0.05) either. No invertebrate juveniles were found on the algal surface.

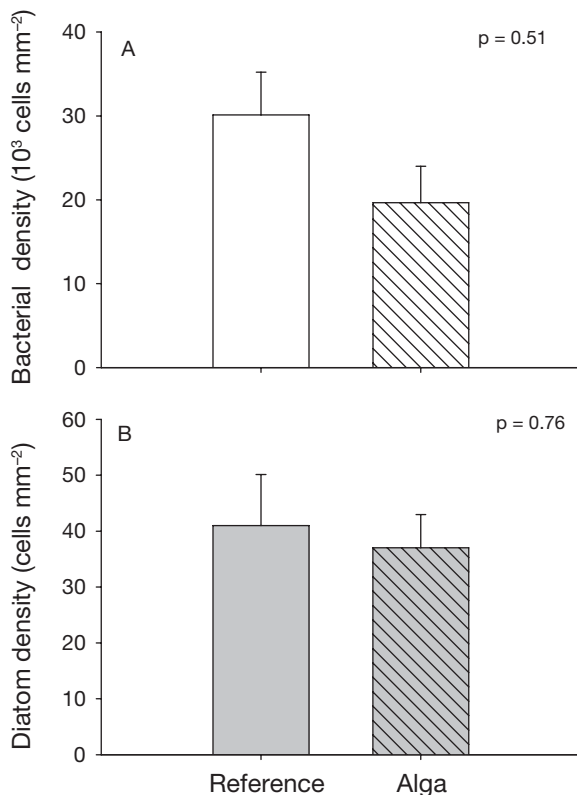


Fig. 1. Densities of (A) bacteria and (B) diatoms on stones (reference) and on frond surfaces of *Caulerpa racemosa* (alga). Bacteria and diatoms were counted using scanning electron microscopy at 5000 and 1000 $\times$  magnification, respectively. Samples were taken from the seafloor next to the pier of the Coastal Marine Lab of the Hong Kong University of Science and Technology, Port Shelter Bay, June 2004. Data plotted are means ( $n = 5$ )  $\pm$  SE.  $p$ -values ( $t$ -test) are given in each panel

### Bacterial community profiles on *Caulerpa racemosa* and reference surfaces

The analysis of Terminal Restriction Fragment Length Polymorphism (T-RFLP) based on 113 discernible TRFs revealed that the bacterial community profiles on the surface of *Caulerpa racemosa* were different from those on the inanimate reference surfaces (i.e. stones in the ambient environment) (Table 1). Cluster analysis of a similarity matrix revealed that the profiles of bacterial communities on replicate algal surfaces were highly similar, forming a cluster distantly related to the bacterial communities on replicate reference surfaces (Fig. 2). The lowest number of TRFs (i.e. bacterial ribotypes) were recorded from the reference surface, while communities which developed on the surfaces of *Caulerpa racemosa* fronds had a higher number of bacterial

Table 1. Terminal restriction fragment length polymorphism profiles of bacterial communities derived from surface of *Caulerpa racemosa* and from stones of ambient environment at a reference site. Presence and fragment sizes (bps) of individual terminal restriction fragments (TRFs) denoted as follows: X, <sup>a</sup>: present in 3 out of 3 replicates; <sup>b</sup>: present in 2 out of 3 replicates; presence in 1 out of 3 replicates omitted; -: absence of particular TRFs in a given biofilm

TRF (bps)	Reference (rock)	<i>Caulerpa</i>
62	-	X <sup>b</sup>
71	X <sup>b</sup>	X <sup>b</sup>
76	-	X <sup>b</sup>
80	X <sup>a</sup>	-
91	-	X <sup>a</sup>
92	X <sup>a</sup>	-
96-123	-	X <sup>a</sup>
127	X <sup>a</sup>	X <sup>b</sup>
128	X <sup>a</sup>	-
131-182	-	X <sup>a</sup>
183-205	X <sup>a</sup>	-
206	-	X <sup>b</sup>
207-209	X <sup>b</sup>	-
217-247	-	X <sup>b</sup>
248	X <sup>b</sup>	X <sup>a</sup>
249	X <sup>b</sup>	X <sup>a</sup>
263-298	-	X <sup>b</sup>
299	X <sup>a</sup>	-
300-312	-	X <sup>a</sup>
314	X <sup>a</sup>	-
315	X <sup>b</sup>	X <sup>a</sup>
369	X <sup>b</sup>	-
370	-	X <sup>b</sup>
371	X <sup>a</sup>	X <sup>b</sup>
372	X <sup>a</sup>	X <sup>b</sup>
373	-	X <sup>b</sup>
374	X <sup>a</sup>	-
375	X <sup>a</sup>	X <sup>b</sup>
424-451	-	X <sup>b</sup>
456-486	X <sup>a</sup>	-

ribotypes representing a higher number of discernible DNA-strands (Table 1). The presence of certain TRFs common to algal and reference surfaces (e.g. 71, 127, 248, 315, 371, 375 bp) indicated that some bacterial types were not affected by algae. The algal-associated bacterial communities were characterized by the absence (e.g. 80, 128, 374 bp) or presence (e.g. 62, 96-123, 131-182 bp) of certain bacterial ribotypes compared to bacterial communities from reference sites (Table 1).

Based on the similarity analysis of 242 discernible TRFs (Table 2), the community profiles in biofilms harvested on artificial substrata (Petri dishes) in the *Caulerpa* habitat and alga-free control sites 50 m away were compared. These communities were characterized by the presence (e.g. 298, 472, 473 bp) or absence (e.g. 131, 181, 370-386, 484 bp) of bacterial ribotypes on Petri-dishes (Table 2). In general, bac-

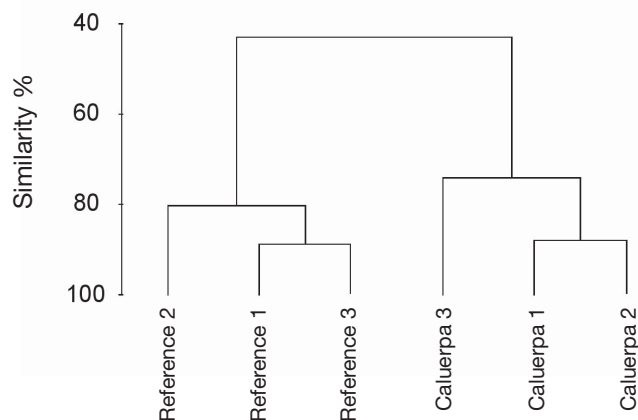


Fig. 2. Dendrogram compiled from cluster analyses of presence and absence of terminal restriction fragments (TRFs) in terminal restriction fragment length polymorphism profiles of epibiotic bacteria on green alga *Caulerpa racemosa* (Caulerpa: 1–3) in comparison to the epibiotic bacteria from a reference surface (Reference: 1–3). Experiments replicated 3 times denoted as 1, 2 and 3. Dendrogram constructed by using similarity matrices of TRFs of bacterial community DNA samples

terial communities developed in the *Caulerpa* stand were different from bacterial communities developed at alga-free control sites (Fig. 3).

#### Inhibition of bacterial growth by metabolites of *Caulerpa racemosa*

CCW did not inhibit growth of any of the 17 bacterial strains tested in disc diffusion assays. A control (AFSW) did not affect bacterial growth either. Similarly, neither surface-associated metabolites at 2× SLC nor a hexane control inhibited the growth of the bacterial strains. Dipping algal thalli in hexane did not have a significant effect on *Caulerpa* cell lyses.

#### Influence of *Caulerpa* extracts on larval development and settlement

CCW caused mortality of early trochophores of *Hydroides elegans* at the concentrations of 1×, 0.5×, and 0.1× CCW (Fig. 4A: ANOVA,  $df = 4$ ;  $F = 12.3$ ,  $p < 0.05$ ). Less than 30% of *H. elegans* larvae developed normally to the nectochaete stage at the original (1×) and 0.5 × CCW concentration (Fig. 4B: ANOVA,  $df = 4$ ;  $F = 18.5$ ,  $p < 0.05$ ). Diluted 100-fold (0.01×), CCW had no toxic effect on early trochophore larvae.

Larval settlement of *Hydroides elegans* induced by IBMX in the presence of different molecular fractions of CCW was different (Fig. 5A; ANOVA,  $df = 5$ ;  $F =$

Table 2. Terminal restriction fragment length polymorphism profiles of bacterial communities derived from Petri dishes exposed to fouling in *Caulerpa racemosa* microhabitats for 24 h and from Petri dishes out of these stands as references. Presence and fragment sizes (bps) of individual terminal restriction fragments (TRFs) are denoted as follows: X, <sup>a</sup>; present in 3 out of 3 replicates; <sup>b</sup>; present in 2 out of 3 replicates; presence in 1 out of 3 replicates omitted; –: absence of particular TRFs in a given biofilm

TRF (bps)	Reference	<i>Caulerpa</i>
62	–	X <sup>b</sup>
64	X <sup>b</sup>	–
78	X <sup>a</sup>	X <sup>b</sup>
80	–	X <sup>b</sup>
91	X <sup>b</sup>	–
92	–	X <sup>b</sup>
127	X <sup>b</sup>	–
128	X <sup>a</sup>	X <sup>a</sup>
131	X <sup>a</sup>	–
141	X <sup>b</sup>	–
144	–	X <sup>b</sup>
149	X <sup>b</sup>	–
180	X <sup>a</sup>	X <sup>b</sup>
181	X <sup>a</sup>	–
182	–	X <sup>b</sup>
184–269	X <sup>b</sup>	–
298	–	X <sup>a</sup>
299	X <sup>a</sup>	X <sup>b</sup>
311	–	X <sup>b</sup>
321	X <sup>b</sup>	–
367	X <sup>a</sup>	X <sup>b</sup>
369	X <sup>b</sup>	X <sup>a</sup>
370–386	X <sup>a</sup>	–
406	X <sup>b</sup>	X <sup>b</sup>
457	–	X <sup>b</sup>
472	–	X <sup>a</sup>
473	–	X <sup>a</sup>
474	X <sup>a</sup>	X <sup>a</sup>
484	X <sup>a</sup>	–
495	–	X <sup>b</sup>

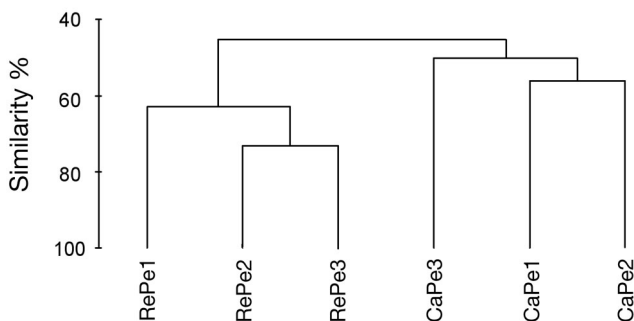


Fig. 3. Dendrogram compiled from cluster analyses of presence and absence of terminal restriction fragments (TRFs) in terminal restriction fragment length polymorphism profiles of epibiotic bacteria on Petri dishes exposed to dense assemblages of the green alga *Caulerpa racemosa* (CaPe1–3) in comparison to epibiotic bacteria from Petri dishes from a reference site (RePe1–3). Experiments replicated 3 times denoted as 1, 2 and 3. Dendrogram constructed by using similarity matrices of TRFs of bacterial community DNA samples

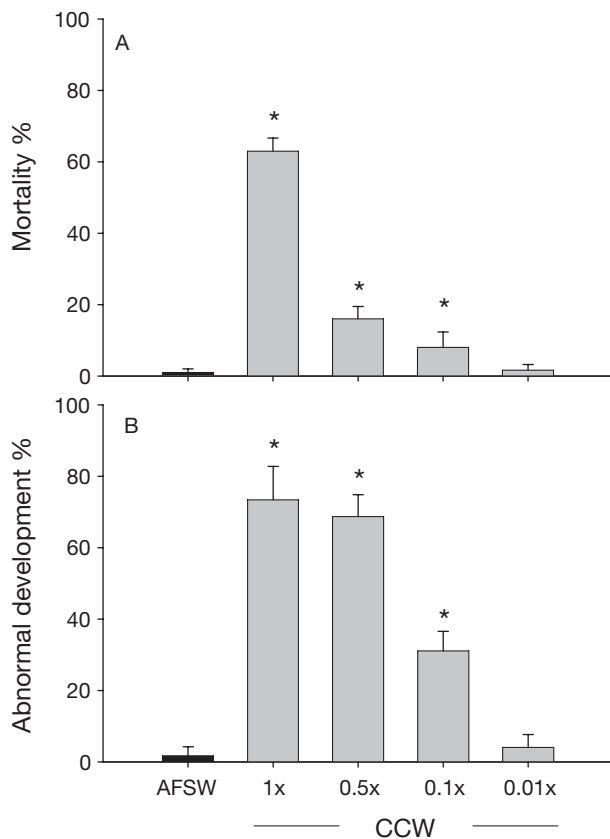


Fig. 4. Effect of *Caulerpa racemosa* conditioned seawater (CCW) and a dilution series on (A) survival of trochophore larvae and (B) larval development of early trochophore larvae of *Hydroides elegans*. Samples assayed under laboratory conditions together with controls (0.22  $\mu\text{m}$ -filtered and autoclaved seawater [AFSW]). Larvae treated with CCW for 24 h. Data provide means of 3 batches done in 5 replicates  $\pm$  SD. \*Significant statistical differences ( $p < 0.05$ , Dunnett's test) between the treatment and the controls

19.8, LSD:  $p < 0.0001$ ). Larval settlement in dishes containing 100 kD, 100–10 kD and 10–1 kD filter residues of CCW was not different from that in AFSW (LSD:  $p > 0.05$ ), while settlement in dishes containing a fraction of <1 kD was significantly lower than that in other dishes (LSD:  $p < 0.01$ ). Similar results were obtained in bioassays with *Bugula neritina* (Fig. 5B). In both experiments, the fraction of <1 kD inhibited larval settlement, whereas a high molecular weight fraction had no effect on the settlement of larvae, except the 1–10 kD fraction for *B. neritina* (Fig. 5).

Surface attached metabolites at a concentration of 2 $\times$  SLC significantly affected (ANOVA,  $df = 5$ ;  $F = 8.3$ ,  $p < 0.05$ ) *Hydroides elegans* larval settlement induced by IBMX when compared to the DMSO control ( $54 \pm 2\%$  and  $74 \pm 5\%$  of larval settlement, respectively). Other concentrations of surface attached metabolites did not have any effect on larval settlement.

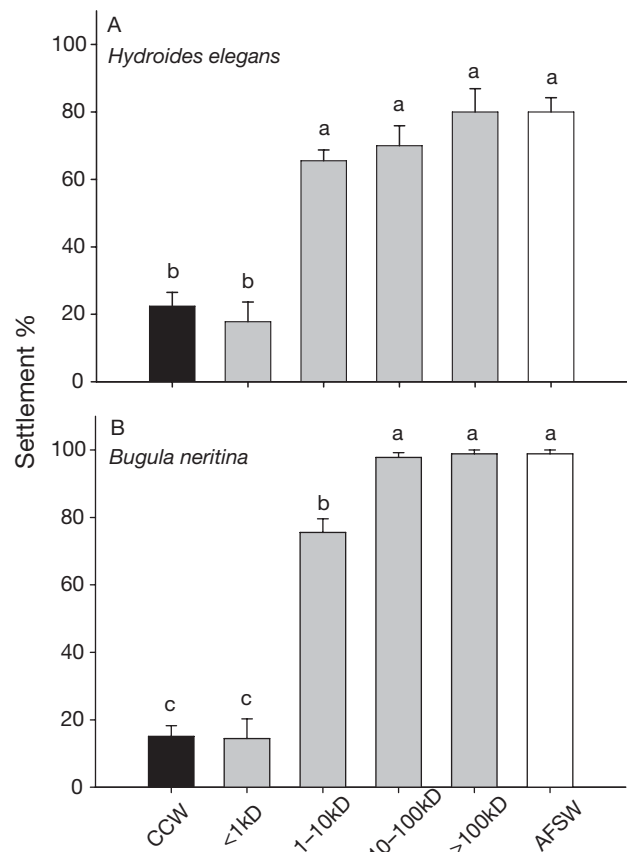


Fig. 5. Effect of *Caulerpa racemosa* conditioned seawater at non-toxic concentrations 0.01 $\times$  (black bars) and molecular size fractions (grey bars) on larval settlement of (A) *Hydroides elegans* and (B) *Bugula neritina*. *H. elegans* larvae treated with  $10^{-4}$  M 3-isobutyl-1-methylxanthine prior to experiment. Filtered (0.22  $\mu\text{m}$ ) and autoclaved seawater (AFSW) used as controls (open bars). Percentage data of larval settlement are means  $\pm$  SD of 6 replicates. a, b, c: data that are significantly different (LSD post-hoc multiple comparison test,  $p < 0.05$ )

At a concentration  $\geq 0.012$  mg  $\text{ml}^{-1}$  in AFSW, CYN inhibited *Hydroides elegans* larval settlement (Fig. 6A). Solutions of CYN in AFSW at the concentration of 0.06 mg  $\text{ml}^{-1}$  and higher were toxic to *H. elegans* larvae (Fig. 6B). CYN at the concentration of 0.006 mg  $\text{ml}^{-1}$  was neither inhibitive nor toxic (Fig. 6).

### Identification of CYN

The largest peak in the HPLC chromatogram of an ethanol extract from *Caulerpa racemosa* represented the compound CYN (indicated by an asterisk in Fig. 7A). The purity of the compound was verified by a comparison of the resultant mass spectrum (Fig. 7A) with published data in Jung & Pohnert (2001). The hexane:ethyl acetate fraction and the ethyl acetate fraction of CCW did not reveal CYN in the chroma-

tograms (Fig. 7B,C). GC-MS revealed the presence of CYN in the surface extracts of *C. racemosa*. A chromatographic dilution series experiment revealed the detection limit of CYN at  $0.01 \mu\text{g ml}^{-1}$ .

## DISCUSSION

The abundance of epibiotic bacteria and diatoms on the frond surfaces of *Caulerpa racemosa* and in the biofilms developed on the reference substrata was similar as revealed by electron microscopic enumeration (Fig. 1). One explanation for this observation was the low physical renewal of epithelial algal tissue or surface sloughing (Wahl 1997, Nylund & Pavia 2005). An alternative explanation was the possibility of a targeted chemical defense against certain microbial species, leading to a numerically similar but compositionally different microbial community profile in comparison to undefended substrates (Harder et al. 2003).

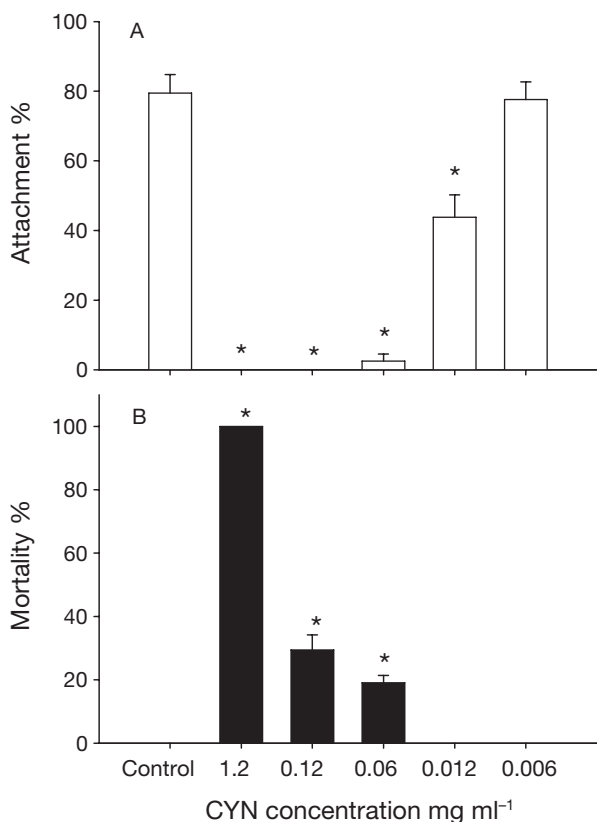


Fig. 6. Effect of caulerpenyne (CYN) on (A) larval settlement and (B) larval mortality of *Hydroides elegans*. Larvae treated with  $10^{-4}$  M 3-isobutyl-1-methylxanthine prior to experiment. Filtered ( $0.22 \mu\text{m}$ ) and autoclaved seawater used as control. Percentage data are means  $\pm$  SD of 6 replicates. \*Data that are significantly different (Dunnett's post-hoc multiple comparison test,  $p < 0.05$ )

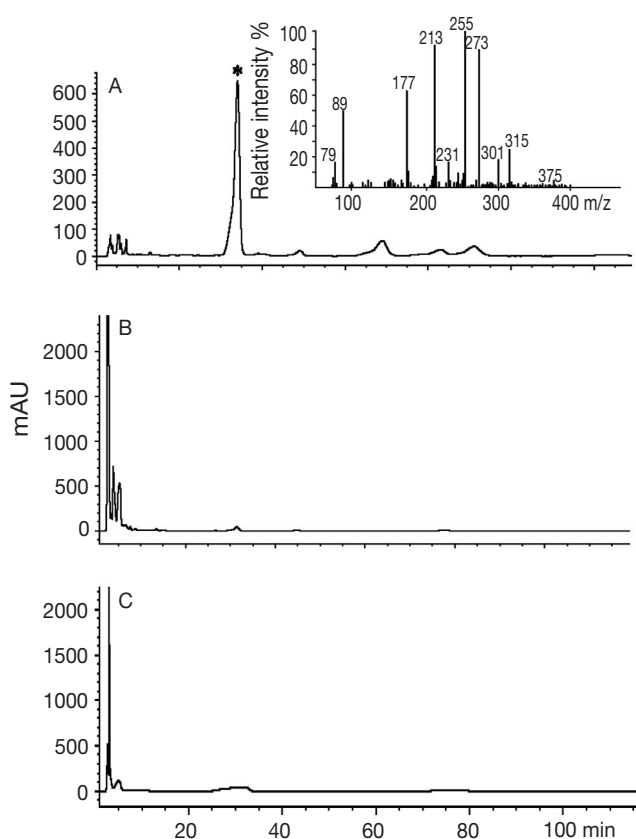


Fig. 7. Normal phase high performance liquid chromatograms of extracts of alga *Caulerpa racemosa* detected at 254 nm. (A) Ethanol extract of *C. racemosa*. Insert shows EI mass spectrum of dominant metabolite caulerpenyne. (B) Hexane:ethyl acetate (95:5%) fraction of *C. racemosa* conditioned seawater. (C) Ethyl acetate (100%) fraction of *C. racemosa* conditioned seawater

Cluster analysis of a similarity matrix demonstrated that the profiles of bacterial communities on replicate algal surfaces were highly similar, forming a cluster distantly related to bacterial communities on replicate reference surfaces. Since we assumed that the algae and the reference rock surfaces had been exposed to the same pool of bacterial colonizers at the experimental site, we hypothesized that the differences in the bacterial communities were the result of chemical alga-related attributes, such as inhibitory agents against bacterial epibiosis or nutritious agents for the enrichment of specific bacteria. The physical defenses, such as mucus production and sloughing of dead algal cells, may also have resulted in the formation of unique bacterial communities on the surface of the alga. However, we did not observe an antimicrobial effect of surface-attached and waterborne metabolites of *Caulerpa racemosa* on bacterial isolates from natural biofilms. This phenomenon could be due to the fact that bacteria may be affected by algal metabolites at any phase

of the colonization process through different mechanisms. The measurement of inhibition zones in the disk-diffusion bioassay, indicating bacteriological or bacteriostatic effects, reflects only one mechanism (inhibition of bacterial growth) by which bacterial abundance can be affected. In the natural environment, algal metabolites may affect bacterial colonization through inhibition of growth, chemotaxis (repulsion), cell motility (spreading) and production of external polymers (attachment). For instance, antibacterial activities of compounds did not necessarily correlate to the prevention of bacterial attachment (Wahl et al. 1994, Boyd et al. 1999, Newbold et al. 1999, Kelly et al. 2003).

The high similarities of bacterial community profiles between algal surfaces were supposed to stem from the uniformity and a similar presence or absence of these attributes. Despite the limitation of the T-RFLP analysis in revealing the complexity of native bacterial communities at the species level (Heuer et al. 2001), the presence or absence of certain bacterial types in the alga-associated bacterial community in comparison to the supposedly ubiquitous bacterial colonizers in the control, indicated possible physical and chemical controlled anti-epibiotic effects of the alga. The observation of bacterial enrichment of certain bacterial types was not surprising when considering the nutritional value of algal exopolymeric secretions (Wotton 2004). Indeed, specific associations between macroalgae and bacteria have previously been shown in *Caulerpa* spp. (Meusnier et al. 2001, Delbridge et al. 2004) and other macroalgae (Egan et al. 2000, Dobretsov & Qian 2002, Matsuo et al. 2003, Harder et al. 2004).

The results of the similarity analysis of the bacterial communities in the biofilms harvested from artificial substrata in the *Caulerpa* habitat and alga-free control sites 50 m away were in good accordance with the results obtained from algal and rock surface communities. Based on the similarity analysis of 242 discernible TRFs, the bacterial community profiles of the reference biofilms formed a cluster distantly related to the bacterial communities of biofilms on artificial surfaces in the *C. racemosa* stand. This suggested that algal waterborne metabolites play an important role in the formation of bacterial communities in the *Caulerpa* microhabitat.

The CCW and surface-attached metabolites did not inhibit bacterial growth in the disc diffusion assay, which could be attributed to several reasons. Firstly, a mechanical defense may be responsible for the formation of unique bacterial communities associated with this alga. Secondly, *Caulerpa* chemical compounds may have effects other than bactericidal that were not revealed in the disk-diffusion bioassay. Thirdly, the concentration of antibacterial waterborne and surface

associated compounds (i.e. CYN) may have been too low to inhibit the bacterial growth in our experiment. However, CCW exhibited a toxic effect on *Hydroïdes elegans* larvae, or evoked abnormal larval metamorphosis in a concentration dependent fashion. At sublethal concentrations, the low molecular weight fraction (<1 kDa) of CCW inhibited larval settlement of both *H. elegans* and *Bugula neritina*. Surface attached metabolites of this alga inhibited *H. elegans* settlement only at 2× SLC. These data suggested that waterborne metabolites are most likely responsible for antilarval defense in the macroalga *Caulerpa racemosa*.

We did not identify the prominent antimicrobial metabolite CYN (Dumay et al. 2002, Paul et al. 2006) in CCW. This finding may be due to either the absence of this compound in seawater or to the quick degradation of CYN in CCW (Amade & Lemée 1998). It has been shown that 50 % of CYN degrades after 4 h in seawater and only 5 % of CYN remains after 24 h in seawater (Amade & Lemée 1998). Our HPLC method identified CYN in seawater at concentrations as low as 0.01 µg ml<sup>-1</sup>, while an effective inhibitive concentration of CYN against *Hydroïdes elegans* larvae was 1000× higher. This suggests that although CCW may contain some CYN the concentration was insufficient to have any biological effect on settling larvae of *H. elegans*. Thus, bioactive metabolites other than CYN seem to be responsible for the lack of settlement response of competent larvae and the toxic effects on pre-competent larvae of *H. elegans*. Interestingly, a similar result was observed by Amade & Lemée (1998); these authors found that while CYN quickly degraded in seawater, this solution remained inhibitive to sea urchin egg cleavage. Further investigations are needed in order to pursue water-soluble antifouling compounds from *Caulerpa* and determine their concentrations as well as release rates under natural conditions.

Hypothetically, the optimal defense strategy of marine organisms would be to conserve energy and exude deterrent metabolites at or close to their surface (Jennings & Steinberg 1997, Steinberg et al. 2001, 2002). However, highly water-soluble, polar metabolites, such as phlorotannins are increasingly discounted as effective natural antifouling agents due to their rapid dissolution from their host organisms (Jennings & Steinberg 1997, Kubanek et al. 2004). Although the causative agents remain unidentified in our study, these arguments may need to be cautiously reconsidered in the light of these and our previous findings (Dobretsov & Qian 2002, Harder et al. 2004). Water-soluble molecules may play an essential chemical ecological role in marine systems, thus exemplifying a distinct concept in comparison to prominent examples of other algal antifoulants (Steinberg et al. 1997, de Nys et al. 1998, Bhadury & Wright 2004).

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