Vol. 318: 153–163, 2006 | MARINE ECOLOGY PROGRESS SERIES
Mar Ecol Prog Ser | Published August 3

Chemically mediated antifouling in the red alga *Delisea pulchra*

S. A. Dworjanyn[1,2,*], R. de Nys[1,3], P. D. Steinberg[1]

[1]School of Biological Earth and Environmental Sciences and Centre for Marine Biofouling and Bio-Innovation, University of New South Wales, Sydney, New South Wales 2052, Australia

[2]*Present address:* Scottish Association for Marine Science, Dunstaffnage Marine Laboratory, Oban, Argyll PA37 1QA, UK

[3]*Present address:* School of Marine Biology and Aquaculture, James Cook University, Townsville, Queensland 4811, Australia

ABSTRACT: Using laboratory assays, we tested whether the secondary metabolites (furanones) on the surface of the red alga *Delisea pulchra* deter the settlement and growth of a range of ecologically relevant fouling organisms. *D. pulchra* and 4 other co-occurring seaweeds were almost exclusively fouled by other algae. Consequently, we carried out laboratory assays using propagules from 4 fouling algae (*Ulva* sp., *Ceramium* sp., *Polysiphonia* sp. and *Ectocarpus siliculosis*) representing the natural fouling community. The crude surface extract of *D. pulchra* at the same concentration as on the surface of the plant, the furanone fraction of this extract, and pure furnaones, deterred the settlement of fouling organisms in ecologically relevant assays. These data, coupled with knowledge of the surface concentration of furanones on *D. pulchra* and a mechanism by which furanones are sequestered onto the surface of the plant, provide a rigorous demonstration of chemically mediated antifouling.

KEY WORDS: Antifouling · Bioassay · Chemical ecology · Surface interaction · Natural products · Surface chemistry · Furanones

INTRODUCTION

Space is often a limiting resource in the marine environment, and consequently plants and animals often overgrow or 'foul' the surface of other organisms. Fouling organisms or epibiota can have a considerable negative impact on their hosts. Epibiota on seaweeds can limit access to light and nutrients, reducing photosynthesis (Littler & Littler 1999), and components of fitness such as growth and reproduction (Dixon et al. 1981, D'Antonio 1985; reviewed by de Nys & Steinberg 1999). Fouling can also increase hydrodynamic drag on algae, causing them to lose blades (Dixon et al. 1981) or be detached from the substratum (D'Antonio 1985). Fouled plants can also attract herbivores, leading to increased tissue loss from herbivory (Bernstein & Jung 1979, Wahl & Hay 1995).

Given the disadvantages of being fouled, if an alga can control fouling on its surface, it should gain an ecological and evolutionary advantage over plants that do not control fouling. Algae can do this by sloughing surface cells (Johnson & Mann 1986, Keats et al. 1997) and by absorbing nutrients from, and excising, fouled thalli (Littler & Littler 1999). There is also evidence that algae are able to chemically inhibit the settlement and growth of fouling organisms by releasing secondary compounds onto or near their surfaces (Schmitt et al. 1995, de Nys et al. 1998, Dworjanyn et al. 1999).

As intellectually appealing as the idea of chemical inhibition of fouling organisms may be, the extent to which this strategy is employed by algae is not well known. Rigorous demonstrations of chemically mediated antifouling in marine organisms are largely absent from the literature, in part because of methodological difficulties (Hay 1996). To show that an alga uses chemicals as antifoulants, several criteria have to be met (Davis et al. 1989, Schmitt et al. 1995, Clare 1996, Hay 1996). The plant should be generally free of

*Email: symon.dworjanyn@sams.ac.uk

fouling in the field. It must be demonstrated that the putative antifouling chemicals reduce fouling at the concentration at which they are found at the plant surface. Assays to determine inhibition by these chemicals need to use ecologically relevant fouling organisms. They must also account for the way in which the chemical(s) are presented by the algae (i.e. on the surface or surrounding water) and any interactions between or among the compounds produced by a host plant. Knowledge of the mechanism by which the alga transports these compounds to its surface confirms that the presence of these chemicals on the surface of the alga is not a methodological artifact. Aside from the assays described herein and in associated publications (de Nys et al. 1995, 1998, Maximilien et al. 1998, Dworjanyn et al. 1999) all these criteria have not been fulfilled for any alga.

Several studies have shown that crude whole plant extracts from a range of algae can deter settlement of potential fouling organisms (e.g. Nylund & Pavia 2003); however, the lack of data on the presence of these compounds on the surface of the algae make it difficult to interpret these studies in an ecological context. Phlorotannins, ubiquitous in brown algae, have been shown to deter the settlement of a range of fouling organisms (reviewed by de Nys & Steinberg 1999). However, when carefully measured in the field, the concentrations of phlorotannins near the surface of brown algae are orders of magnitude too low to act as antifoulants (Jennings & Steinberg 1994, 1997). Some of the criteria for natural antifoulants have been met for the brown alga *Dictyota menstrualis* and the red alga *Delisea pulchra*. The common fouling organism *Bugula neritina* avoids settling on the surface of *D. menstrualis*, and surface rubbings of the latter that contain pachydictyol A and dictyol E also inhibit *B. neritina* when coated onto surfaces in laboratory assays (Schmitt et al. 1995). While the work of Schmitt et al. (1995) suggests an antifouling role for these terpenoids, 2 caveats remain: (1) rubbing the surface of an alga to extract surface compounds can cause lysis in algal surface cells, thus also extracting compounds inside the thallus (Dworjanyn 2001); and (2) the concentrations of these compounds on the surface of the alga are yet to be determined.

For *Delisea pulchra*, furanones have been extracted and quantified from the surface of the plant without disturbing surface cells (de Nys et al. 1998, Dworjanyn et al. 1999). Furanones are also secreted onto the surface of *D. pulchra* via specialised gland cells, confirming a mechanism for presenting furanones to the surface of the alga (Dworjanyn et al. 1999). Furthermore, furanones at natural concentrations inhibit colonisation by bacteria in the field (Maximilien et al. 1998) and settlement of the cosmopolitan fouling alga *Ulva* sp. in laboratory assays (de Nys et al. 1995). *D. pulchra* is generally free of macrofouling (except for shallow plants in summer, for which an increase in fouling is thought to be caused by a drop in furanone concentrations; Dworjanyn 2001), and bacterial abundances on the surface of *D. pulchra* are roughly 1 order of magnitude lower than in co-occurring plants (Maximillien et al. 1998).

Herein, we build on these studies and test the hypothesis that furanones inhibit ecologically relevant fouling organisms at natural concentrations. A survey of organisms fouling *Delisea pulchra* (carried out on shallow plants in summer, when fouling is present) and co-occurring macroalgae was made to select appropriate fouling organisms for use in the assays. Subsequently, the response of these fouling organisms to crude surface extracts and partially purified surface extracts of *D. pulchra* thalli and furanones at surface concentrations were tested in laboratory settlement and growth assays.

MATERIALS AND METHODS

Plant material, extraction and isolation of furanones. *Delisea pulchra* was collected from Cape Banks, Sydney, New South Wales (34° 04′ S, 151° 14′ E). The algal tissue was frozen, freeze-dried, and extracted with dichloromethane, and the resulting crude extract reduced *in vacuo.* Metabolites (see Fig. 1) were purified by vacuum liquid chromatography (VLC) and high performance liquid chromatography, and structures confirmed by ^{1}H and ^{13}C nuclear magnetic-resonance spectroscopy (de Nys et al. 1993). For surface extractions of non-polar secondary metabolites, plants were collected and immediately placed in clip-seal bags with ambient seawater and kept cool for transport back to the laboratory; they were used within 3 h of collection.

Fouling survey. A survey of the epiphytes growing on common seaweeds at Cape Banks was made to determine common fouling organisms in the habitat in which *Delisea pulchra* occurs (see also Jennings & Steinberg 1997). We randomly collected 5 obviously fouled plants each of *Corallina officianalis*, *Ecklonia radiata*, *Sargassum linearifolium*, *Zonaria diesingiana* and *D. pulchra*. To determine the percentage cover of different fouling organisms on the macroalgae, ten 1 cm^2 squares were chosen at random on each of the 5 replicate plants, and cover assessed using the point-intercept method (100 points) viewed under a stereomicroscope. The taxonomic groupings of fouling organism ranged from family to species level, depending on ease of identification, and were identified using various taxonomic keys (Womersley 1984, 1987, 1994,

1996, Farrant & King 1989, Millar 1990). These data were then used in selecting ecologically relevant species for the laboratory fouling assays.

Algae settlement assays. The most abundant organisms fouling the 5 species of host macroalgae were other algae (see 'Results; Fouling survey'). We tested the effects of surface extracts and furanones on the settlement and growth of 4 of the most common of these epiphytic algae (*Ulva* sp., *Ceramium* sp., *Polysiphonia* sp. and *Ectocarpus siliculosus*). Fertile epiphytic plants for fouling assays were collected from the intertidal at Shark Point, Sydney, New South Wales (33° 54′ S, 151° 16′ E). Bioassays were conducted by inducing the algae to shed propagules (tetraspores, carpospores or gametes) and then placing the propagules in petri dishes whose surfaces were coated with the test compounds and which contained sterile, filtered (0.2 µm, Millipore) seawater. All assay dishes were incubated at 18°C, 30 to 35 mmol photons m^{-2} s^{-1} in a dark:light cycle of 8:16 h. Details of assays for each species follow.

Ulva sp.: The effects of *Delisea pulchra* surface extracts and secondary metabolites on the settlement and development of gametes of *Ulva* sp. were examined following de Nys et al. (1995). Fertile *Ulva* sp. (distinguishable by discolouration of the margin of the thallus) were collected in the field. The plants were washed in sterile filtered (0.2 µm, Millipore) seawater (SFS) and then left to dry for approximately 2 h. Placing the plants in SFS near a light source induced the release of swarmers. The positively phototactic swarmers concentrated on the side of the container closest to the light source, where they were easily collected. A sample of these swarmers was observed under a microscope to identify whether they were gametes (biflagellated) or spores (quadra-flagellated). Only unfertilised gametes were used in this assay; in this population, these readily grow without fertilisation (Bold & Wynne 1985).

To remove contaminants, the gametes were twice 'raced' in SFS across a watchglass towards a light source. The gametes were diluted in SFS until the green colour of the gamete solution was barely discernible by eye. The solution was placed on a magnetic stirrer to maintain a uniform suspension, and 0.5 ml of gamete suspension was added to each test dish. The dishes were then placed in the dark for 2 h to allow the gametes to settle, and cultured as described above. After 5 d (at which time it was easily discernible which of the gametes had settled and were growing), the dishes were examined microscopically and the number of growing plants in 5 haphazardly selected 100 μm^2 areas (field of view) on each dish was counted. The results are expressed as the number of settled plants per field of view.

Red algae: The effects of *Delisea pulchra* surface extracts and furanones on the settlement and development of red algae epiphytes representing 2 life history stages were tested using fertilised carpospores from *Polysiphonia* sp. and tetraspores from *Ceramium* sp. The same assay method was used for both species:

Carposporophytes of *Polysiphonia* sp. and tetrasporophytes of *Ceramium* sp. were collected from the field as epiphytes. Fertile filaments were washed 3 times in SFS and placed in shallow dishes containing SFS. The algae spontaneously released non-motile spores after 2 to 3 h. The spores (from both species) are negatively buoyant and were collected from the bottom of the dish using a glass pipette. Approximately 100 ± 10 spores were placed in each test dish. The spores were cultured as described (earlier) for 24 h to allow settlement and germination. The test dishes were then examined microscopically and settlement and germination success was scored for all spores. Successful settlement and germination was defined as spores that were attached to the surface of the dish and divided. Spores were scored as not settled when they were either not attached, or attached but not germinated. The results are expressed as percentage settled.

Ectocarpus siliculosus: The effects of surface extracts of *Delisea pulchra* and furanones on the settlement and growth of *E. siliculosus* was also tested in laboratory assays. Gametes were obtained from cultured *E. siliculosus* originally collected as epiphytes from Shark Point and cultured as described earlier. To induce the release of gametes, the culture media was replaced with a small volume of SFS and the culture vessel placed near a light source. Motile gametes were usually released after 30 min. The SFS containing gametes was decanted from the culture vessel and a uniform gamete suspension was maintained using a mechanical stirrer; 0.5 ml of this suspension was added to each test dish. The test dishes were placed in the dark for 2 h to allow even settlement of the phototactic gametes and cultured as described earlier. After 48 h culture, the settled *E. siliculosus* had under gone several cell divisions and were easily counted under a microscope (×40 magnification). The number of settled plants was counted in each dish; non-settled gametes were not counted. The results are expressed as the number of settled plants per test dish.

Testing effects of crude surface extracts from *Delisea pulchra*. The non-polar secondary metabolites on the surface of *D. pulchra* were quantitatively harvested following the method of de Nys et al. (1998). This extract, which represents the concentration and composition of metabolites found naturally on the surface of the alga, was then assayed against propagules from the 4 epiphytic algae. To make surface extracts, freshly collected *D. pulchra* was dried in a salad spin-

ner (3 revolutions), and pieces of tissue with a surface area of approximately 9 cm^2 (weight, determined by wet weight:area regression: approx. 130 mg) were removed and vortexed in 20 ml of distilled hexane for 30 s (de Nys et al. 1998). The tissue was then removed and the hexane taken to dryness in air. The resulting crude extracts were redissolved in 0.5 ml of ethanol (99.7% + purity) and pipetted into plastic solvent-resistant petri dishes with a surface area of 9 cm^2. The petri dishes were placed on a shaker until the ethanol had evaporated, leaving an even cover of surface extract on the petri dish at approximately the same concentration as on the plant. SFS (4.5 ml) enriched with Provasoli enrichment solution (PES, 10 ml l^{-1}) was added to each dish. Control dishes (solvent control) were prepared by drying 20 ml of distilled hexane, adding 0.5 ml of ethanol (99.7+% purity), and preparing dishes as for extracts. Untreated dishes containing only seawater enriched with PES (seawater control) were also prepared. We used 5 replicate dishes (n = 5) for each treatment (surface extract, solvent control and seawater control) for each algal species tested.

Testing effects of furanone and lipid fractions from *Delisea pulchra*. The non-polar surface extract of *D. pulchra* consists primarily of furanones and lipids (as determined by ^1H nuclear magnetic-resonance spectroscopy and gas chromatography-mass spectrometry). The surface extract of *D. pulchra* was separated into lipid and furanone fractions, a portion of each was recombined at the same ratio as on the plant, and the activity of these 3 fractions (furanones, lipids, furanones plus lipids) was tested against carpospores from *Polysiphonia* sp. This was done to test whether furanones alone were responsible for the antifouling activity of surface extracts or if lipids play any role in the plant's antifouling defence. The hexane surface extract from several plants of known surface area was pooled and the hexane extract was air-dried. The resultant crude extract was redissolved in 200 µl of distilled hexane and separated by VLC using a 1 g normal phase silica-gel column (Alltech). The lipid fraction was eluted with 1.3 ml distilled hexane. The furanone fraction was eluted with 2 ml of distilled hexane:ethyl acetate (1:4). Each fraction was taken to dryness in air. The absence of furanone from the lipid fraction and the presence of Furanones 1 to 4 (Fig. 1) in the furanone fraction was confirmed using thin-layer chromatography (TLC) (1:10 hexane:ethyl acetate) with purified furanones (1 to 4, Fig. 1) as standards. Other compounds are present in *D. pulchra*, some of which were evident in the TLCs; however, they occur in minute concentrations and none have been found to possess antifouling activity (de Nys et al. 1993).

We prepared 3 test solutions containing either (1) the furanone fraction, (2) the lipid fraction or (3) equal

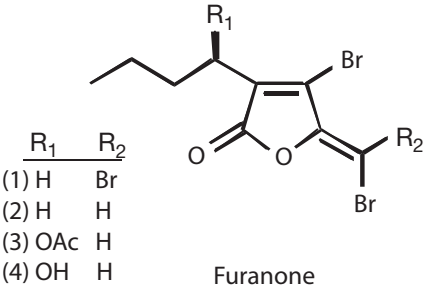

Fig. 1. *Delisea pulchra.* Structures of 4 major furanones (1 to 4)

parts of the furanone and lipid fractions in distilled hexane. Assay dishes were prepared by adding 0.5 ml of this solution to petri dishes (9 cm^2), placing them on a shaker, and allowing the hexane to dry in air; this resulted in an even cover of the extract. A dilution series of each test solution was prepared, such that addition of 0.5 ml of each treatment to the petri dishes resulted in a final surface concentration range of 100, 50, 10, 5, 1 and 0.5% of the natural concentration of each fraction found on the surface of *Delisea pulchra*. We tested 3 replicates of each concentration for each extract fraction, solvent controls consisting of dishes coated with distilled hexane, and an SFS control against carpospores of *Polysiphonia* sp.

Assays using purified metabolites. To test the effect of individual secondary metabolites produced by *Delisea pulchra* on the settlement and development of epiphytes, Furanones 2 and 3 (Fig. 1) were assayed against the 4 algal epiphytes. Furanones 2 and 3 were selected because they comprised (respectively) the least and most active furanones in previous assays (de Nys et al. 1995). Compound 3 is also consistently the most abundant compound found on the surface of *D. pulchra* (Dworjanyn 2001). The ability of furanones to act additively or synergistically was also tested. To do this, a mixture of Furanones 1 to 4 (Fig. 1) in the same ratio as found on the surface of the plant (Dworjanyn et al. 1999) were assayed against the 4 epiphytic algae and compared to the results of assays using Furanones 2 and 3. For all assays, furanones were applied to the surface of petri dishes, with a surface area of 9 cm^2. Stock solutions of Furanones 2 and 3 and a mixture of Furanones 1 to 4 (Fig. 1) in the ratio of 1:3.5:37.5:58 (Furanones 1:2:3:4) (Dworjanyn et al. 1999) were dissolved in ethanol (99.7+% purity). Serial dilutions prepared such that addition of 0.5 ml of each treatment to the petri dishes resulted in a surface concentration range of 10 µg cm^{-2}, 1 µg cm^{-2}, 100 ng cm^{-2}, 10 ng cm^{-2}, and 1 ng cm^{-2}. We tested 3 replicates of each treatment, controls consisting of dishes coated with distilled hexane, and an SFS control against the 4 algal epiphytes.

Statistical analysis. The data from the crude surface extract assays and partially purified extract assays on *Polysiphonia* sp. carpospores were analysed by 1-way and 2-way ANOVAs (as specified in the relevant 'Results' sections) followed by Tukey's Honestly Significant Different (HSD)-test. The assumptions of normality and heterogeneity of variance were examined using frequency histograms of residuals and plots of residuals versus means, respectively. The significance level was taken as $p < 0.05$. The occurrence of many zeros (e.g. no settlement) in data from the assays using furanones meant that the data did not fulfil the assumption of homogeneity of variance (Cochran's *C*-test), even when the data was transformed. These data were thus not formally analysed for significant differences. Non-parametric tests were also not used in these cases as the assumptions of these tests were also violated by the data (Underwood 1997).

RESULTS

Fouling survey

The most abundant organisms fouling the macroalgae surveyed were other algae. More than 99% (by area) of the fouling on all plants sampled were algae, with invertebrates largely absent from these fouling assemblages (Fig. 2). All 3 algal divisions (Rhodophyta, Chlorophyta and Phaeophyta) were represented as epiphytes, with filamentous forms being the most common. *Polysiphonia* spp., *Ceramium* sp., and members of the family Ectocarpaceae were consistently abundant across all host species. *Polysiphonia* sp., *Ceramium* sp., and *Ectocarpus siliculosus* were selected for use in the laboratory settlement assays. *Ulva* sp. was also used as an assay organism because it was present on several hosts and has been found to be

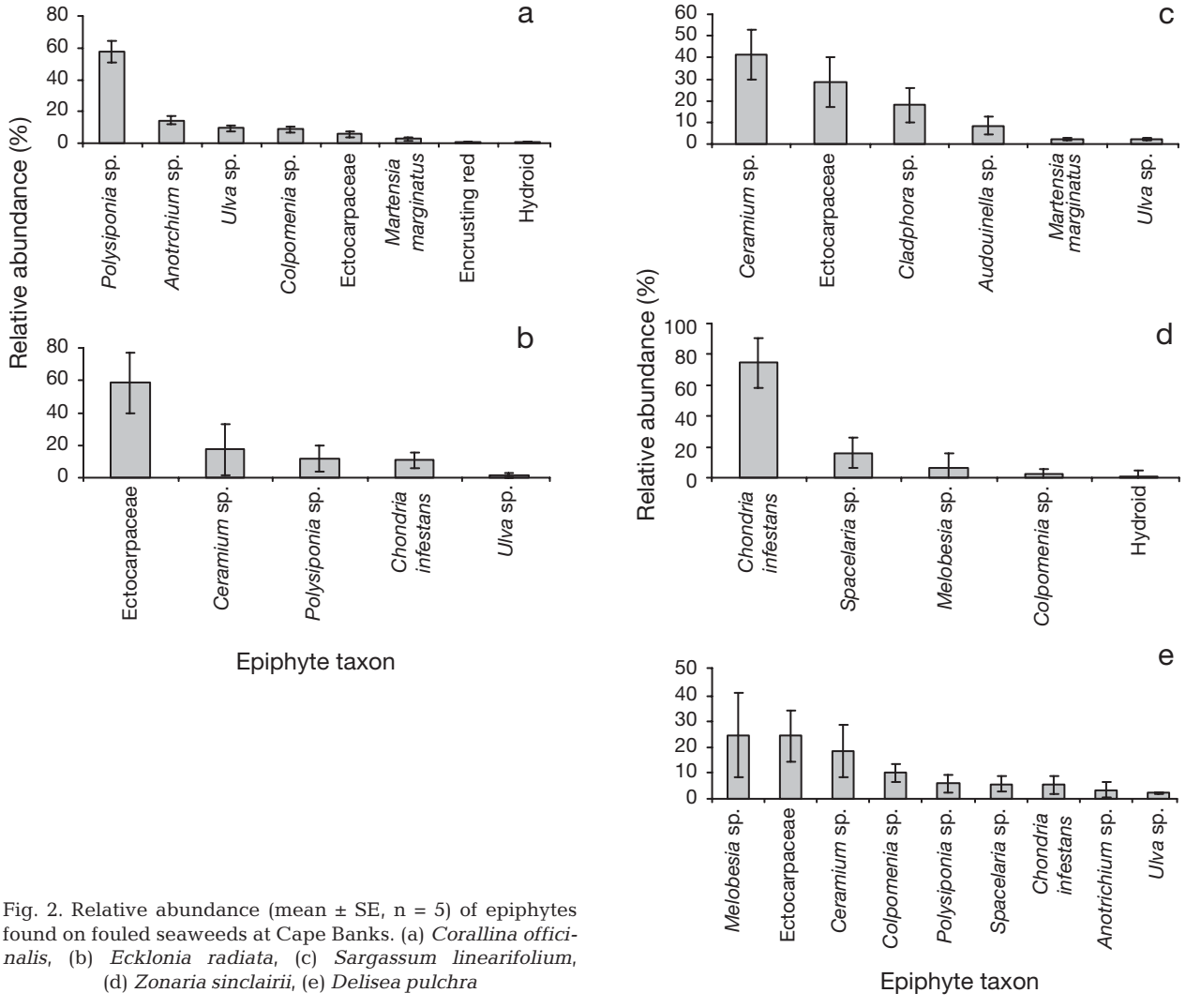

Fig. 2. Relative abundance (mean ± SE, n = 5) of epiphytes found on fouled seaweeds at Cape Banks. (a) *Corallina officinalis*, (b) *Ecklonia radiata*, (c) *Sargassum linearifolium*, (d) *Zonaria sinclairii*, (e) *Delisea pulchra*

common in other surveys of fouling on *Delisea pulchra* (Dworjanyn 2001).

Effect of crude surface extract from *Delisea pulchra*

Crude surface extracts of *Delisea pulchra* strongly deterred the settlement and growth of *Polysiphonia* sp. carpospores, *Ulva* sp. gametes, *Ceramium* sp. tetraspores and *Ectocarpus siliculosus* gametes (Fig. 3, Table 1). There was no settlement or growth of *Ulva* sp., *Polysiphonia* sp. or *E. siliculosus* in the dishes containing surface extract, and low settlement and growth of *Ceramium* sp. (3.25% ± 3.3 SE relative to settlement in controls of 50 to 60%) (Fig. 3, Table 1). The solvent controls and seawater controls were not significantly different from each other in any of the assays (Fig. 3, Table 1 ANOVA, followed by Tukey's HSD-test $\alpha < 0.005$).

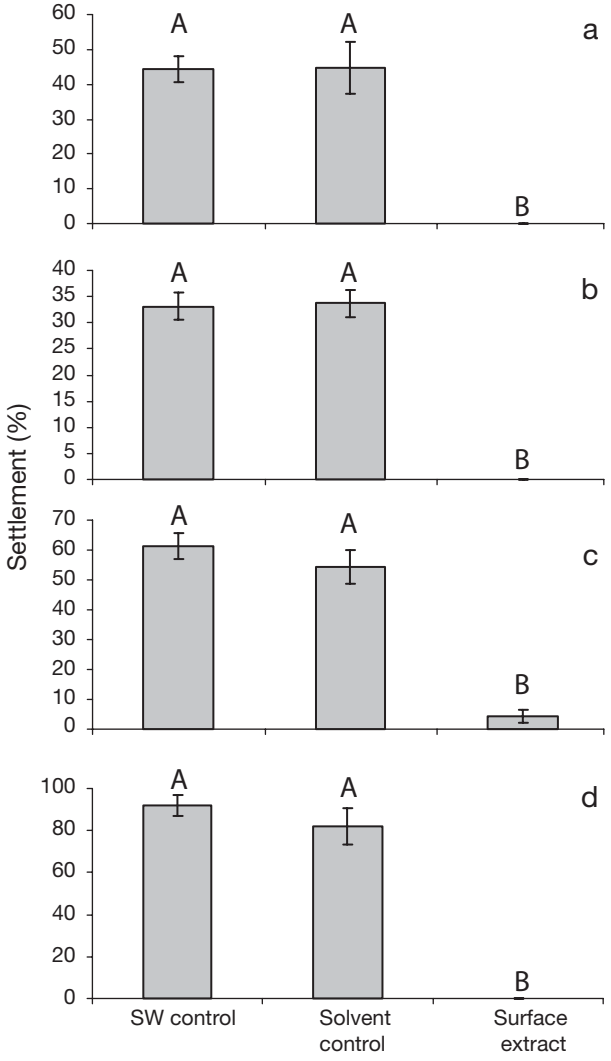

Fig. 3. *Delisea pulchra.* Effect (mean ± SE, n = 5) of crude surface extract of *D. pulchra* on settlement and growth of (a) *Polysiphonia* sp. carpospores, (b) *Ulva* sp. gametes, (c) *Ceramium* sp. tetraspores, and (d) *Ectocarpus siliculosus* gametes. Different letters indicate significant differences (Tukey's Honestly Significant Different [HSD]-test $\alpha < 0.05$). SW: seawater

Table 1. *Delisea pulchra.* Results of 1-way ANOVA testing effects of crude surface extract assays on settlement and growth of fouling organisms. $p < 0.0001$

Source	df	MS	*F*
Polysiphonia sp.			
Treatment	2	3157.07	27.8
Error	10	113.6	
Ulva sp.			
Treatment	2	1793.87	82
Error	12	21.89	
Ceramium sp.			
Treatment	2	3934.81	49.1
Error	10	80.14	
Ectocapus siliculosus			
Treatment	2	3157.07	27.8
Error	10	113.6	

Effect of furanone and lipid and fractions from *Delisea pulchra*

The TLC of the 2 fractions of the crude surface extract showed that the first fraction, the 'lipid fraction', contained no furanones and the second fraction, the 'furanone fraction', contained the 4 common furanones (1 to 4; Fig. 1). The lipid fraction, at natural or lower concentrations than on the plant, had no effect on the settlement and growth of the *Polysiphonia* sp. carpospores compared to the controls (Fig. 4, Table 2). The furanone fraction and the furanone fraction

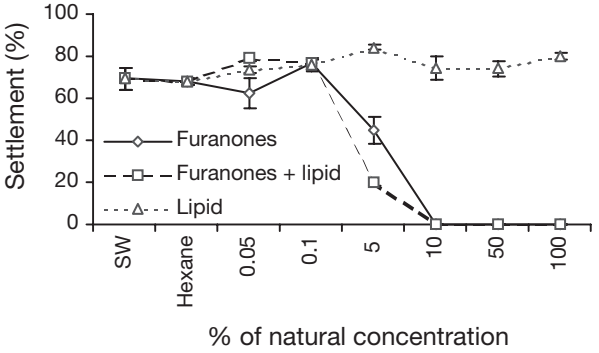

Fig. 4. *Delisea pulchra.* Effect (mean ± SE, n = 3) of furanone and lipid fractions of crude surface extract of *D. pulchra* and furanone fraction with lipids combined on settlement and growth of *Polysiphonia* sp. carpospores. SW and hexane are controls containing no surface extract

recombined with the lipid fraction completely inhibited the settlement of all the carpospores at 100, 50 and 10% of the concentration found on the surface of the plant (Fig. 4, Table 2). The lipid-furanone mixture inhibited the settlement of a significantly greater percentage of carpospores at 5% of the concentration naturally found on the surface of the plant than did the furanone fraction alone (Fig. 4, Table 2, ANOVA followed by Tukey's HSD-test $\alpha = 0.05$) but otherwise did not differ from the effect of furanones alone.

Table 2. *Delisea pulchra*. Results of 2-way ANOVA on the effect of the furanone fraction of the surface extract and furanone fraction combined with lipid fraction on settlement and growth of *Polysiphonia* sp. carpospores

Source	df	MS	*F*	p
Concentration	5	7554.2	215.5	<0.001
Treatment	1	18.0	0.5	0.481
Interaction	5	487.2	12.5	<0.001
Error	24	35.0		

Effects of furanones

Furanones 2 and 3, and the mix of the 4 most common furanones in the ratio found on the surface of the plant were very effective at inhibiting the settlement and growth of the 4 epiphytes tested (Fig. 5). Furanone 3 was marginally more effective at inhibiting settlement than the mix of furanones (Fig. 5). All the epiphytes were completely inhibited from settling and growing by Furanone 3 at 100 ng cm^{-2} (Fig. 5). Furanone 3 also had a strong effect (i.e. ~90% inhibition) on settlement of *Ulva* sp. gametes at 10 ng cm^{-2} (Fig. 5a) The mix of furanones at the same ratio as on the surface of the plant inhibited the settlement of *Ulva* sp. gametes and *Ceramium* sp. tetraspores at 100 ng cm^{-2}, and completely inhibited the settlement of *Polysiphonia* sp. carpospores and *Ectocarpus siliculosus* gametes at 1 µg cm^{-2} (Fig. 5). The mixture of furanones also had a strong effect on the settlement gametes of *Ulva* sp. at 10 ng cm^{-2} and carpospores of *Polysiphonia* sp. and gametes *E. siliculosus* at 100 ng cm^{-2} (Fig. 5).

The activity of Furanone 2 against the settlement of epiphytes was lower than that of the other 2 furanone treatments. Compound 2 completely inhibited the settlement of *Ulva* sp. gametes, *Ceramium* sp. tetraspores and *Polysiphonia* sp. carpospores at 1 µg cm^{-2}, and completely inhibited the settlement of *Ectocarpus siliculosus* gametes at 10 µg cm^{-2} (Fig. 5).

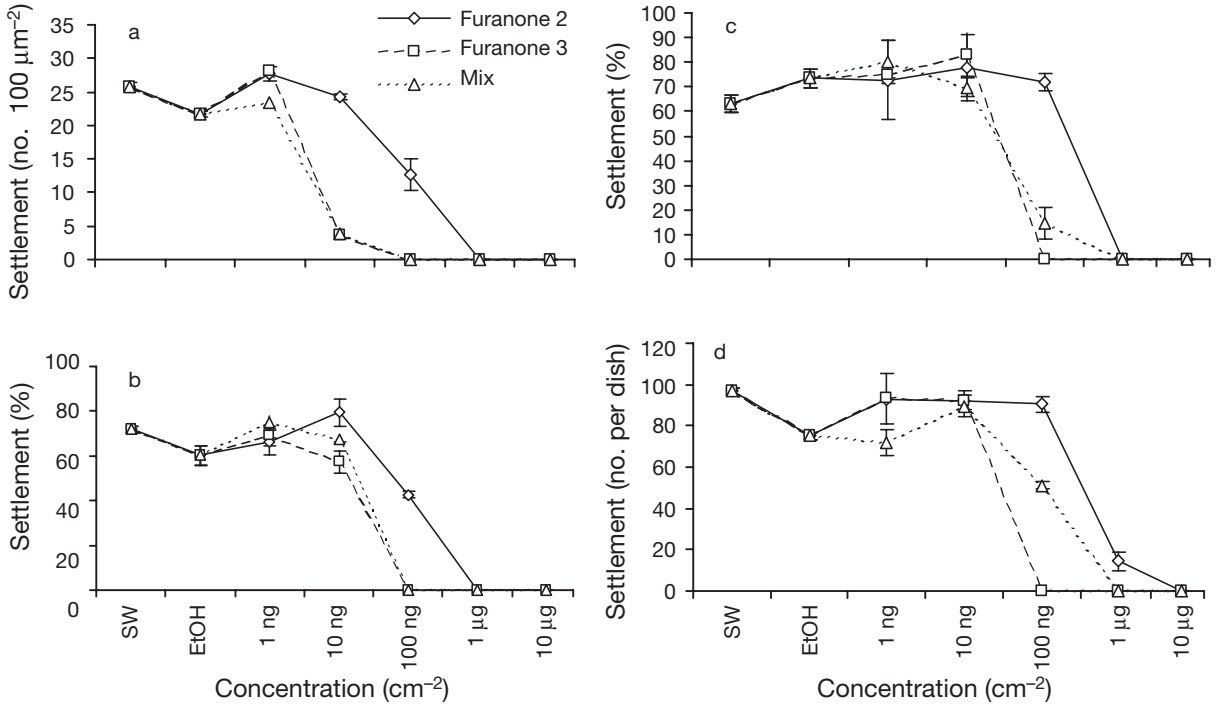

Fig. 5. *Delisea pulchra*. Effect (mean ± SE, n = 3) of Furanones 2 and 3 and mix of the 4 most abundant furanones (1 to 4), in the same ratio as present on the surface of *D. pulchra*, on settlement and growth of (a) *Ulva* sp. gametes, (b) *Ceramium* sp. tetraspores, (c) *Polysiphonia* sp. carpospores, and (d) *Ectocarpus siliculosus* gametes. SW and EtOH are controls containing no furanones

DISCUSSION

The concept of chemically mediated antifouling in marine organisms has been proposed for more than 50 yr (Walker & Smith 1948). Rigorous demonstrations of this phenomenon however, have until recently been hindered by a lack of methods to accurately harvest and measure surface compounds (Davis et al. 1989, Hay 1996, de Nys et al. 1998). Any such demonstration must provide evidence that a compound(s) is naturally found on the surface of an organism in sufficient concentrations to deter the settlement of ecologically relevant fouling organisms. The red alga *Delisea pulchra* produces a suite of secondary metabolites, brominated furanones, that are typically found in the plant at concentrations of between approx. 5 and 15 mg g^{-1} (dry wt) (de Nys et al. 1996a, Wright et al. 2000); these compounds are located in vesicles in specialised gland cells (Dworjanyn et al. 1999). These gland cells are distributed throughout the alga, but are mostly found amongst the cortical cells, where they release furanones onto the surface of the alga (Dworjanyn et al. 1999). Furanones have been extracted from the surface of *D. pulchra* using a method that leaves surface cells intact, allowing non-polar (hexane-soluble) compounds on the surface of the alga to be accurately measured (de Nys et al. 1998, Dworjanyn et al. 1999). Surface concentrations of furanones vary on the plant, between plants, and over time, but are typically present at about 100 ng cm^{-2} (Dworjanyn et al. 1999, Dworjanyn 2001). These concentrations are sufficient to deter the attachment of mixed bacterial communities (Maximilien et al. 1998), and deterred the settlement of *Ulva* sp., a potential fouling alga (de Nys et al. 1995). *D. pulchra* is free of fouling in the field, except in shallow waters during summer, when colonisation by fouling organisms coincides with a decrease in the concentration of furanones on the surface of the plant (Dworjanyn 2001). The present study adds to previous work on chemically mediated antifouling in *D. pulchra* by testing whether compounds at concentrations found naturally on the surface of the alga deter the settlement of a range of fouling organisms that the plant would encounter in the field.

One of the critical issues in demonstrating ecological roles for secondary metabolites is testing them against ecologically relevant natural enemies. However, antifouling roles are often attributed to secondary metabolites based on assays against organisms that are selected for their convenience, or commercial importance, rather than for their ecological relevance (Clare 1996). To test the validity of such assays, we surveyed the fouling community on *Delisea pulchra* and 4 other common co-occurring seaweeds, using representatives of this community in assays. The fouling community on these algae consisted of a diverse range of algal genera from all 3 algal divisions and a range of life history stages (i.e. tetrasporophytes, carposporophytes etc.). Notably, invertebrates were largely absent from the fouling assemblage. Our results were similar to those of other studies at this site, Cape Banks (Jennings & Steinberg 1997), although at other locations and for other algae, invertebrates such as bryozoans, tube-building animals and sponges are also common (e.g. Bernstein & Jung 1979, Schmitt et al. 1995). This variation between fouling assemblages suggests that fouling assays should be tailored to the host and environment in question.

Another important issue in maintaining ecological relevance in antifouling assays is testing compounds that are actually present on the surface of the plant at the same concentrations that they are present on the plant. As noted by several authors (Davis et al. 1989, Schmitt et al. 1995, Clare 1996, Hay 1996), tests of surface-mediated interactions such as antifouling and allelopathy are often made with compounds extracted from within the organisms, with no knowledge of whether these compounds are present on the surface of the organism or, if so, at what concentration. The importance of this issue has been highlighted in studies of the red alga *Laurencia obtusa*, which produces secondary metabolites that are effective in inhibiting fouling organisms at low concentrations (de Nys et al. 1996b). However, the ultrastructure of the alga and measurement of the compounds actually found on the surface of the plant revealed that these compounds are not present on the surface of the alga at active concentrations (de Nys et al. 1998).

In this study, we tested the activity of compounds found on the surface of *Delisea pulchra* at natural concentrations in 2 ways:

• Firstly we used crude-surface extracts of *Delisea pulchra* obtained using the same method developed for measurements of surface concentrations (de Nys et al. 1998, Dworjanyn et al. 1999), whereby the alga is dipped into hexane, a highly non-polar solvent, which extracts the non-polar compounds from the surface of the alga without disturbing any surface cells. Natural concentrations were then simulated in assays by coating the surface of assay dishes with the extract from a piece of algae with the same surface area as the assay dish. Crude surface extracts of *D. pulchra* at natural concentrations almost totally inhibited the settlement of the 4 algal epiphytes tested; *Polysiphonia* sp. carpospores, *Ulva* sp. gametes, *Ceramium* sp. tetraspores and *Ectocarpus siliculosus* gametes (Fig. 3). The compounds responsible for the antifouling activity of the crude extract were confirmed to be furanones, as only the fraction of the surface extract that contained these compounds had antifouling activity (Fig. 4). Further-

more, this fraction was found on the plant in excess of the concentration needed to deter the settlement of *Polysiphonia* sp. carpospores (Fig. 4). It is important to note that this extraction technique will only harvest compounds that are hexane-soluble; thus, more polar compounds that may have antifouling activity were not tested in these assays. However, it is known that this technique extracts all furanones (de Nys et al. 1998), which are by far the most abundant secondary metabolites in this plant (de Nys et al. 1993) and the only compounds that have been found to have antifouling activity (de Nys et al. 1995)

• Secondly, we used pure furanones in assays to test whether the concentration of secondary metabolites on the surface of *Delisea pulchra* is sufficient to deter the settlement of fouling organisms. The concentration of these compounds needed to deter the settlement of fouling organisms was then compared to previously measured concentrations on the surface of *D. pulchra* (de Nys et al. 1998, Dworjanyn et al. 1999, Dworjanyn 2001). Furanones that differed only by the substitution of a single functional group displayed different activities in antifouling assays. Furanone 3 (Fig. 1) was active at the lowest concentration in all assays, inhibiting the settlement of the 4 epiphytic algae tested at between 10 and 100 ng cm^{-2}. Furanone 3 is consistently also 1 of the 2 most abundant compounds on the surface of *D. pulchra*, being found on healthy plants at concentrations of approx. 30 to >100 ng cm^{-2} (Dworjanyn et al. 1999, Dworjanyn 2001). Hence, the concentration of this compound alone on the surface of *D. pulchra* would be sufficient to deter the settlement of many fouling organisms. Conversely, Furanone 2 is typically present on the surface of the plant at concentrations of <40 ng cm^{-2} (Dworjanyn et al. 1999, Dworjanyn 2001). This compound is effective at 1 order of magnitude higher concentration than Furanone 3 and would probably not perform an antifouling role alone on the surface of the plant. Thus, not all furanones necessarily play an important role as antifoulants on the surface of the plant. Similar structure–activity differences for algal metabolites have been observed for defences against herbivory (Hay et al. 1987) and, for sponge metabolites, for multiple ecological functions including fouling deterrence (Kubanek et al. 2002).

By mixing the furanones at approximately the same ratios as on the surface of *Delisea pulchra*, we simulated the chemical 'cocktail' that fouling organisms would encounter when settling on the plant. Although the mix was perhaps marginally less effective than Furanone 3 alone against 2 of the epiphytes tested, it inhibited the settlement of the 4 epiphytes at 100 ng cm^{-2} . This inhibitory concentration is again similar to the concentration of furanones on the surface of the plant (Dworjanyn et al. 1999, Dworjanyn 2001).

The function of compounds other than furanones, such as lipids, on the surface of *Delisea pulchra* are unknown. In general, little is known of the identity or function of compounds adhering to algal surfaces. However, in terrestrial plants, secondary metabolites on leaves are presented in a wax matrix that is believed to reduce the loss of these compounds from the leaf surfaces (Bernays & Chapman 1994). Similarly, in *D. pulchra*, the presence of lipids on its surface may form part of the 'delivery system' for the furanones. These lipids may increase the efficacy of the furanones by assisting their even spread and reducing the probability of furanone-free pockets on the plant. This is especially important in an antifouling defence because even minute 'chinks in the armour' (sensu McKey 1979) may be quickly exploited by the very small propagules of fouling organisms (in particular bacteria). The presence of the furanones in a lipid matrix may also slow their degradation or dissolution in the water column, thus extending the effective life of these compounds. The reintroduction of surface lipids to the furanone fraction of the surface extract at 1 concentration (5%) slightly but significantly increased the efficacy of furanones in inhibiting the settlement and growth of *Polysiphonia* sp. tetraspores.

One criticism of the type of laboratory antifouling assay used herein, is that because of the small volume of water used, assay organisms may be exposed to higher concentrations of the test chemicals in the aqueous phase than they would in nature (Henrikson & Pawlik 1995). This should not have affected the present results, since both the furanones and the crude surface extract from *Delisea pulchra* were used are relatively non-polar, and thus tend to remain on the surface of petri dishes, as in nature they would remain on the surface of the plant. Indeed, experiments on the solubility of furanones in closed systems similar to the petri dishes used herein have shown that the low concentration of furanones that do enter the aqueous phase remain very low in seawater because of chemical and microbial degradation (T. Charlton pers. comm.). Perhaps the most convincing evidence that these artefacts do not compromise laboratory assays is the results from bacterial assays (Maximilien et al. 1998). Bacterial attachment assays involving coating furanones onto surfaces in the laboratory in restricted water volume yielded results similar to those of short-term assays in the field (Maximilien et al. 1998). In fact, as these laboratory assays do not allow the propagules to choose between different substrata (as they would in nature) but force them to attempt to settle on only 1 surface, they may be a conservative measure of deterrence. Caveats must, however, be attached to any laboratory assay in a closed system. Lack of flushing may result in a build-

up of metabolites excreted from the test organism as well as the test compound, hydrodynamic cues possibly used by the test organism in the settlement process will be absent, and density-dependent behaviour such as gregariousness may interact with the test compound. Results from laboratory assays should only be seen as a broad indication of the effects of antifouling compounds.

Given the potential artifacts of laboratory assays, field assays where a known surface concentration of a putative antifoulant is exposed to a natural fouling community would be an ideal test of chemically mediated antifouling. Some progress in this direction has been made (Henrikson & Pawlik 1995, Kubanek et al. 2002). The technology to do this in an ecologically relevant manner, however, has not been broadly developed for anything except short-term bacterial attachment assays (Maximilien et al. 1998). Furanones have been tested in long-term field assays in applied studies where they were incorporated into extruded polymer matrices and tested in the field (Dworjanyn 2001, G. B. Christie et al. pers. comm.). These studies demonstrated that furanones strongly deter fouling by mixed assemblages of fouling organisms for a period of months. The ecological interpretation of the results is restricted, however, because surface concentrations of antifoulants on these artificial surfaces were not measured. The solvents used to extract them from the surface also tend to interact with the matrix containing the compounds, restricting surface measurements. Further difficulty in the use of artificial matrices in field assays is that the leaching rates of these materials are consistently non-linear. The approximately exponential reduction in release rate over time is a poor approximation of the natural release pattern expected for marine organisms.

Although a common theme in the marine chemical ecology literature, rigorous demonstrations of chemically mediated antifouling are limited. Interestingly, demonstrations of non-chemical strategies for dealing with fouling are numerous (e.g. cell sloughing: Johnson & Mann 1986; blade abandonment: Littler & Littler 1999; intercalery growth: Jennings & Steinberg 1997), and are exhibited by several taxa (corallines, fucoids, laminarians). This may reflect the relative ease of experimentally testing non-chemical antifouling strategies compared to chemically mediated antifouling, or perhaps indicate of the rarity of the chemical strategy in nature. There is potential for chemically mediated antifouling in seaweeds. There are many compounds produced by algae that deter many types of fouling organisms (e.g. see review by Clare 1996). There are also several algal taxa that have specialised cells that may have an excretory function; for example, gland cells are common in the Bonnemaisoniales (the order to which *Delisea pulchra* belongs) and the Ceramiales. The use of crude surface extracts in antifouling assays, as in the present study, comprises a method that can be used to assess how common chemically mediated fouling is in marine algae.

Acknowledgements. This work was supported by an Australian Postgraduate Award to S.A.D. and the Centre for Marine Biofouling and BIO-Innovation.

LITERATURE CITED

Bernays EA, Chapman RF (1994) Host–plant selection by phytophagous insects. Contemporary topics in entomology. 2. Chapman & Hall, New York

Bernstein BB, Jung N (1979) Selective pressures and coevolution in a kelp canopy community in southern California. Ecol Monogr 49:335–355

Bold HC, Wynne MJ (1985) Introduction to the algae: structure and reproduction, 2nd edn. Prentice-Hall, Englewood Cliffs, NJ

Clare AS (1996) Marine natural product antifoulants: status and potential. Biofouling 9:211–229

D'Antonio C (1985) Epiphytes on the rocky intertidal alga *Rhodomela larix* (Turner) C. Agardh.: negative effects on the host and food for herbivores. J Exp Mar Biol Ecol 86: 197–218

Davis AR, Targett NM, McConnell OJ, Young CM (1989) Epibiosis of marine and benthic invertebrates: natural products chemistry and other mechanisms inhibiting settlement and overgrowth. In: Scheuer PJ (ed) Bioorganic marine chemistry, Vol 3. Springer, Berlin, p 85–114

de Nys R, Steinberg PD (1999) Role of secondary metabolites from algae and seagrasses in biofouling control. Recent advances in marine biotechnology, Vol 3. Biofilms, bioadhesion, corrosion and biofouling. 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 49:11 213–11 220

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, Steinberg PD, Rogers CN, Charlton TS, Duncan MW (1996a) Quantitative variation of secondary metabolites in the sea hare *Aplysia parvllia* and its host plant, *Delisea pulchra*. Mar Ecol Prog Ser 130:135–146

de Nys R, Leya T, Maximilien R, Asfar A, Nair PS, Steinberg PD (1996b) The need for standardised broad scale bioassay testing: a case study using the red alga *Laurencia rigida*. Biofouling 10:213–224

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

Dixon J, Schroeter SC, Kastendiek J (1981) Effects of the encrusting bryozoan *Membranipora membranacea* on the loss of blades and fronds by the giant kelp *Macrocystis pyrifera* (Laminariales). J Phycol 17:341–345

Dworjanyn SA (2001) Chemically mediated antifouling and the cost of producing secondary metabolites in a marine alga. 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

Farrant PA, King RJ (1989) The Dictyotales (Algae: Phaeophyta) of New South Wales. Proc Linn Soc NSW 110: 369–405

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

Hay ME, Fenical W, Gustafson K (1987) Chemical defense against diverse coral reef herbivores. Ecology 68: 1581–1591

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

Jennings JG, Steinberg PD (1994) *In-situ* exudation of phlorotannins by the sublittoral kelp *Ecklonia radiata*. Mar Biol 121:349–354

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

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

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

Kubanek J, Whalan K E, 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

Littler MM, Littler OS (1999) Blade abandonment/proliferation: a novel mechanism for rapid epiphyte control in marine macrophytes. Ecology 80:1736–1746

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

McKey D (1979) The distribution of secondary compounds within plants. In: Rosenthal GA, Janzen OH (eds) Herbivores: their interaction with secondary plant metabolites. Academic Press, New York, p 55–133

Millar AJK (1990) Marine red algae of the Coffs Harbour region, Northern New South Wales. Aust Syst Bot 6: 1–90

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

Schmitt TM, Hay ME, Linquist N (1995) Constraints on chemically mediated coevolution: multiple functions for seaweed secondary metabolites. Ecology 76:107–123

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

Walker FT, Smith MM (1948) Seaweed culture. Nature 162: 31–32

Womersley HBS (1984) The marine benthic flora of Southern Australia. Part I. Flora and Fauna of South Australia Handbook Committee, Adelaide

Womersley HBS (1987) The marine benthic flora of Southern Australia. Part II**.** Flora and Fauna of South Australia Handbook Committee, Adelaide

Womersley HBS (1994) The marine benthic flora of Southern Australia. Part IIIA. Australian Biological Resources Study, Canberra

Womersley HBS (1996) The marine benthic flora of Southern Australia. Part IIIB. Australian Biological Resources Study, Canberra

Wright JT, de Nys R, Steinberg PO (2000) Geographic variation in halogenated furanones from the red alga *Delisea pulchra* and associated herbivores and epiphytes. Mar Ecol Prog Ser 207:227–241

Editorial responsibility: Anthony J. Underwood (Contributing Editor), Sydney, Australia

Submitted: February 23, 2005; Accepted: November 25, 2005
Proofs received from author(s): July 11, 2006