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Surface chemistry versus whole-cell extracts: antifouling tests with seaweed metabolites

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ABSTRACT: Development of research in the field of chemical inhibition of colonisation of seaweed surfaces has been constrained by the lack of appropriate methods for testing realistic concentrations of potentially deterrent compounds. Here we extend earlier work (de Nys et al. 1998) on the red alga *Delisea pulchra* to 6 other Australian seaweed species to investigate whether these methods could be used more generally in studies of natural defences against biofouling. We compared the effects of surface extracts of *D. pulchra*, *Caulerpa filiformis*, *Dictyopteris acrostichoides*, *Dilophus marginatus*, *Laurencia rigida*, *Solieria robusta* and *Pterocladia capillacea* on the settlement of 2 ecologically relevant fouling species, and further compared the effects of surface extracts to those of non-polar, whole-cell extracts of the 7 seaweeds. We also measured the natural biofouling cover of these seaweeds in a field survey and examined whether levels of biofouling on the seaweeds in the field are predicted by the activity of either the surface extracts or the whole-cell extracts of these species. The results from settlement tests with surface extracts at natural concentrations showed that 2 species, *D. pulchra* and *C. filiformis*, had non-polar metabolites on their surfaces in sufficient quantities to significantly inhibit settlement. These species also had significantly lower biofouling cover in the field compared to the other seaweeds. The results of the settlement tests with whole-cell extracts, however, demonstrated that all the seaweeds contain non-polar metabolites that inhibit settlement at concentrations lower than total whole tissue content and that no individual whole-cell extract was generally more inhibitory than the others. Therefore, we conclude that results from settlement assays with whole-cell extracts are poor predictors of natural antifouling roles of seaweed metabolites, and that such bioassays are of little use if the objective is to explore the chemical mediation of interactions between seaweeds and fouling organisms. We also conclude, that with careful choice of solvent and extraction time, the surface extraction procedure described here may be broadly useful for investigating the deterrent effects of seaweed surface metabolites against fouling organisms.

KEY WORDS: Biofouling · Surface extraction · Seaweeds · Dipping technique · Chemical defence · Epiphytes

INTRODUCTION

Secondary metabolites play a major role in mediating ecological interactions between marine organisms (Hay 1996). For seaweeds, most evidence for chemical mediation of ecological interactions comes from studies on herbivory (Paul et al. 2001). However, research is focusing increasingly on chemical mediation of biofouling (epiphytism). Biofouling can affect seaweeds negatively (de Nys & Steinberg 1999), and thus should exert selection for the development of deterring properties against biofouling. Antifouling adaptations may be physical, such as sloughing of the outermost cell layers (Johnson & Mann 1986, Keats et al. 1997, Nylund & Pavia 2005), physiological, such as oxidative bursts (Collen et al. 1995, Weinberger et al. 1999, Weinberger & Friedlander 2000), or chemical, i.e. the production of secondary metabolites that prevent

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settlement and growth of fouling organisms (Steinberg et al. 2001).

Over the last decade, a significant number of researchers have reported that crude chemical extracts or isolated compounds from seaweeds deter fouling organisms. For example, metabolites from brown algae reduce the settlement and normal development of the common fouling bryozoan species *Bugula neritina* (Schmitt et al. 1995, Walters et al. 1996, Steinberg et al. 1998), inhibit metamorphosis of larvae of the polychaete *Hydroides elegans* (Walters et al. 1996, Lau & Qian 1997), inhibit settlement of cyprid larvae of the barnacle *Balanus improvisus* (Wikström & Pavia 2004), and inhibit spore settlement of the green alga *Enteromorpha prolifera* (Cho et al. 2001). Furthermore, metabolites from some red algae inhibit settlement of cyprid larvae of the barnacles *Balanus improvisus* and *B. amphitrite* (de Nys et al. 1995, Konig et al. 1999, Nylund & Pavia 2003), and of larvae of *B. neritina* and *H. elegans* (Walters et al. 1996, Steinberg et al. 1998). In addition they inhibit the settlement and germling development of gametes of the green algae *Ulva* spp. (de Nys et al. 1995, Steinberg et al. 1998, Dworjanyn et al. 2006), the red algae *Polysiphonia* sp. and *Ceramium* sp., and the brown alga *Ectocarpus siliculosus* (Dworjanyn et al. 2006). Additionally, some green algal species produce settlement-inhibiting metabolites against certain fouling invertebrates and algae (Walters et al. 1996, Steinberg et al. 1998, Harder et al. 2004).

Although a number of researchers have reported antifouling properties of algal metabolites, ecologically relevant roles of secondary metabolites as biofouling inhibitors have only been rigorously demonstrated for 4 seaweed species: *Dictyota menstrualis*, *Delisea pulchra*, *Bonnemaisonia hamifera* and *Asparagopsis armata* (Schmitt et al. 1995, Steinberg et al. 2001, Nylund et al. 2005, Dworjanyn et al. 2006, Paul et al. 2006). For a metabolite to be a natural antifoulant it must either be present at the surface of the host or released to the surrounding water at a concentration that deters ecologically relevant fouling organisms (Davis et al. 1989). Many researchers on the biofouling ecology of seaweed secondary metabolites have used crude extracts or purified compounds from whole plant material without showing that the metabolites are present at surface concentrations sufficient to deter fouling organisms (Lau & Qian 1997, Konig et al. 1999, Wisespongpand & Kuniyoshi 2003). In the few instances where this has been explicitly examined, the inhibitory effects of whole plant crude extracts were not good predictors of the ability of seaweeds to deter and prevent biofouling (Nylund & Pavia 2003).

The lack of a standard method for testing ecologically relevant concentrations of surface metabolites against fouling organisms has been a significant constraint on the advancement of research on the chemical mediation of surface colonisation of seaweeds. Here we address this issue by extending the surface extraction 'dipping' technique developed by de Nys et al. (1998) to a variety of other seaweeds, including species containing biologically active secondary metabolites. We developed protocols for extraction of surface-associated metabolites from these seaweeds and tested these extracts against settlement of 2 ecologically relevant fouling species in laboratory bioassays. We compared the effect of these non-polar surface extracts with whole-cell extracts for 7 Australian seaweed species and examined whether the levels of biofouling of the seaweeds in the field are predicted by the activity of either the surface extracts or the whole-cell extracts of these species. The more general goal was to investigate if antifouling tests using whole-cell extracts could be a useful predictor of the function of an algal metabolite as an ecologically relevant antifoulant.

MATERIALS AND METHODS

Study organisms and collection of seaweeds for extractions. Seven seaweed species common in the Sydney area were chosen to compare the antifouling effects of surface extracts with whole-cell extracts. The species were the brown algae *Dictyopteris acrostichoides* (J. Agardh) Bornet 1885 and *Dilophus marginatus* J. Agardh ex J. Agardh, 1985; the green alga *Caulerpa filiformis* (Suhr) K. Hering, 1841; and the red algae *Delisea pulchra* (Greville) Montagne, 1844, *Laurencia rigida* J. Agardh, 1876, *Pterocladia capillacea* (S. G. Gmelin) Bornet, 1876 and *Solieria robusta* (Greville) Kylin, 1932. The seaweeds were collected by snorkelling and on SCUBA from 3 localities in the Sydney area (Bare Island, Shark Bay and Shark Point) during May and June 2004. The seaweeds were placed in individual plastic bags under water and immediately transported to the laboratory in abundant water. At the laboratory, the seaweeds collected for surface extractions were placed in individual aerated containers filled with freshly collected seawater and extracted on the same day. The algae collected for whole-cell extractions were dried in a salad spinner, weighed and placed in a freezer.

The fouling test organisms used in this study were gametes of the green alga *Ulva australis* and carpospores of the red alga *Polysiphonia* sp. These species are part of the natural fouling community on seaweeds (Wahl & Hay 1995, Jennings & Steinberg 1997, Wahl et al. 1997, Dworjanyn 2000, Karez et al. 2000).

Surface extractions: determination of solvents and extraction times. Surface-associated metabolites from the algal species were extracted using the 'dipping'

technique (de Nys et al. 1998). This method allows surface-associated non-polar compounds to be extracted without co-extracting the interior of the cells. We used 100% hexane for extracting surface-associated metabolites of *Delisea pulchra*, *Pterocladia capillacea* and *Solieria robusta*. Appropriate solvents for extractions of surfaces of the other seaweeds, i.e. *Caulerpa filiformis*, *Dictyopteris acrostichoides*, *Dilophus marginatus* and *Laurencia rigida* were determined by extracting pieces of algal thalli in different mixtures of dichloromethane (DCM) and hexane (0 to 10% DCM, depending on the species) and assessing the effect on the lysis of epithelial cells using epifluorescent microscopy at ×1000 (Zeiss Axiophot microscope with a 360 to 395 nm long-pass filter, dichromic mirror at 395 nm, and band-pass filter of 420 nm). Pieces of algae (n = 5 for each species), carefully dried with precision wipes, were added to 20 ml glass vials containing 5 ml of different DCM-hexane mixtures and vortexed for 30 s. Vials containing seawater only served as controls. Following extraction, the effect of different DCM-hexane mixtures on the lysis of surface cells for all of the algal species, except *C. filiformis*, was directly assessed by counting the number of lysed vs. intact cells for 10 randomly-chosen fields (0.1 mm^2) for each DCM-hexane mixture. The effect of different DCM-hexane mixtures on cell lysis of *C. filiformis*, which is siphonous and lacks cell walls, was qualitatively assessed by comparing the shape of the chloroplasts for tissues extracted with different DCM-hexane mixtures.

After appropriate solvents had been determined for each algal species, we extracted 5 individual pieces (n = 5) respectively of *Caulerpa filiformis* for 0, 30 and 60 s ; *Dilophus marginatus* and *Solieria robusta* for 0, 20, 30, 40, 50 and 60 s; *Laurencia rigida* and *Pterocladia capillacea* for 0, 20, 30, 40 and 50 s; or *Delisea pulchra* and *Dictyopteris acrostichoides* for 0, 30, 40, 50 and 60 s. Following the extractions, the effect of extraction time was immediately assessed by counting the number of lysed vs. intact cells for 10 randomly-chosen fields (0.1 mm^2) for each extraction time. The effect of extraction time on *C. filiformis* was qualitatively assessed by observing changes of the chloroplasts. These extractions gave us information as to when the algal epithelial cells began to lyse. Appropriate immersion times in the solvents were then decided by choosing the longest possible immersion time when no significant cell lysis occurred, compared to controls, to maximise surface extraction duration and efficiency.

Surface extraction for bioassays. ***Variable solvent and time extraction:*** Surface extracts for bioassays were obtained by extracting pieces of individual algal thalli on a vortex using solvents and immersion times based on the results of the preceding tests. To use concentrations of surface extracts in the bioassays that would correspond to the natural concentration of surface metabolites of the algal species, surface area:wet weight ratios were calculated for all of the algae. Based on the surface area:wet weight ratios, algal pieces with surface areas (determined by weighing the algal pieces), equal to and twice as large as the area of the test dishes (9 cm^2) used in the bioassays, were extracted. We extracted algal pieces with surface areas both 1 and 2 times the area of the test dishes because we did not know how efficient the extraction procedure was for all the non-polar surface-associated metabolites. This procedure resulted in several replicate units of surface extract of each algal species, corresponding to 1 and 2 times the amount of surface extracts yielded from algal tissue with the same surface area as the test dishes. This is hereafter termed 'natural' and 'twice the natural' concentration of surface metabolites. The solvents used in the surface extractions were evaporated in a fume cupboard overnight, and the resulting extracts were bioassayed the following day.

Fixed solvent and time extraction: As detailed above, the 'dipping' technique requires that specific extraction solvent and immersion times are determined, which can be labour-intensive (de Nys et al. 1998). Other techniques such as the swabbing technique (Schmitt et al. 1995) can be difficult to apply on small filamentous algae. Thus, we investigated whether a simpler technique of extraction of fresh algal material in DCM could function as an alternative method for initial screenings of surface-associated natural antifoulants in seaweeds. Fresh pieces of algae with surface areas equal to the area of the test dishes (9 cm^2) were extracted in 100% DCM for 30 s on a vortex as described above. Due to its polarity, DCM should extract surface-associated metabolites more efficiently than using hexane (de Nys et al. 1998). Since DCM was used for whole-cell extractions, the extraction of fresh material in DCM also served to control any differences in antifouling activity between the DCM hexane surface extracts and whole cell extracts. Differences here could result from the extraction of surface associated vs. interior metabolites or the extraction of metabolites with dissimilar polarities. The extractions with DCM were repeated to obtain several replicate units of extracts of each algal species, hereafter termed 'DCM surface extracts'. After the extractions, the DCM was evaporated in a fume cupboard overnight and the resultant extracts were bioassayed the following day.

Whole-cell extractions. Assays of whole-cell extracts were also done for each seaweed species. The algae collected for whole-cell extractions were freeze-dried, ground and extracted 3 times for 2 h with DCM on a shaking table; then the resulting solutions were fil-

tered, and the DCM was reduced by rotary evaporation. Extracts were then transferred to pre-weighed glass vials, dried under a stream of nitrogen, weighed and stored in a freezer until used in bioassays. Total tissue contents of extracts, in terms of extract weight per algal surface area, were calculated for each seaweed species by using the weights of the extracts and individual algal surface area:dry weight ratios.

Bioassays. ***Preparation of test dishes:*** *Surface extracts:* In all the bioassays, the surface extracts of the 7 seaweed species with selected solvents and extraction times were tested at natural and twice the natural concentration. The DCM surface extracts were tested at concentrations corresponding to the amount of extract yielded from algal tissue with the same surface area as the test dishes. The surface extracts at natural and twice the natural concentration and the DCM surface extracts were re-dissolved in 0.5 ml hexane, then the solutions were transferred to Petri dishes (surface area 9 cm^2) and left to evaporate on a shaking table. Solvent controls were prepared by evaporating the same amounts of solvents that were used in the extractions and by re-dissolving in 0.5 ml hexane. In the bioassays both solvent controls and pure filtered seawater (FSW) controls were used. However, the solvent controls in the assays with surface extracts and the whole-cell extracts had no significant effect on the settlement of any of the test organisms, compared to FSW controls (data not shown). Therefore, only solvent controls were used in the statistical analyses of the bioassay results. After evaporation, bioassays were immediately performed on the extract. For each test organism, the surface extracts of the different algal species were tested with replication (n = 8) in separate assays.

Whole-cell extracts: Whole-cell extracts from freeze-dried material were dissolved in ethanol, and a 0.5 ml aliquot of each extract was added to Petri dishes. The Petri dishes were dried on a shaking table resulting in a coating of whole-cell extracts at concentrations ranging from 10 ng to 10 µg cm^{-2}. Solvent controls were prepared accordingly. After evaporation, bioassays were immediately performed on the extract. The whole-cell extracts of all the seaweed species were tested with replication (n = 4) in a single experiment for each test organism.

Inhibition of algal settlement and germling development: Inhibition of algal settlement and germling development by the seaweed surface and whole-cell extracts was examined using carpospores of *Polysiphonia* sp. and gametes of *Ulva australis*. Plants of *Sargassum* sp. with epiphytic *Polysiphonia* sp. were collected at Shark Point, Clovelly at low tide and placed in a bucket filled with seawater. In the laboratory, branches of *Polysiphonia* sp. bearing cystocarps were removed by forceps under a dissecting microscope and placed in a glass container filled with FSW. The branches were left until spores appeared on the bottom of the container (ca. 10 to 20 min). The spores were removed with a plastic pipette, and 20 to 40 spores were transferred to treatment and control dishes filled with 4 ml of FSW. The dishes were incubated overnight at 20 to 22°C at a light:dark cycle of 15:9 h. After incubation, percent settlement was determined by counting the number of settled and unsettled spores using a dissecting microscope. Surface and whole-cell extracts of all the 7 seaweeds were tested against *Polysiphonia* sp. spores.

Fertile *Ulva australis* thalli were collected from Shark Point at low tide. The thalli were manually shaken to remove excess water and placed in individual plastic bags on ice. In the laboratory, the individual thalli were placed in separate glass beakers containing FSW. The beakers were exposed to a bright light source, and within a few minutes positively phototactic swarmers were concentrated at the light side of the beaker. The swarmers were identified as gametes based on the number of flagella (Fletcher 1989, de Nys et al. 1995). Swarmers confirmed to be gametes were pipetted to a beaker containing 100 ml FSW, and stirred with a magnetic stirrer to obtain a uniform suspension of gametes. Gametes were added to the beaker until the colour of the solution turned light green. A 1.0 ml aliquot of the resulting gamete suspension and 4 ml of nutrient-enriched FSW were added to treatment and control dishes (McLachlan 1973). Dishes were placed in the dark for 2 h to allow for even settlement of gametes. After 6 d of incubation at 20 to 22°C and a light:dark cycle of 15:9 h, the growing germlings were counted. Five fields (1.75 mm^2) were counted for each dish using an inverted binocular microscope. Whole-cell extracts of all the 7 seaweed species were used in the tests against *U. australis* gametes. Surface extracts of only 6 of the 7 seaweed species were tested against *U. australis* gametes as *Laurencia rigida* could not be found in sufficient quantities at the time of the experiments.

Biofouling in field. To quantify the natural biofouling cover of the seaweeds, we conducted a field survey at different localities at Shark Bay, Bare Island and Shark Point early in August 2004. Only 5 seaweed species were included in the survey, since the remaining 2 species were senescent at the time of the study. Eight individuals (n = 8) of *Caulerpa filiformis*, *Dictyopteris acrostichoides*, *Delisea pulchra*, *Solieria robusta* and *Pterocladia capillacea* were randomly collected by snorkelling and on SCUBA and placed in separate plastic bags under water. In the laboratory, the seaweeds with associated fouling organisms were photographed, and the abundance of the total biofouling and of different biofouling groups were later deter-

mined using the image analysis software ImageJ 1.32j (Wayne Rasband, National Institutes of Health, USA), for each algal species.

Statistical analyses. The homogeneity of variances for all the obtained data were tested with Cochran's *C*-test and arc-sin transformed as required to fulfil the requirement of homogenous variance; this was done prior to further statistical analyses (Underwood 1997). The results from the tests on (1) the effect of extraction in different solvents and for different time periods on the lysis of epithelial cells, (2) the settlement experiments with surface extracts, and (3) the survey of natural biofouling cover, were all analysed by analysis of variance (1-factor ANOVA) followed by Student-Newman-Keuls test (SNK) (Underwood 1997). The results from the settlement experiments with whole-cell extracts were analysed by 1-factor ANOVA followed by Dunnett's test (Dunnett 1955). Dunnett's test was used because we were only interested in comparing the effects of the different extracts with the control. The use of 2 controls in the settlement experiments with surface extracts (for surface and DCM surface extracts), however, precluded the use of Dunnett's test.

RESULTS

Effects of solvents and extraction time on cell viability

Dipping pieces of algal thalli for 30 s in increasing concentrations of DCM in hexane resulted in a significant effect on lysis of surface cells of *Dictyopteris acrostichoides* (ANOVA, $F_{4,20} = 11.17$, $p < 0.0001$) (Fig. 1A), *Dilophus marginatus* (ANOVA, $F_{5,24} = 105.08$, $p < 0.0001$) (Fig. 1B) and *Laurencia rigida* (ANOVA, $F_{3,16} = 20.06$, $p < 0.0001$) (Fig. 1C). Dipping pieces of *Caulerpa filiformis* for 30 s in 2, 4, and 5% DCM resulted in no visually detectable changes in the shape of the chloroplasts compared to the control. When algal pieces were dipped in 6% DCM, however, bright red background fluorescence made it impossible to focus on any chloroplasts, which we interpreted as interior damages as a consequence of the dipping.

Based on the experiments with the DCM-hexane mixtures, the following solvents were used for surface extraction: 1% DCM for *Laurencia rigida*, 4% DCM for *Dictyopteris acrostichoides*, 5% DCM for *Caulerpa filiformis* and 6% DCM for *Dilophus marginatus*. *Delisea pulchra*, *Pterocladia capillacea* and *Solieria robusta* were extracted in 100% hexane, based on results from findings in previous studies (de Nys et al. 1998, G. M. Nylund unpubl. data). Dipping pieces of the algal species in the chosen solvents for different time periods resulted in a significant effect of immersion time on lysis of surface cells for all tested species (Fig. 2). Dipping pieces of *C. filiformis* in 5% DCM for 60 s caused the chloroplasts to swell, compared to pieces dipped for 30 s and control pieces dipped in seawater. Therefore, 30 s was decided to be an appropriate immersion time for extraction of surface-associated metabolites of *C. filiformis*. Appropriate immersion times, based on these data (Fig. 2) for the other 6 species were: 30 s for *L. rigida*, *P. capillacea* and *S. robusta*; and 40 s for *D. pulchra*, *D. marginatus* and *D. acrostichoides*.

Total whole tissue content of extracts

The total tissue concentration of extracts per algal surface area for all 7 seaweeds ranged from 50% to >17 times the highest-tested concentration used in the bioassays with whole-cell extracts (10 µg cm^{-2}). The following whole tissue concentrations per cm^2 algal surface were calculated: *Caulerpa filiformis* (88.2 µg), *Delisea pulchra* (17.6 µg), *Dictyopteris acrostichoides* (20.7 µg), *Dilophus marginatus* (41.1 µg),

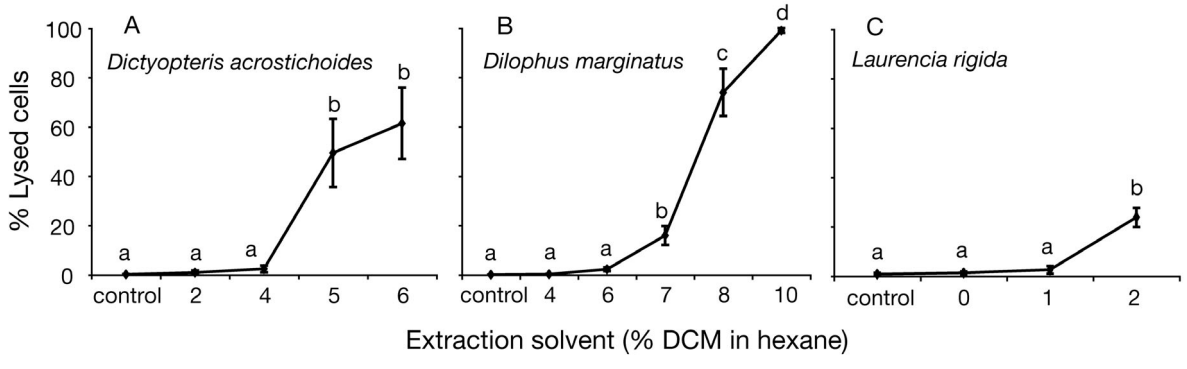

Fig. 1. *Dictyopteris acrostichoides*, *Dilophus marginatus* and *Laurencia rigida*. Percentage of lysed surface cells following extraction (immersion) for 30 s in hexane with increasing concentrations of dichloromethane (DCM). Means ± SE are shown (n = 5). Significant differences are indicated by a, b, c, d (SNK, $\alpha = 0.05$)

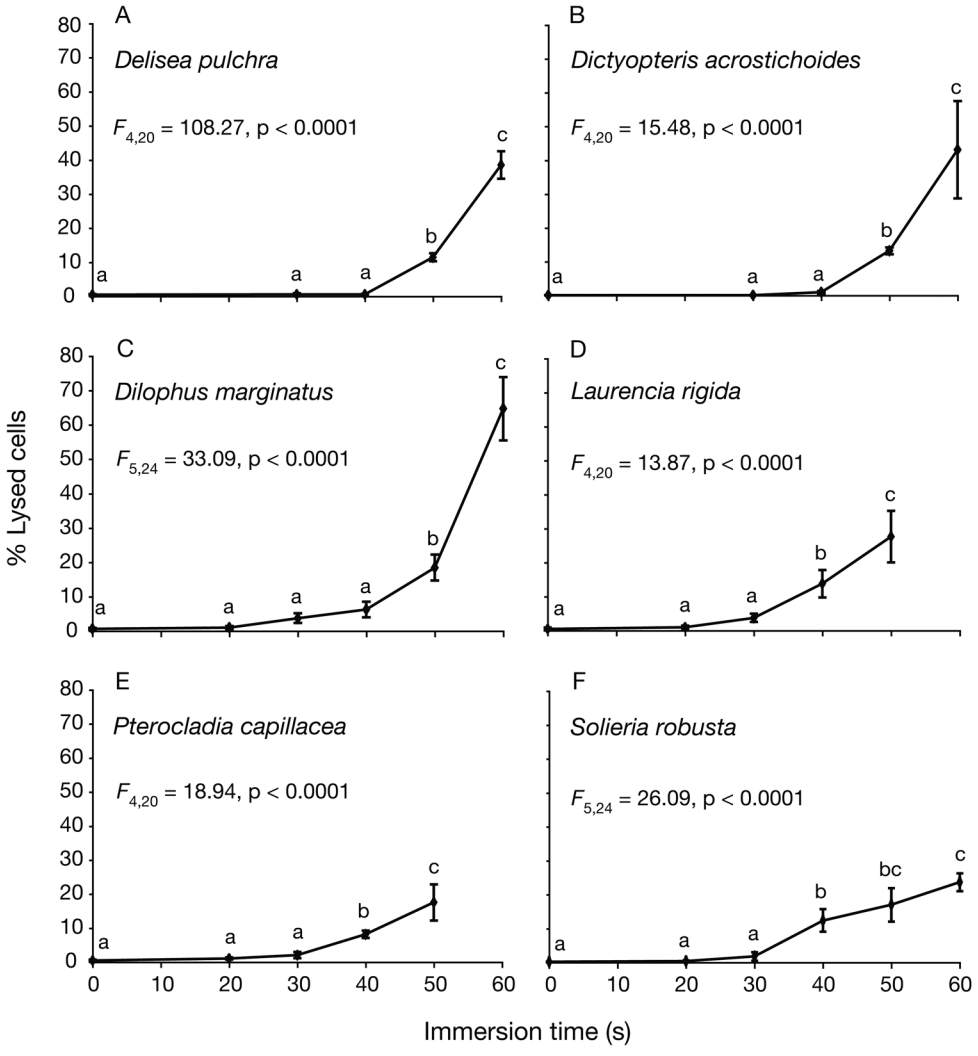

Fig. 2. Percentage of lysed surface cells (A) *Delisea pulchra*, (B) *Dictyopteris acrostichoides*, (C) *Dilophus marginatus*, (D) *Laurencia rigida*, (E) *Pterocladia capillacea* and (F) *Solieria robusta* following extraction (immersion) in mixtures of dichloromethane (DCM) and hexane for different time periods. Solvent mixtures: 100% hexane for *D. pulchra*, *P. capillacea* and *S. robusta*; 1% DCM for *L. rigida*; 4% DCM for *D. acrostichoides*; 6% DCM for *D. marginatus*. Means ± SE are shown (n = 5). Significant differences are indicated by a, b, c (SNK, α = 0.05). *F*- and p-values from ANOVA analyses on effect of immersion time on cell lysis are shown for each species

Laurencia rigida (175.1 µg), *Pterocladia capillacea* (4.7 µg) and *Solieria robusta* (6.5 µg).

Inhibition of settlement of *Polysiphonia* spores

Surface extracts tested at natural and twice the natural concentrations and DCM surface extracts of *Caulerpa filiformis* and *Delisea pulchra* significantly inhibited spore settlement of *Polysiphonia* sp. (Table 1, Fig. 3A). Surface and DCM surface extracts of the remaining 5 tested seaweeds had no significant effects on spore settlement compared to the controls. The settlement success in the solvent controls averaged over all assays was 70.8 ± 12.3% of carpospores added (means ± SD).

At the highest-tested concentration (10 µg cm^{-2}), whole-cell extracts of all 7 species significantly inhibited spore settlement of *Polysiphonia* sp. (ANOVA, $F_{28,87}$ = 74.03, p < 0.0001, Dunnett's test, α = 0.05, Fig. 3B). At 1 µg cm^{-2}, extracts of all seaweeds except *Laurencia rigida* significantly inhibited spore settlement compared to the control, while extracts of 2 species, *Delisea pulchra* and *Dilophus marginatus*, significantly inhibited the settlement at 100 ng cm^{-2}. None of the extracts were inhibitory at the lowest test concentration (10 ng cm^{-2}). The settlement success in the solvent control was 55.6 ± 2.4% of carpospores added (means ± SD).

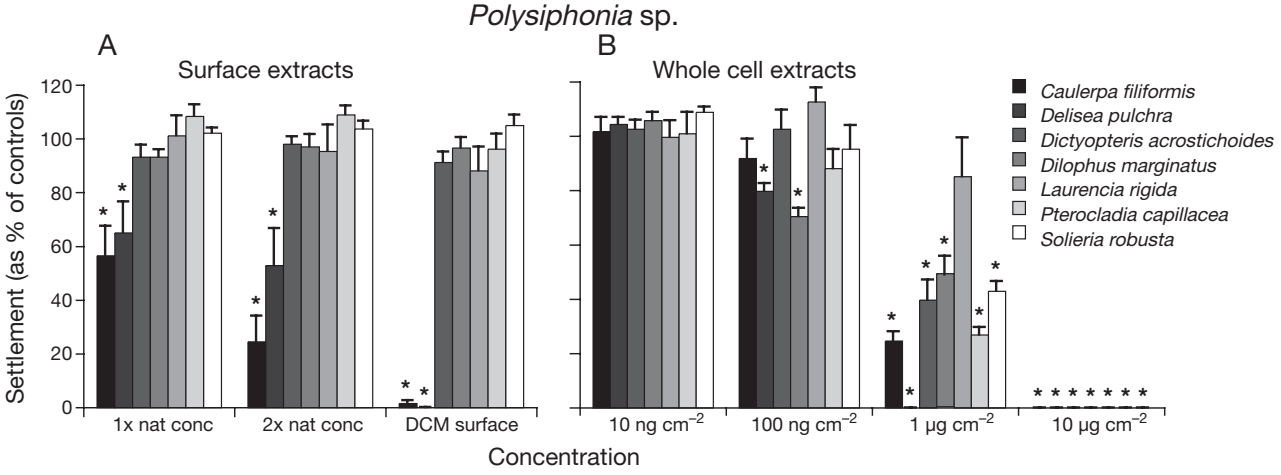

Fig. 3. *Polysiphonia* sp. Effect of (A) surface and (B) whole cell extract of *Caulerpa filiformis*, *Delisea pulchra*, *Dictyopteris acrostichoides*, *Dilophus marginatus*, *Laurencia rigida*, *Pterocladia capillacea* and *Solieria robusta* on the settlement of spores of *Polysiphonia* sp. Means + SE are shown (n = 8 and 4 for surface and whole-cell extract, respectively). *Significant differences; SNK and Dunnett's test (α = 0.05) for surface and whole-cell extracts, respectively

Inhibition of settlement and germling development of *Ulva australis* gametes

Surface extracts of *Delisea pulchra* tested at twice the natural concentration significantly inhibited settlement and germling development of gametes of *Ulva australis*. (Table 2, Fig. 4A). Surface extracts of the remaining 5 seaweed species had no significant effect on the settlement and germling development at any of the 2 tested concentrations. DCM surface extracts of *Caulerpa filiformis*, *D. pulchra* and *Dilophus marginatus* significantly inhibited settlement and germling development of the gametes, while the DCM surface extracts of *Dictyopteris acrostichoides*, *Pterocladia capillacea* and *Solieria robusta* had no significant effect, compared to the controls (Table 2, Fig. 4A). The number of growing germlings in the solvent controls averaged over all the assays was 14.7 ± 4.6 mm^{-2} (means ± SD).

Whole-cell extracts of all of the 7 tested seaweeds significantly inhibited settlement and germling development of gametes of *Ulva* sp. (ANOVA, $F_{28,87} = 17.77$, $p < 0.0001$, Dunnett's test, $\alpha = 0.05$) (Fig. 4B) at the highest tested concentration (10 µg cm^{-2}). At 1 µg cm^{-2}, extract of one species, *Delisea pulchra*, significantly inhibited settlement and germling development compared to the control, while none of the extracts were inhibitory at 10 and 100 ng cm^{-2}. The number of growing germlings in the solvent control was 30.5 ± 1.9 mm^{-2} (means ± SD).

Table 1. One-factor ANOVAs for the effects of surface and DCM surface extracts from *Caulerpa filiformis*, *Delisea pulchra*, *Dictyopteris acrostichoides*, *Dilophus marginatus*, *Laurencia rigida*, *Pterocladia capillacea* and *Solieria robusta* on the settlement of spores of *Polysiphonia* sp. The factor 'Treatment' consists of 5 levels; surface extracts tested at natural and twice the natural concentration; DCM surface extracts tested at 1 concentration; DCM-hexane and pure DCM solvent controls. Significant values in **bold**

Seaweed species	Factor	df	MS	*F*	p
Caulerpa filiformis	Treatment	4	1.43	35.03	**<0.0001**
	Residual	35	0.041		
Delisea pulchra	Treatment	4	7824.27	22.23	**<0.0001**
	Residual	35	351.95		
Dictyopteris acrostichoides	Treatment	4	74.19	1.32	0.28
	Residual	35	56.40		
Dilophus marginatus	Treatment	4	44.99	0.53	0.72
	Residual	35	85.72		
Laurencia rigida	Treatment	4	98.80	0.62	0.65
	Residual	35	159.23		
Pterocladia capillacea	Treatment	4	57.26	0.66	0.63
	Residual	35	87.07		
Solieria robusta	Treatment	4	21.79	0.28	0.89
	Residual	35	76.96		

Biofouling in the field

The field survey showed that there were significant differences in the total biofouling cover between the different seaweed species (ANOVA, $F_{4,35} = 12.16$, $p < 0.0001$) (Fig. 5A). Both *Caulerpa filiformis* and *Delisea pulchra* had significantly lower biofouling cover compared to the other investigated species (SNK, $\alpha = 0.05$).

Furthermore, the seaweeds were dominated by different fouling species: filamentous brown algae (mainly *Ectocarpus* sp.) on *Dictyopteris acrostichoides*, encrusting algae and colonial bryozoans (mainly *Membranipora membranacea*) on *Pterocladia capillacea* and encrusting algae and filamentous red algae including *Polysiphonia* sp. on *Solieria robusta* (Fig. 5B). *C. filiformis* was dominated by encrusting algae, *Ectocarpus* sp. and hydroids, while *D. pulchra* was dominated by encrusting algae. However, individual biofouling groups on *C. filiformis* and *D. pulchra* were not dominating to the same extent as on the other seaweed species. *Ulva* spp. were only found on *D. acrostichoides* and *P. capillacea* (Fig 5B).

Table 2. One-factor ANOVAs for the effects of surface and dichloromethane (DCM) surface extracts from *Caulerpa filiformis*, *Delisea pulchra*, *Dictyopteris acrostichoides*, *Dilophus marginatus*, *Pterocladia capillacea* and *Solieria robusta* on the settlement and germling development of gametes of *Ulva australis*. The factor 'Treatment' consists of 5 levels; surface extracts tested at natural and twice the natural concentration, DCM surface extracts tested at 1 concentration, and DCM-hexane and pure DCM solvent controls. Significant values in **bold**

Seaweed species	Factor	df	MS	*F*	p
Caulerpa filiformis	Treatment	4	169.11	5.55	**0.0014**
	Residual	35	30.48		
Delisea pulchra	Treatment	4	375.98	16.19	**<0.0001**
	Residual	35	23.22		
Dictyopteris acrostichoides	Treatment	4	12.37	1.074	0.38
	Residual	35	11.51		
Dilophus marginatus	Treatment	4	131.51	5.32	**0.0019**
	Residual	35	24.70		
Pterocladia capillacea	Treatment	4	7.05	0.68	0.61
	Residual	35	10.40		
Solieria robusta	Treatment	4	60.30	2.97	**0.033**
	Residual	35	20.33		

DISCUSSION

Despite many studies reporting antifouling effects of seaweed extracts and metabolites (Konig et al. 1999, Da Gama et al. 2002, Nylund & Pavia 2003) only a few studies have successfully described a natural antifouling role of a seaweed metabolite or extract (Schmitt et al. 1995, Steinberg et al. 2001, Nylund et al. 2005, Dworjanyn et al. 2006, Paul et al. 2006). This is probably a consequence of the lack of a general method for testing ecologically relevant concentrations of metabolites against fouling organisms. The determination of ecological roles for natural products usually requires information about the intra-individual distribution of secondary metabolites and how they are presented to interacting organisms (Hay 1996, Steinberg et al. 2001, Pavia et al. 2002). This information is crucial in ecological antifouling research, since the active compounds need to be present on the surface of the host or released to the surrounding water to exert an effect on the foulers (Davis et al. 1989). In this study, we investigated if the 'dip-

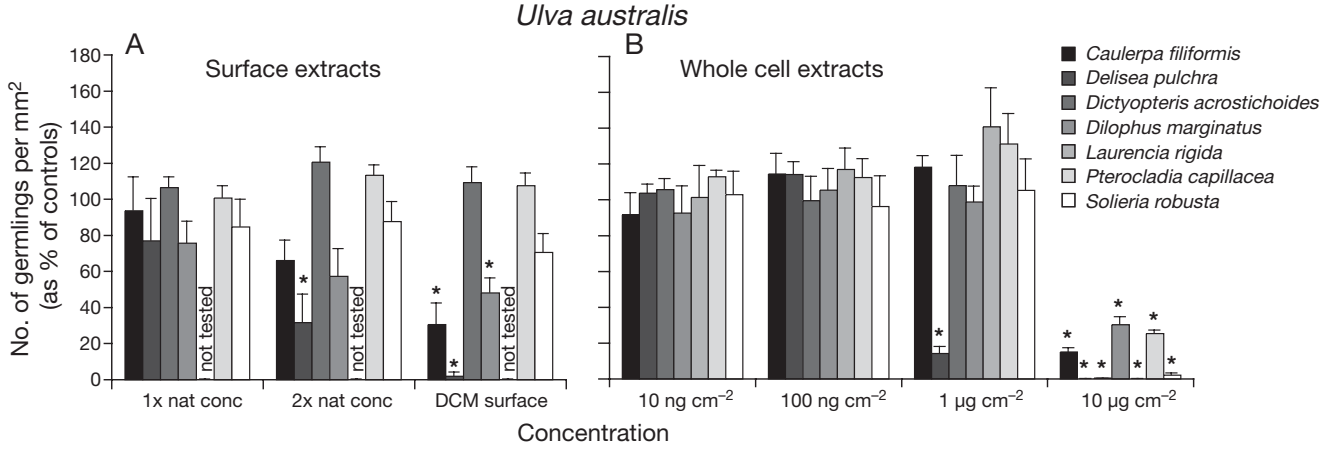

Fig. 4. *Ulva australis*. Effect of (A) surface and (B) whole cell extract of *Caulerpa filiformis*, *Delisea pulchra*, *Dictyopteris acrostichoides*, *Dilophus marginatus*, *Laurencia rigida* (only whole cell extract), *Pterocladia capillacea* and *Solieria robusta* on the settlement and germling development of gametes of *U. australis*. Means + SE are shown (n = 8 and 4 for surface and whole-cell extract, respectively). *L. rigida* = only whole-cell extract. *Significant differences above the bars; SNK and Dunnett's test (α = 0.05) for surface and whole-cell extracts, respectively

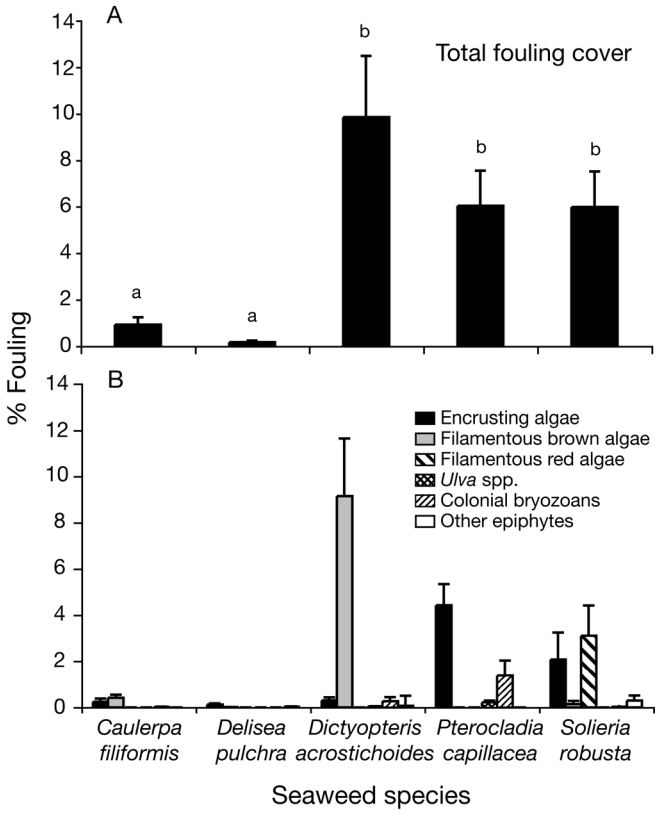

Fig. 5. Natural fouling (% seaweed surface area) of *Caulerpa filiformis*, *Delisea pulchra*, *Dictyopteris acrostichoides*, *Pterocladia capillacea* and *Solieria robusta*. (A) Total fouling cover; (B) fouling cover partitioned into different fouling groups. Means + SE are shown (n = 8). Significant differences are indicated by a, b (SNK, $\alpha = 0.05$)

ping' technique described by de Nys et al. (1998) can be used more generally in studies of natural defences against biofouling in seaweeds by developing protocols for extraction of surface-associated metabolites of 7 seaweed species. The result from the experiments with different extraction solvents and immersion times shows that mixing hexane with different amounts of DCM is a broadly applicable procedure to find suitable extraction times and solvents needed for the 'dipping' technique. By extracting the seaweeds in different mixtures of the solvents for different time periods, we were able to determine the longest possible extraction time before the surface cells started to lyse in order to maximise the extraction efficiency of surface-associated metabolites, without co-extracting the interior of the plants.

The result from the bioassays shows that the activity of surface extracts obtained by the 'dipping' technique was the best predictor of the natural biofouling abundance of the seaweeds. Surface extracts of only *Caulerpa filiformis* and *Delisea pulchra*, tested at natural and twice the natural concentrations, were inhibitory in the bioassays; and these seaweeds also had significantly lower biofouling cover in the field compared to the other investigated species. Since the surface extracts were obtained by immersing seaweed pieces in mixtures of DCM and hexane, this pattern may in theory be explained by a correlation between the presence of inhibitory non-polar compounds and the production of waterborne inhibitors and/or an effective physical defence. However, we find this an unlikely explanation for our present findings. In contrast to the assays with the surface extracts, the tests with the whole-cell extracts showed that all 7 seaweeds produce non-polar metabolites that inhibit ecologically relevant fouling species at concentrations lower than the whole tissue content. However, no individual whole-cell extract was generally more inhibitory than the others. For example, whole-cell extracts of 6 of the 7 tested seaweeds significantly inhibited settlement of *Polysiphonia* sp. spores at a concentration of 1 µg cm^{-2}. In principle, this difference in activity may be due to physicochemical effects of hydrophobic metabolites or chemistry per se, i.e. metabolites functioning as chemical inhibitors or toxins (Steinberg et al. 1998). Previous studies on *D. pulchra* have shown that settlement inhibition of purified furanones (the active metabolites of *D. pulchra*) applied to similar surfaces as used in this study was not due to any general physicochemical modification of the surfaces. When 3 concentrations of 2 different furanones were coated on glass and plastic, the wettability of the surfaces were nearly identical, while the inhibitory effects of these metabolites on *Ulva lactuca* and *Balanus amphitrite* differed by an order of magnitude or more (de Nys et al. 1995, Steinberg et al. 1998). Furthermore, it was shown that wettability was completely uncorrelated to the settlement of gametes of *Ulva australis* and larvae of *Bugula neritina*. This was for glass and plastic surfaces that were coated with different concentrations of extracts of 10 seaweed species, broadly overlapping with those used in this study (Steinberg et al. 1998). These findings suggest that the observed differences in the activity of the extracts in this study were due to chemical inhibition of settlement by different metabolites and/or different concentrations of metabolites.

The combined results from the settlement tests with whole-cell extracts and surface extracts obtained by the 'dipping' technique suggest that the metabolites responsible for the inhibition of settlement in the bioassays with whole-cell extracts are not present on the surfaces of most of the tested seaweeds in sufficient quantities to deter biofouling under natural conditions. The whole-cell extracts were obtained by extracting whole plants in a non-polar solvent; this consequently resulted in an extraction of both interior and surface-

associated non-polar compounds. Obviously, the whole-cell extractions with DCM may have extracted metabolites with a broader range of polarity than the surface extractions using mixtures of DCM and hexane. This could potentially bias our conclusion that the active metabolites in the whole-cell extracts are not present on the surfaces of most of the tested seaweeds in sufficient quantities to deter settlement. However, the results from the settlement tests with surface extracts obtained by extracting fresh pieces of seaweeds in DCM for 30 s, were generally comparable to the results from the tests with the surface extracts obtained by the 'dipping' technique, but not with results from tests with whole-cell extracts. This strongly implies that the active metabolites of the whole-cell extracts were not present at the surface of most of the tested seaweeds in sufficient quantities to deter settlement. Consequently, these laboratory assays suggest that results from settlement assays with whole-cell extracts are poor predictors of the more ecologically relevant effects of surface extracts obtained by the 'dipping' technique. Furthermore, surface extracts of several seaweeds had no effect on the fouling species tested here, even though whole-cell extracts of the same species inhibited settlement at concentrations less than 1% of the total whole tissue content. This implies that testing whole-cell extracts even at a very low proportion of the total tissue content does not guarantee ecologically relevant test concentrations. Hence, we conclude that bioassays with whole-cell extracts are generally of little use if the objective is to describe the ecological effects of natural antifoulants in seaweeds. Even though this study only considered extracts of seaweeds, our results suggest that whole-cell extractions may also result in erroneous conclusions about the function of secondary metabolites as natural antifoulants for other organisms. In several previous studies on marine invertebrates, it was suggested that extracts or isolated compounds have ecologically relevant antifouling roles based on bioassays where whole animal samples have been extracted (Teo & Ryland 1995, Bryan et al. 2003, Kelly et al. 2003). However, it is possible or even likely that some of these metabolites are never presented to fouling organisms under natural conditions and that they have other ecological functions besides fouling deterrence, even though the extracted metabolites show inhibitory activity in laboratory settlement assays. An exception to this ambiguity is found in the work on the sponge *Erylus formosus*. This sponge produces several triterpene glycosides. It has been shown that formoside, the major triterpene glycoside found in *E. formosus*, significantly inhibits biofouling in the field at concentrations corresponding to the surface concentration of the sponge (Kubanek et al. 2000, 2002).

The activity of the surface extracts obtained by extracting fresh pieces of seaweeds in DCM for a fixed time period (e.g. 30 s) corresponded better with the natural biofouling cover and the activity of the surface extracts obtained by the 'dipping' technique, compared to the activity of the whole-cell extracts. Obviously, extractions of fresh seaweeds in DCM cannot replace the 'dipping' method, since the DCM surface extraction procedure lyses surface cells as determined by our study and de Nys et al. (1998); this may result in the co-extraction of some intracellular secondary metabolites. Nevertheless, the DCM surface extraction may be a useful method in the initial screening of seaweeds for potential surface-associated natural antifoulants. If the DCM surface extract of a seaweed species inhibits the settlement of ecologically relevant fouling organisms, the seaweed can be further extracted with the 'dipping' technique, adapted specifically to each seaweed species, to more rigorously evaluate the ecological relevance of the inhibitory activity. If, on the other hand, no inhibition occurs, it would imply that the seaweed does not use non-polar surface-associated metabolites to deter biofouling. In that case, it would not be necessary to determine a specific extraction solvent and immersion time needed for the 'dipping' technique, which is a very labour-intensive and time-consuming procedure.

The combined results from the bioassays with surface extracts at natural concentrations and the field survey suggest that *Caulerpa filiformis* and *Delisea pulchra*, but not the other 5 investigated species, naturally deter biofouling by producing non-polar surface-associated secondary metabolites. These results are in agreement with previous studies on antifouling by *D. pulchra* (de Nys et al. 1995, Maximilien et al. 1998, Dworjanyn et al. 2006). This alga produces a series of structurally related non-polar metabolites, halogenated furanones (Kazlauskas et al. 1977, de Nys et al. 1993), and it has been shown that the furanones are localised within the alga to enable their release to the surface in sufficient quantities to deter colonising organisms (de Nys et al. 1998, Dworjanyn et al. 1999, Dworjanyn et al. 2006). *C. filiformis* belongs to a chemically rich genera, which is known to produce an array of different diterpenoids and sesquiterpenoids (Paul & Fenical 1987). However, we are not aware of any studies that have focused on either the secondary chemistry or the antifouling properties of *C. filiformis*. Since we only tested crude extracts in the settlement assays, we do not know which specific compounds caused the observed inhibition in our study.

In conclusion, our study has shown that the 'dipping' technique can be used in combination with bioassays of ecologically relevant fouling organisms as a general method for studies on natural antifouling roles of non-

polar seaweed metabolites. Alternatively, the simpler and faster technique of extracting fresh pieces of seaweeds in DCM for a short time period (e.g. 30 s) can be used in initial screening studies. Furthermore, this study has shown that bioassays with whole-cell extracts are generally of little use if the objective is to explore the chemical mediation of interactions between seaweeds and fouling organisms. Finally, the combined results from the antifouling assays with surface extracts and the fouling survey suggest that *Caulerpa filiformis* and *Delisea pulchra*, but not the 5 other investigated seaweed species, produce non-polar surface-associated metabolites that naturally deter biofouling.

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LITERATURE CITED

Bryan PJ, McClintock JB, Slattery M, Rittschof DP (2003) A comparative study of the non-acidic chemically mediated antifoulant properties of 3 sympatric species of ascidians associated with seagrass habitats. Biofouling 19:235–245

Cho JY, Kwon EH, Choi JS, Hong SY, Shin HW, Hong YK (2001) Antifouling activity of seaweed extracts on the green alga *Enteromorpha prolifera* and the mussel *Mytilus edulis*. J Appl Phycol 13:117–125

Collen J, Delrio MJ, Garciareina G, Pedersen M (1995) Photosynthetic production of hydrogen peroxide by *Ulva rigida* C. Ag. (Chlorophyta). Planta 196:225–230

Da Gama BAP, Pereira RC, Carvalho AGV, Coutinho R, Yoneshigue-Valentin Y (2002) The effects of seaweed secondary metabolites on biofouling. Biofouling 18:13–20

Davis A, Targett N, McConnell O, Young C (1989) Epibiosis of marine algae and benthic invertebrates: natural products chemistry and other mechanisms inhibiting settlement and overgrowth. In: Scheuer PJ (ed) Bioorganic marine chemistry, Vol. 3. SpringerVerlag, Berlin, p 85–114

de Nys R, Steinberg PD (1999) Role of secondary metabolites from algae and seagrasses in biofouling control. In: Fingerman M, Nagabhushanam R, Thompson MF (eds) Recent advances in marine biotechnology, Vol. 3. Science Publishers, Enfield, NH, p 223–244

de Nys R, Wright AD, Konig GM, Sticher O (1993) New halogenated furanones from the marine alga *Delisea pulchra* (cf *fimbriata*). Tetrahedron 49:11213–11220

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

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

Dunnett CW (1955) A multiple comparison procedure for comparing several treatments with a control. J Am Stat Assoc 50:1096–1121

Dworjanyn S (2000) Chemically mediated antifouling and the cost of producing secondary metabolites in seaweeds. PhD thesis, University of New South Wales, Sydney

Dworjanyn SA, de Nys R, Steinberg PD (1999) Localisation and surface quantification of secondary metabolites in the red alga *Delisea pulchra*. Mar Biol 133:727–736

Dworjanyn SA, de Nys R, Steinberg PD (2006) Chemically mediated antifouling in the red alga *Delisea pulchra*. Mar Ecol Prog Ser 318:153–163

Fletcher RL (1989) A bioassay technique using the marine fouling green alga *Enteromorpha*. Int Biodeterior Biodegrad 25:407–422

Harder T, Dobretsov S, Qian PY (2004) Waterborne polar macromolecules act as algal antifoulants in the seaweed *Ulva reticulata*. Mar Ecol Prog Ser 274:133–141

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

Jennings JG, Steinberg PD (1997) Phlorotannins versus other factors affecting epiphyte abundance on the kelp *Ecklonia radiata*. Oecologia 109:461–473

Johnson CR, Mann KH (1986) The crustose coralline alga, *Phymatolithon* Foslie, inhibits the overgrowth of seaweeds without relying on herbivores. J Exp Mar Biol Ecol 96: 127–146

Karez R, Engelbert S, Sommer U (2000) 'Co-consumption' and 'protective coating': two new proposed effects of epiphytes on their macroalgal hosts in mesograzer-epiphyte-host interactions. Mar Ecol Prog Ser 205:85–93

Kazlauskas R, Murphy PT, Quinn RJ, Wells RJ (1977) A new class of halogenated lactones from the red alga *Delisea fimbriata* (Bonnemaisoniaceae). Tetrahedron Lett 1:37–40

Keats DW, Knight MA, Pueschel CM (1997) Antifouling effects of epithallial shedding in three crustose coralline algae (Rhodophyta, Coralinales) on a coral reef. J Exp Mar Biol Ecol 213:281–293

Kelly SR, Jensen PR, Henkel TP, Fenical W, Pawlik JR (2003) Effects of Caribbean sponge extracts on bacterial attachment. Aquat Microb Ecol 31:175–182

Konig GM, Wright AD, de Nys R (1999) Halogenated monoterpenes from *Plocamium costatum* and their biological activity. J Nat Prod 62:383–385

Kubanek J, Pawlik JR, Eve TM, Fenical W (2000) Triterpene glycosides defend the Caribbean reef sponge *Erylus formosus* from predatory fishes. Mar Ecol Prog Ser 207:69–77

Kubanek J, Whalen KE, Engel S, Kelly SR, Henkel TP, Fenical W, Pawlik JR (2002) Multiple defensive roles for triterpene glycosides from two Caribbean sponges. Oecologia 131: 125–136

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

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

McLachlan J (1973) Growth media-marine. In: Stein JR (ed) Handbook of phycological methods: culture methods and growth measurements. Cambridge University Press, Cambridge

Nylund GM, Pavia H (2003) Inhibitory effects of red algal extracts on larval settlement of the barnacle *Balanus improvisus*. Mar Biol 143:875–882

Nylund GM, Pavia H (2005) Chemical versus mechanical

inhibition of fouling in the red alga *Dilsea carnosa*. Mar Ecol Prog Ser 299:111–121
Nylund GM, Cervin G, Hermansson M, Pavia H (2005) Chemical inhibition of bacterial colonization by the red alga *Bonnemaisonia hamifera*. Mar Ecol Prog Ser 302:27–36
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, Fenical W (1987) Natural products chemistry and chemical defence in tropical marine algae of the phylum Chlorophyta. In: Scheuer PJ (ed) Bioorganic marine chemistry, Vol 1. Springer-Verlag, Berlin Heidelberg, p 1–29
Paul VJ, Cruz-Rivera E, Thacker RW (2001) Chemical mediation of macroalgal-herbivore interactions: ecological and evolutionary perspectives. In: McClintock JB, Baker BJ (eds) Marine chemical ecology. CRC Press, Boca Raton, FL, p 227–265
Pavia H, Toth GB, Aberg P (2002) Optimal defense theory: elasticity analysis as a tool to predict intraplant variation in defenses. Ecology 83:891–897
Schmitt TM, Hay ME, Lindquist N (1995) Constraints on chemically mediated coevolution: multiple functions for seaweed secondary metabolites. Ecology 76:107–123
Steinberg PD, De Nys R, Kjelleberg S (1998) Chemical inhibition of epibiota by Australian seaweeds. Biofouling 12: 227–244
Steinberg P, De Nys R, Kjelleberg S (2001) Chemical mediation of surface colonization. In: McClintock JB, Baker BJ (eds) Marine chemical ecology. CRC Press, Boca Raton, FL, p 355–87
Teo SLM, Ryland JS (1995) Potential antifouling mechanisms using toxic chemicals in some British ascidians. J Exp Mar Biol Ecol 188:49–62
Underwood AJ (1997) Experiments in ecology: their logical design and interpretation using analysis of variance. Cambridge University Press, Cambridge
Wahl M, Hay ME (1995) Associational resistance and shared doom: effects of epibiosis on herbivory. Oecologia 102: 329–340
Wahl M, Hay ME, Enderlein P (1997) Effects of epibiosis on consumer-prey interactions. Hydrobiologia 355:49–59
Walters LJ, Hadfield MG, Smith CM (1996) Waterborne chemical compounds in tropical macroalgae: positive and negative cues for larval settlement. Mar Biol 126:383–393
Weinberger F, Friedlander M (2000) Response of *Gracilaria conferta* (Rhodophyta) to oligoagars results in defense against agar-degrading epiphytes. J Phycol 36:1079–1086
Weinberger F, Friedlander M, Hoppe HG (1999) Oligoagars elicit a physiological response in *Gracilaria conferta* (Rhodophyta). J Phycol 35:747–755
Wikström S, Pavia H (2004) Chemical settlement inhibition versus post-settlement mortality as an explanation for differential fouling of two congeneric seaweeds. Oecologia 138:223–230
Wisespongpand P, Kuniyoshi M (2003) Bioactive phloroglucinols from the brown alga *Zonaria diesingiana*. J Appl Phycol 15:225–228

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