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Isolated thallus-associated compounds from the macroalga *Fucus vesiculosus* mediate bacterial surface colonization in the field similar to that on the natural alga

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This study investigated whether surface-associated compounds isolated from the macroalga *Fucus vesiculosus* had the potential to mediate microbial and/or macrobial epibiosis similar to that on the natural alga. To selectively yield thallus-associated compounds and avoid contamination by intracellular algal compounds, cell lysis was monitored by surface microscopy of algal cells and chemical profiling of algal surface extracts by coupled gas chromatography mass spectroscopy. The optimized extraction resulted in polar and non-polar algal surface extracts. The non-polar surface extract was immobilized in hydrogel, the polar surface extract was homogeneously perfused through the gel to ensure a temporally constant delivery of polar extract components. During a 7 day field trial, bacterial biofilms were formed on control gels and gels featuring polar and/or non-polar extract components. PERMANOVA revealed that bacterial community profiles on controls and on gels featuring polar or non-polar extract were significantly different from the profile on *F. vesiculosus*, while the profile on the gels bearing both polar and non-polar extracts was not. Moreover, the polar surface extracts inhibited the settlement of barnacle cyprids. Considering the pronounced effects of bacterial biofilms on invertebrate larval settlement, these results suggest that algal surface chemistry affects macrofouling not only directly but also indirectly, *via* its control of biofilm formation and composition.

Keywords: surface chemistry; biofilm; macroalgae; epibiosis; *Fucus vesiculosus*

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Introduction

Almost any substratum immersed in seawater is rapidly colonized by bacteria, fungi, diatoms, protozoa, and larvae of marine invertebrates (Wahl 1989; Abarzua et al. 1999). In contrast, the thalli of many marine macroalgae are often remarkably free from macroscopic fouling organisms. As such animate surfaces are particularly susceptible to attachment of planktonic colonizers because of their predominance in the photic zone where conditions for the growth of many fouling organisms are optimal (De Nys et al. 1995, Hellio et al. 2000, 2001; Steinberg and De Nys 2002; Kubanek et al. 2003, Lam et al. 2008), antifouling control mechanisms can be expected in macroalgae.

The surface chemistry of algal thalli not only mediates associations with beneficial microorganisms (Fries 1988; Croft et al. 2005, 2006; Matsuo et al. 2005; Tsavkelova et al. 2006), but also may attract microbial pathogens (Correa and Mclachlan 1994; Weinberger et al. 1997). More often, antimicrobial effects of extracts of macroalgae against microbial target organisms have been observed (Steinberg et al. 1997; Hellio et al. 2000; Steinberg and De Nys 2002; Pasmore and Costerton 2003; Blunt et al. 2006; Paul et al. 2006, 2007; Puglisi et al. 2007; Wahl 2008). However, the observation of host-specific biofilms on a variety of macroalgae (Lachnit et al. 2009) suggests that the mode of action of algal surface chemistry in the mediation of biofilm formation is more complex than just 'antimicrobial'. Selective recruitment of bacterial epibionts may result from both pro- and antifouling effects of surface-associated compounds on algal thalli and biofilm components. At this stage, an understanding of the chemical ecology of colonization of surfaces in marine systems by bacteria is still in its infancy. This is mainly due to difficulties in sampling and characterizing the microscale distribution of chemical cues on or near a surface, where the efficacies of such cues are crucially determined (Steinberg and De Nys 2001).

There are only a few examples where metabolites at or near the surface of the producing organism have been identified, quantified and tested in realistic concentrations against ecologically meaningful epibiota either in the laboratory or the field (De Nys et al. 1998; Maximilien et al. 1998; Kubanek et al. 2003, Nylund et al. 2006, 2008; Paul et al. 2006). The majority of experiments demonstrating antifouling effects of marine macroalgae have been carried out

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with whole algal tissue extracts (Lau and Qian 1997; Maximilien et al. 1998; Hellio et al. 2001) resulting not only in ecologically irrelevant assessments of intracellular algal metabolites, but also in an over- or underestimation of biologically active threshold concentrations of algal metabolites as extract components have been usually expressed in terms of the wet weight of the extracted algal biomass (Hay 1996).

To test the antimicrobial effects of crude algal extracts against the natural spectrum of planktonic colonizers in field assays, both polar and non-polar extracts have been immobilized in aqueous hydrogels, which slowly release the extracted components into the surrounding water (Henrikson and Pawlik 1995, Vasishtha et al. 1995; Da Gama et al. 2003; Maida et al. 2006). As the diffusion characteristics of chemically diverse crude extract components strongly depend on their polarity, delivery rates to the hydrogel surface may differ substantially among extract components. Thus, compounds with differing polarity will be delivered to the surface with considerable temporal shifts. Consequently, hydrogel-based immobilization and diffusion techniques of chemically diverse whole tissue extracts are inadequate to mimic the natural chemical environment at the algal surface over prolonged periods of time.

To minimize the short-comings of classical resin-based fouling assays under field conditions, the first objective of this study was to (a) optimize the selective extraction of surface-associated algal compounds and to (b) deliver these surface extracts to a test surface irrespective of compound polarities at a constant rate. These methodological improvements served to address the main objective of this study, which was to investigate if surface-associated compounds isolated from the thallus of the brown macroalga *Fucus vesiculosus* had the potential to mediate microbial and macrobial epibiosis similar to that observed on the natural algal surface.

Algal compounds were extracted from the thallus surface with minimal rupture of the outermost algal cell layer. To non-discriminatively and simultaneously deliver the chemically diverse extract components to an experimental gel surface, a perfusion apparatus was developed that ensured a temporally constant composition of an extract of *F. vesiculosus* at the experimental test surface. The formation of biofilms on the 'artificial thallus surface' by the pool of naturally occurring bacterial colonizers was tested in a field experiment over a period of 1 week.

Materials and methods

Collection of algae

The brown alga *F. vesiculosus* was collected near Kiel (Baltic Sea, 54°27′4 N/10°12′ E) at 0.5 to 1 m depth in May 2008. Upon collection, thalli of different individuals were individually packed in plastic bags and transferred to the laboratory in a cooler box at <10°C. Apical thalli (5 to 10 cm) were spin-dried at 600 rpm for 30 s, leaving only a boundary layer of water on the thallus surface. To avoid desiccation and damage, algal thalli they were stored until extraction in a seawater-saturated atmosphere for a maximum of 1 h.

Extraction and verification of algal surface-associated compounds

To determine the optimal extraction conditions, ie without lysing the outer cell membrane, different batches of algal thalli were extracted in hexane/methanol (1:1) in a time series. Cell lysis and the release of intracellular compounds were monitored by surface microscopy and chemical profiling of extracts by coupled gas chromatography mass spectroscopy (GC-MS), respectively.

Microscopic investigation

Algal tips (~ 3 cm^2 surface area) of three different individuals were dipped into a continuously stirred solvent mixture for 1, 5, 10,15, 20, 25, 30, 35, 50 or 60 s with replication ($n = 9$). Subsequently, algal tips were rinsed with sterile-filtered seawater and stained for 30 min with 0.05% Evans Blue in seawater. This dye selectively stains lysed cells (Weinberger et al. 2005). To measure the change in color composition due to increased incorporation of the reporter dye into lysed algal cells, fields of view (0.036 mm^2) were analyzed for their red green blue (RGB) composition on color photographs of algal thalli taken under the same light conditions with the computer program Adobe Photoshop Elements 2.0. RGB is a color model comprising the colors red, green, and blue. Each color is scored within a range from 0 to 255. The median of color values was calculated for the different extraction times and statistically compared by ANOVA to detect differences in color composition indicative of cell lysis.

Chemical profiling of algal surface extracts by GC-MS

The apical tips of several individuals were pooled to a total of 900 g (spin-dry WW). This material was divided into 15 portions of 60 g each. Batches of thalli were dip extracted in 300 ml of a stirred mixture of 1:1 methanol/hexane for 1, 5, 10, 40 or 120 s with replication ($n = 3$). After phase separation of the immiscible solvents, 1 ml of the methanol fraction was dried *in vacuo* and derivatized with 80 μl *N*-methyl-*N*-(trimethylsilyl) trifluoroacetamide (Sigma-Aldrich, Munich, Germany) at 60°C for 30 min. Derivatized

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extract components were analyzed on a Varian 3900 gas chromatograph equipped with a WCOT VF-5ms capillary column (Varian, USA; 30 m × 0.25 mm × 0.25 μm film thickness) coupled to a Saturn 2100T (Varian, USA) ion trap mass selective detector. Samples were injected in splitless mode with an inlet pressure of 72 kPa. The injection port and the interface were held at 250°C. The gas chromatograph was held at 80°C for 5 min and ramped with 15°C min^{-1} to 150°C, held at this temperature for 3 min, ramped to 200°C with 50°C min^{-1} and held for 2 min, followed by 100°C min^{-1} to 310°C. Helium was used as the carrier gas. The mass selective detector was operated in scan mode (m/z 40-650). The electron impact ion-spectra of extract components were compared with entries in the National Institute of Standards and Technology (NIST) mass spectral library (NIST 05). The relative quantity of each peak was normalized as a percentage of the entire integrated peak area of the chromatogram. Two concentration series with external standards (ie glucose and phenylalanine; Sigma-Aldrich, Germany) were used to quantify sugars and amino acids as equivalents of glucose and phenylalanine, respectively.

Preparation of algal surface extracts for field experiments

To calculate the total surface boundary layer of pooled thalli, each thallus was spread without overlap on a contrasting background, photographed and the surface area determined by image analysis (ImageJ 1.37v; NIH, USA). The combined surface area of 1550 g *F. vesiculosus* was determined as 33,790 cm^2. The total mass of pooled thalli (1550 g) was then divided into 10 groups of *ca* 150 g (spin-dry weight at 600 rpm for 30 s). The previous time-series extraction revealed that thalli could be safely surface-extracted for 10 s without being contaminated by intracellular compounds (see Results). Therefore, each group of thalli was extracted for 10 s in 300 ml of a stirred 1:1 mixture of hexane and methanol. Methanol was required to dissolve the adherent aqueous boundary layer on the wet algal material. The organic solvents were removed under vacuum resulting in a mainly aqueous remainder and a precipitated non-polar fraction. The volume of the aqueous residue (in total 110 ml) which contained the polar fraction of algal surface-associated compounds was assumed to approximate to the volume of the total surface boundary layer of extracted algal material, indicating a boundary layer thickness of 32.5 μm. This aqueous residue, subsequently referred to as the polar surface extract, was stored at −80°C. Water insoluble extract components were dissolved in 2 ml of dimethylsulfoxide, subsequently referred to as the non-polar surface extract.

Field experiment with algal surface extracts

A custom-made perfusion device ensured constant delivery of algal surface extracts to an artificial settling substratum of hydrogel. Briefly, liquid and warm hydrogel (3%; Phytagel, Sigma, Germany) was cast into a circular mould with a diameter of 2 cm and a depth of 1 cm containing a stainless steel micromesh (1 mm mesh size). Upon solidification, the mould was unscrewed on one side resulting in a gel cylinder supported by the micromesh. The gel cylinder was evenly perforated with 300 μm pins in a grid pattern 1 mm apart. The opposite side of the casting mould was screwed onto a 10 ml stainless steel container that served as an extract reservoir for the polar surface extract. The container was connected to a perfusion pump (Perfusor VII, Braun, Germany) with silicon tubing enabling a continuous delivery of extract components through the perforated hydrogel matrix (Figure 1). In a preliminary trial using a water-soluble ink, the pump rate for polar, water-soluble compounds at the hydrogel surface was determined as 64 μl cm^{-2} h^{-1}. This delivery rate compensated for diffusive loss of polar algal extract components. The non-polar surface extract was immobilized in 3% hydrogel prior to polymerization. Gels containing the respective volume of dimethlysulfoxide only served as controls. The concentration of the non-polar extract in the hydrogels was adjusted on a volumetric basis equivalent to the resulting volume of the polar surface extract.

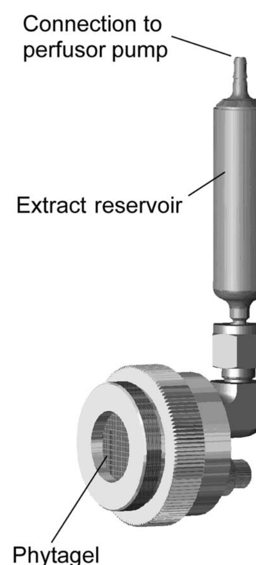

Figure 1. Extract perfusion apparatus. Up to 10 ml of extract were stored in the reservoir connected to the perfusor pump. The extract was continuously delivered through a hydrogel matrix which served as the settling substratum for bacteria and invertebrate larvae.

The experimental devices were anchored in the Kiel Bight at 1 m depth for 7 days. Extracts and controls were assayed using individual perfusion devices. Polar and non-polar surface extracts were assayed individually and in combination. In total, four experiments were set up with replication ($n = 3$): Polar surface extract combined with immobilized non-polar extract; polar surface extract combined with immobilized solvent control; sterile seawater combined with immobilized non-polar surface extract; sterile seawater combined with immobilized solvent control. Upon retrieval, the gel surfaces were rinsed with sterile seawater and barnacle settlement as well as the bacterial community composition were analyzed.

Analysis of bacterial community composition

The bacterial biofilm on algae and on experimental gel surfaces was swabbed with sterile cotton tips with replication ($n = 3$). Bacterial community DNA was extracted from swabs with the QiaAmp DNA mini kit (Qiagen, Hilden, Germany) following the manufacturers protocol for buccal swabs. 16S rRNA gene fragments were polymerase chain reaction (PCR)-amplified using the primers 341F (5′-CTA CGG GAG GCA GCA G-3′) with GC clamp (5′-CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG GC-3′) and 534R (5′-ATT ACCGCGGCTGC TGG-3′) (Muyzer et al. 1993). Reactions were carried out in a volume of 25 μl. For amplification, pure Taq Ready-To-Go PCR Beads (Amersham Biosciences Europe GmbH, Germany) were used. PCR was performed in a Techne TC-3000 thermal cycler at 94°C for 2 min; 10 touchdown cycles of 95°C for 30 s, 65°C for 40 s, and 72°C for 40 s. The annealing temperature started at 65°C and was reduced to 55°C in increments of 1°C $cycle^{-1}$; followed by 30 cycles of 95°C for 30 s, 55°C for 40 s and 72°C for 40 s; and 1 cycle 42°C for 60 s and 72°C for 5 min. All reactions yielded correct-sized amplicons with no additional by-products.

Bacterial community composition was analyzed by denaturing gradient gel electrophoresis (DGGE). PCR products were loaded onto a double gradient poly-acrylamide gel (Petri and Imhoff 2001) in 0.5× TAE. The gel was prepared with denaturing gradients ranging from 40% (15 ml acrylamide-bis 37.5:1; 16.8 g urea; 16 ml formamide; 1 ml 50× TAE; ad 100 ml H_2O) to 80% (20 ml bis-acrylamide 37.5:1; 33.625 g urea; 32 ml formamide; 1 ml 50× TAE; 100 ml H_2O) and acrylamide gradients ranging from 6 (40% denaturing gel) to 8% (80% denaturing gel). Gels were developed at 60°C for 14.5 h at 80 V. After electrophoresis, gels were stained for 45 min in Sybr–Gold® (Invitrogen GmbH, Germany), rinsed for 45 min in 1× TAE buffer and photographed under UV illumination.

Statistical analysis

Experiments to evaluate the optimal extraction time and barnacle settlement on chemically manipulated surfaces were analyzed by one-factor ANOVA followed by Tukey's honest significant differences (HSD) test using the computer program (Statistica, USA, Tulsa). The fixed factor was 'time', the dependent variables in two separate tests were the red and green values of the RGB analysis, respectively. Shapiro-Wilk's W statistic was used to test for normal distribution. Levene's test (in the case of non-normal distributed data) was used to test for homogenous variances or Cochran's C test (for normal distributed data), respectively. Some data did not fulfil the criteria of normality or homoscedasticity. Since ANOVA is relatively robust against violation of the assumption of homoscedasticity (Underwood 1997), data were analyzed parametrically at a lowered α-level of 0.01 (Wakefield and Murray 1998) to compensate for the increased probability of type 1 errors.

The similarity of DGGE banding patterns was analyzed by the computer program Primer (Primer Ltd, UK, Plymouth). Single band positions were assimilated to 'phylotypes' and band positions were transformed into a binary presence-absence matrix. Similarities between individual samples were calculated by Bray-Curtis analysis. Multidimensional scaling analysis (MDS) was applied to ordinate samples in three dimensions according to the Bray-Curtis similarity matrix, and projected onto a plane. Differences between groups were analyzed by Permutational Multivariate Analysis of variance (PERMANOVA, Anderson 2001). PERMANOVA relies on comparing the observed value of a test statistic (pseudo F-ratio) against a recalculated test statistic generated from random permutation of the data. The stated advantage of the permutation approach is that the resulting test is 'distribution free' and not constrained by many of the typical assumptions of parametric statistics. Due to limited replication ($n = 3$), the analysis of banding patterns did not yield sufficient permutations to get a reasonable test statistic. Instead, Monte Carlo p-values were generated as a test statistic (Anderson and Robinson 2003).

Results

Optimization of algal surface extraction

Evans Blue staining of extracted algal surface tissue demonstrated that a 1 s exposure to the solvent mixture already changed the algal surface cell morphology. This effect increased after 5 s; however, cells were not lysed. Incorporation of the reporter dye into cells, indicative of cell lysis, was observed only after 10 s of

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extraction (Figure 2). RGB analysis was a useful technique to measure incorporation of Evans Blue into algal surface cells as *R* and *G*-values changed significantly with increased extraction times. *B*-values were zero irrespective of the extraction time. There were no significant changes in color composition after extraction for 1 s compared to the non-extracted control. Significant differences in color composition were detected after 10 s extraction time for the *R*-value ($n = 9$, $F = 87$, $p < 0.001$, one-way ANOVA, Tukey's HSD) and already after 5 s for the *G*-value ($n = 9$, $F = 87$, $p < 0.001$, one-way ANOVA, Tukey's HSD) (Figure 3).

Cell lysis and the concomitant release of intracellular cell components could also be followed by GC-MS analysis of algal surface extracts. There was a clear positive correlation of the number of detected algal surface-associated compounds and increased extraction time (Figures 3 and 4). The outer surface of intact algal epidermal cells featured only two amino acids (L-serine and L-threonine) and two sugars (beta-D-galactofuranose and glycitol), which were present in

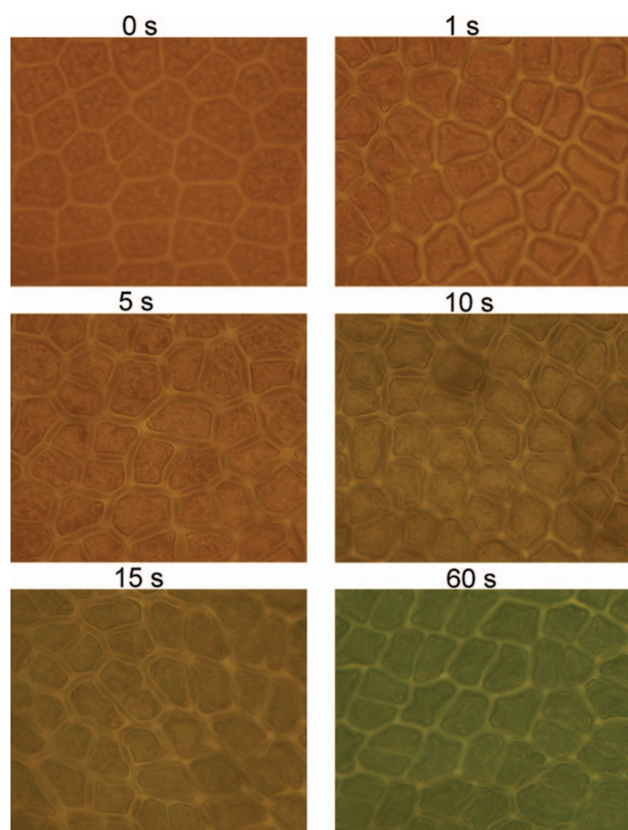

Figure 2. Thallus surface of *F. vesiculosus* after extraction in hexane/methanol (1:1) for 0, 1, 5, 10 and 60 s. Incorporation of the reporter dye Evans Blue indicated cell lysis of outer cells after 10 s of dip extraction.

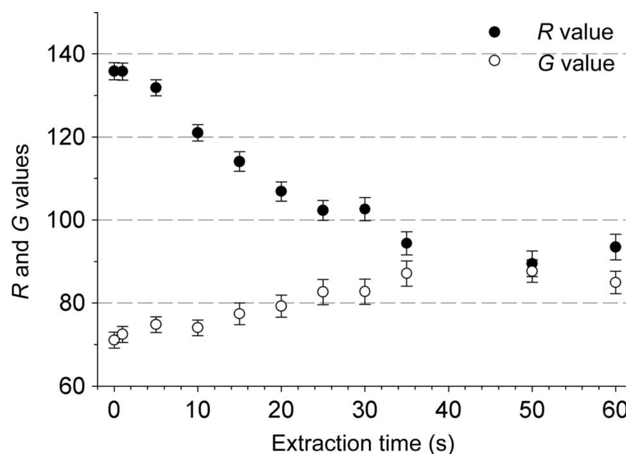

Figure 3. *R* (red) and *G* (green) values of surface cell layers of *F. vesiculosus* after different extraction times and staining with Evans blue. The *y*-axis represents means ± SE of RG readings ($n = 3$).

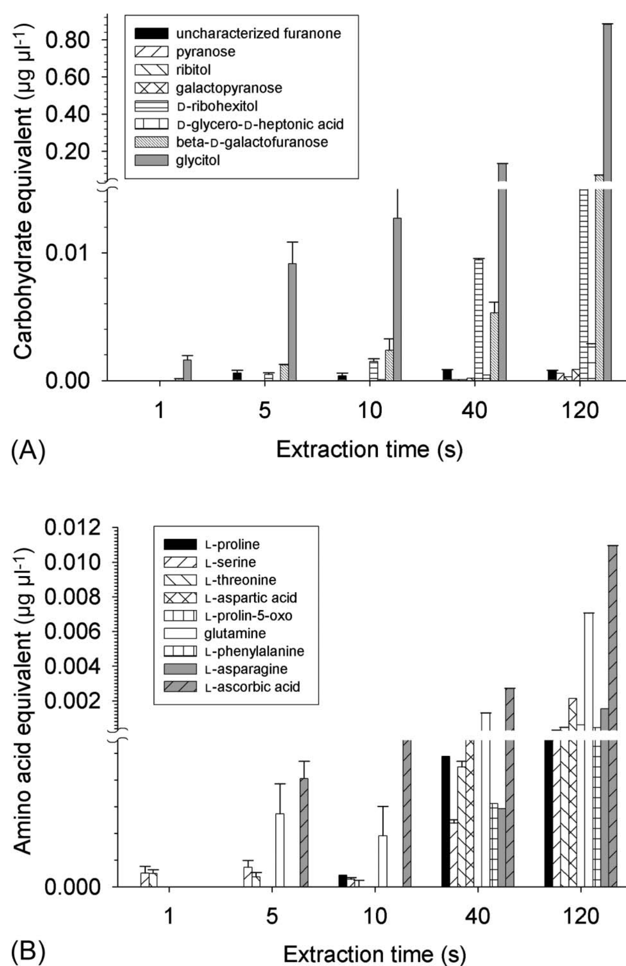

Figure 4. A/B. GC-MS analysis of *F. vesiculosus* surface extracts after different extraction times with replication ($n = 3$). Concentrations of sugars (A) and amino acids (B) were quantified by external calibration with glucose and phenylalanine, respectively.

surface extracts obtained after 1 s. Extraction for 10 s revealed three additional sugars and amino acids, respectively, in the concentration range of less than 5 ng μl^{-1}. Any extraction longer than 10 s not only increased the concentration of compounds already present in the short time extracts *ca* 20-fold, but also revealed additional compounds in these extracts. No additional compounds appeared after extraction for 120 s (Figures 3 and 4). Based on these results, the maximim duration of a non-destructive surface extraction was determined to be 10 s for *F. vesiculosus* and algal samples were extracted accordingly.

Effect of F. vesiculosus *surface extracts on colonization by barnacles and bacteria*

A 7 day field trial with polar and non-polar fractions of the algal surface extracts, obtained and delivered according to the methodology outlined above, resulted in significantly lower levels of colonization by barnacles compared to hydrogel control substrata. The antifouling effect was observed with the polar fraction as well as the combination of polar and non-polar fractions of the algal surface extract ($n = 3$, $F = 8$, $p < 0.001$, one-way ANOVA, Tukey's HSD, Figure 5), but not with the apolar fraction alone.

The community composition of bacterial colonizers on experimental hydrogel substrata was also influenced by algal surface extracts (Figure 6). Similarity, analysis grouped the bacterial communities on experimental substrata into three main groups. One distinctive community pattern was shared by the gels chemically influenced by the non-polar fraction of algal surface extract (A) and on control gels (C). Another distinct community pattern was found on gels influenced by the polar fraction of algal surface extract (P), the combination of polar and non-polar surface extracts (PA), and the natural bacterial biofilm community on *F. vesiculosus* replicate FV 1. A third distinct community pattern was observed on *F. vesiculosus* replicates FV 2 and FV 3 (Figure 6). While the bacterial community profiles on control gels (C) and on gels treated with polar (P) or non-polar (A) extract were significantly different from the profile on the alga (FV), the profile on the gels treated with both, polar and non-polar extracts (PA), was not (PERMANOVA, p_MC = 0.25, Table 1).

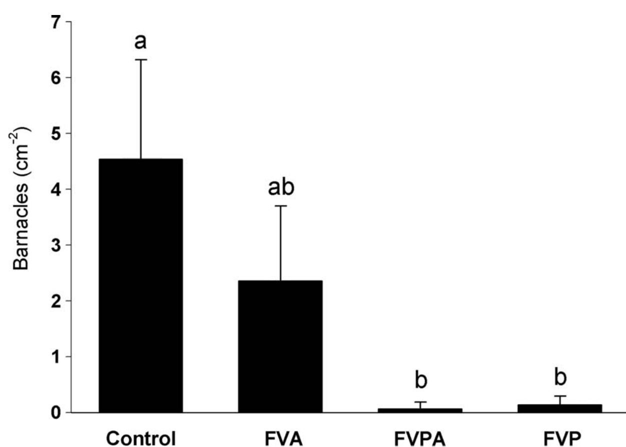

Figure 5. Barnacle cyprid settlement on chemically manipulated hydrogels (mean ± SE, $n = 3$). Statistical differences are indicated by different letters above the bars ($\alpha = 0.01$, Tukey's test). Control: sterile seawater control combined with immobilized solvent; FVA, non-polar surface extract; FVP, polar surface extract; FVPA, combined polar and non-polar surface extract.

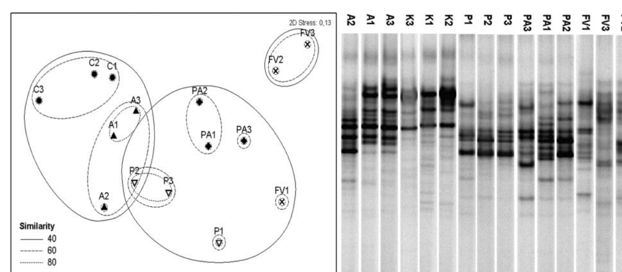

Figure 6. DGGE gel and non-metric multidimensional scaling (NMDS) plot showing the bacterial community composition on *F. vesiculosus* (FV) and chemically manipulated hydrogels ($n = 3$). Multidimensional scaling of DGGE band pattern was performed using the Bray-Curtis similarity index. The stress value was 0.13. C, sterile seawater control combined with immobilized solvent; A, non-polar surface extract; P, polar surface extract; PA, combined polar and non-polar surface extract.

Table 1. Pairwise comparison of bacterial communities on different individuals of *F. vesiculosus* and hydrogels chemically manipulated with algal surface extracts derived from DGGE banding pattern analysis.

Tests among levels of the factor surface				
Group	t	P_PERM	P_MC	# UP
FV/PA	1.3810	0.2730	0.2520	10
FV/P	2.9055	0.0990	0.0430	10
FV/C	3.9146	0.0890	0.0240	10
FV/A	3.9336	0.0960	0.0170	10
PA/P	3.3066	0.1010	0.0220	10
PA/C	4.8354	0.0910	0.0070	10
PA/A	2.9172	0.0980	0.0300	10
P/C	4.6347	0.1010	0.0050	10
P/A	4.4549	0.0930	0.0090	10
C/A	4.1123	0.1080	0.0140	9

FV, *F. vesiculosus*; PA, polar combined with non-polar; P, polar; A, non-polar; C, control.

Similarities of DGGE banding patterns of bacterial communities were analyzed within and between group identities.

Permutation p-values (P_PERM) and Monte Carlo p-values (P_MC) as well as the number of unique permutations (#UP) obtained by PERMANOVA based on 999 permutations are presented.

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Discussion

Many marine macroalgae contain compounds with the potential to inhibit colonization by macrobial (Fusetani 2004; Blunt et al. 2006; Paul et al. 2007) and microbial foulers (Steinberg et al. 1998; Boyd et al. 1999; Hellio et al. 2000, 2001; Puglisi et al. 2007; Dubber and Harder 2008). Most of the studies used crude algal tissue extracts comprising both intra- and extra- cellular compounds, and laboratory based fouling assays. Owing to this experimental approach, the ecological relevance of the findings remain unclear as to whether the causative compounds would occur in sufficient concentrations to exert a similar effect on the algal surface in nature.

The present study tested the hypothesis that the colonization of algal thalli by bacteria and invertebrate larvae is influenced and directed by the surface chemistry present on the alga. This assumption was stimulated by previous observations demonstrating that bacterial community compositions on the macroalgae *F. vesiculosus, Saccharina latissima, Delesseria sanguinea, and Ulva compressa* significantly differed from each other (Lachnit et al. 2009), thus suggesting a specific association of bacteria with algae that is controlled at least in part by the surface chemistry of algae.

Conceptually, the surface chemistry of a macroalga is the sum of exuded secondary metabolites and extracellular exopolymeric substances (EPS) present on the thallus. This complex mixture of compounds is presumably in equilibrium with the ambient water body. Depending on their solubility in seawater, compounds may get more or less concentrated in the diffusion boundary layer (DBL) surrounding the thallus, which is a thin layer of stationary water ranging between 40 and 100 μm not mixed with ambient water (Wahl 1997; Hurd 2000). The *in situ* concentration of exuded algal metabolites on the thallus surface can be regarded as the sum of molecular adhesion to the thallus surface, diffusion out of the DBL and bacterial degradation within the DBL. Once algal metabolites are degraded by thallus-associated bacteria, the chemical cocktail in the DBL may be further enriched by bacterial metabolites.

It is presumably the chemical composition in the DBL and the gradients reaching outwards which will attract or repel colonizers and, thus, mediate recruitment of fouling organisms on the thallus (Steinberg and De Nys 2001). The objective of this study was to harvest the cocktail of bioavailable thallus-associated compounds and test their effect on surface colonization by bacteria and barnacle cyprids. To avoid the contamination of surface-associated compounds of the alga by intracellular material during extraction, potential cell lysis was carefully monitored by surface microscopy of stained algal cells. The analysis of the chemical profile of algal surface extracts by GC-MS was suitable to detect changes in concentration and composition of indicator molecules (sugars and amino acids) as a function of extraction time and algal cell lysis.

To simulate the chemistry at the algal surface, non-polar compounds were embedded in hydrogel while polar compounds diffusing out of the gel were continuously replaced at the hydrogel surface with a perfusion apparatus delivering the polar algal surface extract. The non-polar surface extract could not be delivered in the same way due to its poor water solubility and the toxicity of organic solvents to bacteria. Therefore, the approach adopted was an experimental compromise to simulate the chemistry at the algal surface by embedding non-polar compounds with low diffusion characteristics, while continuously replacing lost polar compounds (with presumably high diffusion characteristics) to the hydrogel surface through the perfusion apparatus. The colonization of this artificial 'thallus surface' by bacteria resulted in a community profile that was not significantly different from the profile on the alga (FV), whereas the bacterial community profiles on control gels (C) and on gels treated with either polar (P) or non-polar (A) extract were significantly different from the profile on the alga (Table 1). This outcome indirectly suggests that the combined presence of polar and non-polar algal surface extract components shaped the pattern of bacterial colonization in a manner similar to the natural alga. Even though the bacterial community composition on the treatments was not identical to the composition on the natural algal surface, four to twelve bacterial phylotypes, indicated by the same gel bands, recruited to the experimental gel surfaces featuring surface-available compounds on the alga. Other different biological and physical surface properties between the gel and the thallus surface, such as difference in biofilm age, surface topography, texture, and wettability may have contributed to differences in community composition between natural thalli and chemically manipulated gel surfaces.

In addition to the pronounced effect of algal surface extracts on the community composition of microcolonizers, a significant inhibitory effect of polar surface extracts on barnacle cyprid settlement was observed. This observation was in accordance with a previous study demonstrating an anti-settlement effect on barnacles of waterborne compounds from *F. vesiculosus* (Brock et al. 2007).

In summary, algal surface-associated compounds appear to mediate bacterial colonization of the thallus surface of *F. vesiculosus* to a remarkable degree.

It currently remains a matter of speculation whether the surface chemistry of the alga influences different colonizers directly or if subsequent epibionts are attracted or repelled by earlier recruits. In the light of the large body of literature on the pronounced effects of bacterial biofilms on invertebrate larval settlement (Dobretsov et al. 2006), the capacity of *F. vesiculosus* to control its bacterial biofilm deserves attention.

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References

Abarzua S, Jakubowski S, Eckert S, Fuchs P. 1999. Biotechnological investigation for the prevention of marine biofouling II. Blue-green algae as potential producers of biogenic agents for the growth inhibition of microfouling organisms. Bot Mar 42:459–465.

Anderson MJ. 2001. Permutation tests for univariate or multivariate analysis of variance and regression. Can J Fish Aquat Sci 58:626–639.

Anderson MJ, Robinson J. 2003. Generalized discriminant analysis based on distances. Aust NZ J Stat 45:301–318.

Blunt JW, Copp BR, Munro MHG, Northcote PT, Prinsep MR. 2006. Marine natural products. Nat Prod Rep 23:26–78.

Boyd KG, Adams DR, Burgess JG. 1999. Antibacterial and repellent activities of marine bacteria associated with algal surfaces. Biofouling 14:227–236.

Brock E, Nylund GM, Pavia H. 2007. Chemical inhibition of barnacle larval settlement by the brown alga *Fucus vesiculosus*. Mar Ecol Prog Ser 337:165–174.

Correa JA, Mclachlan JL. 1994. Endophytic algae of *Chondrus crispus* (Rhodophyta). 5. Fine-structure of the infection by *Acrochaete operculata* (Chlorophyta). Eur J Phycol 29:33–47.

Croft MT, Warren MJ, Smith AG. 2006. Algae need their vitamins. Eukaryot Cell 5:1175–1183.

Croft MT, Lawrence AD, Raux-Deery E, Warren MJ, Smith AG. 2005. Algae acquire vitamin B-12 through a symbiotic relationship with bacteria. Nature 438:90–93.

Da Gama BAP, Pereira RC, Soares AR, Teixeira VL, Yoneshigue-Valentin Y. 2003. Is the mussel test a good indicator of antifouling activity? A comparison between laboratory and field assays. Biofouling 19:161–169.

De Nys R, Dworjanyn SA, Steinberg PD. 1998. A new method for determining surface concentrations of marine natural products on seaweeds. Mar Ecol Prog Ser 162:79–87.

De Nys R, Steinberg PD, Willemsen P, Dworjanyn SA, Gabelish CL, King RJ. 1995. Broad-spectrum effects of secondary metabolites from the red alga *Delisea pulchra* in antifouling assays. Biofouling 8:259–271.

Dobretsov S, Dahms HU, Harder T, Qian PY. 2006. Allelochemical defense against epibiosis in the macroalga *Caulerpa racemosa* var. *turbinata*. Mar Ecol Prog Ser 318:165–175.

Dubber D, Harder T. 2008. Extracts of *Ceramium rubrum, Mastocarpus stellatus* and *Laminaria digitata* inhibit growth of marine and fish pathogenic bacteria at ecologically realistic concentrations. Aquaculture 274:196–200.

Fries L. 1988. *Ascophyllum nodosum* (Phaeophyta) in axenic culture and its response to the endophytic fungus *Mycosphaerella ascophylli* and epiphytic bacteria. J Phycol 24:333–337.

Fusetani N. 2004. Biofouling and antifouling. Nat Prod Rep 21:94–104.

Hay ME. 1996. Marine chemical ecology: what's known and what's next? J Exp Mar Biol Ecol 200:103–134.

Hellio C, Bremer G, Pons AM, Le Gal Y, Bourgougnon N. 2000. Inhibition of the development of microorganisms (bacteria and fungi) by extracts of marine algae from Brittany, France. Appl Microbiol Biotechnol 54:543–549.

Hellio C, De La Broise D, Dufosse L, Le Gal Y, Bourgougnon N. 2001. Inhibition of marine bacteria by extracts of macroalgae: potential use for environmentally friendly antifouling paints. Mar Environ Res 52:231–247.

Henrikson AA, Pawlik JR. 1995. A new antifouling assay method: results from field experiments using extracts of four marine organisms. J Exp Mar Biol Ecol 194:157–165.

Hurd CL. 2000. Water motion, marine macroalgal physiology, and production. J Phycol 36:453–472.

Kubanek J, Jensen PR, Keifer PA, Sullards MC, Collins DO, Fenical W. 2003. Seaweed resistance to microbial attack: a targeted chemical defense against marine fungi. Proc Natl Acad Sci USA 100:6916–6921.

Lachnit T, Blümel M, Imhoff JF, Wahl M. 2009. Specific epibacterial communities on macroalgae: phylogeny matters more than habitat. Aquat Biol 5:181–186.

Lam C, Grage A, Schultz D, Schulte A, Harder T. 2008. Extracts of North Sea macroalgae reveal specific activity patterns against attachment and proliferation of benthic diatoms: a laboratory study. Biofouling 24:59–66.

Lau SCK, Qian PY. 1997. Phlorotannins and related compounds as larval settlement inhibitors of the tube-building polychaete *Hydroides elegans*. Mar Ecol Prog Ser 159:219–227.

Maida M, Sammarco PW, Coll JC. 2006. A diffusion chamber for assessing efficacy of natural anti-fouling defenses in marine organisms. J Exp Mar Biol Ecol 337:59–64.

Matsuo Y, Imagawa H, Nishizawa M, Shizuri Y. 2005. Isolation of an algal morphogenesis inducer from a marine bacterium. Science 307:1598–1598.

Maximilien R, de Nys R, Holmstrom C, Gram L, Givskov M, Crass K, Kjelleberg S, Steinberg PD. 1998. Chemical mediation of bacterial surface colonisation by secondary metabolites from the red alga *Delisea pulchra*. Aquat Microb Ecol 15:233–246.

Muyzer G, Dewaal EC, Uitterlinden AG. 1993. Profiling of complex microbial populations by denaturing gradient gel-electrophoresis analysis of polymerase chain reaction amplified genes coding for 16s ribosomal RNA. Appl Environ Microbiol 59:695–700.

Nylund GM, Gribben PE, de Nys R, Steinberg PD, Pavia H. 2006. Surface chemistry versus whole-cell extracts: antifouling tests with seaweed metabolites. Mar Ecol Prog Ser 329:73–84.

Nylund GM, Cervin G, Persson F, Hermansson M, Steinberg PD, Pavia H. 2008. Seaweed defence against bacteria: a poly-brominated 2-heptanone from the red alga *Bonnemaisonia hamifera* inhibits bacterial colonisation. Mar Ecol Prog Ser 369:39–50.

Pasmore M, Costerton JW. 2003. Biofilms, bacterial signaling, and their ties to marine biology. J Ind Microbiol Biotechnol 30:407–413.

Paul NA, de Nys R, Steinberg PD. 2006. Chemical defence against bacteria in the red alga *Asparagopsis armata*: linking structure with function. Mar Ecol Prog Ser 306:87–101.

Paul VJ, Arthur KE, Ritson-Williams R, Ross C, Sharp K. 2007. Chemical defenses: from compounds to communities. Biol Bull 213:226–251.

Petri R, Imhoff JF. 2001. Genetic analysis of sea-ice bacterial communities of the Western Baltic Sea using an improved double gradient method. Polar Biol 24:252–257.

Puglisi MP, Engel S, Jensen PR, Fenical W. 2007. Antimicrobial activities of extracts from Indo-Pacific marine plants against marine pathogens and saprophytes. Mar Biol 150:531–540.

Steinberg PD, De Nys R. 2001. Chemical mediation of surface colonization.Marine chemical ecology. Boca Raton: CRC Press. p. 355–387.

Steinberg PD, De Nys R. 2002. Chemical mediation of colonization of seaweed surfaces. J Phycol 38:621–629.

Steinberg PD, Schneider R, Kjelleberg S. 1997. Chemical defenses of seaweeds against microbial colonization. Biodegradation 8:211–220.

Steinberg PD, De Nys R, Kjelleberg S. 1998. Chemical inhibition of epibiota by Australian seaweeds. Biofouling 12:227–244.

Tsavkelova EA, Klimova SY, Cherdyntseva TA, Netrusov AI. 2006. Microbial producers of plant growth stimulators and their practical use: a review. Appl Biochem Microbiol 42:117–126.

Underwood AJ. 1997. Experiments in ecology. Cambridge, UK: Cambridge University Press.

Vasishtha N, Sundberg DC, Rittschof D. 1995. Evaluation of release rates and control of biofouling using monolithic coatings containing an isothiazolone. Biofouling 9:1–16.

Wahl M. 1989. Marine epibiosis. 1. Fouling and antifouling – some basic aspects. Mar Ecol Prog Ser 58:175–189.

Wahl M. 1997. Living attached: aufwuchs, fouling, epibiosis. In: Nagabhushanam R, Thompson MF, editors. Fouling organisms of the Indian Ocean: biology and control technology. New Delhi, India: Oxford & IBH Publ. Co. Pvt Ltd. p. 31–83.

Wahl M. 2008. Ecological lever and interface ecology: epibiosis modulates the interactions between host and environment. Biofouling 24:427–438.

Wakefield RL, Murray SN. 1998. Factors influencing food choice by the seaweed-eating marine snail *Norrisia norrisi* (Trochidae). Mar Biol 130:631–642.

Weinberger F, Hoppe HG, Friedlander M. 1997. Bacterial induction and inhibition of a fast necrotic response in *Gracilaria conferta* (Rhodophyta). J Appl Phycol 9:277–285.

Weinberger F, Pohnert G, Berndt ML, Bouarab K, Kloareg B, Potin P. 2005. Apoplastic oxidation of L-asparagine is involved in the control of the green algal endophyte *Acrochaete operculata* Correa & Nielsen by the red seaweed *Chondrus crispus* Stackhouse. J Exp Bot 56:1317–1326.

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