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A new method for determining surface concentrations of marine natural products on seaweeds

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ABSTRACT: To understand the role of marine natural products in surface mediated ecological interactions—such as biofouling—we need to be able to quantify metabolites at or near the relevant surfaces. We describe a new technique for determining the concentration of natural products on the surface of marine algae. Surface metabolites were quantified for 2 red algae, *Delisea pulchra* and *Laurencia obtusa*, by dipping the algae in hexane for 20 to 40 s at room temperature. This allowed for quantitative extraction of non-polar natural products from the surface of the thalli without disrupting cells (as determined by epifluorescence microscopy). More stringent extraction procedures using other solvents, or longer extraction times in hexane (>50 s), caused significant quantifiable cell damage. Natural products in the surface extracts were then measured using gas chromatography-mass spectroscopy (gc-ms). Mean total surface concentration of natural products from *D. pulchra* were 250 ng cm^{-2}, but <1 ng cm^{-2} for *L. obtusa*. These results contrast to whole plant levels of total secondary metabolites in the 2 algae, which were significantly higher in *L. obtusa* (7 µg mg^{-2} dry weight) than in *D. pulchra* (3.4 µg mg^{-2} dry weight). Dipping thalli in hexane for 30 s also caused no cell lysis in 8 other species of macroalgae. This suggests that the procedure is more broadly applicable for the quantification of non-polar surface metabolites on seaweeds, and other organisms with resistant surface cells. Our results highlight the need to determine where compounds occur, and at what concentration, if we are to understand their ecological roles.

KEY WORDS: Surface mediated interactions · Biofouling · Marine natural products · *Delisea pulchra* · Marine algae · Furanones · Chemical ecology

INTRODUCTION

Natural products, also known as secondary metabolites, mediate a wide range of ecological interactions between marine organisms (Hay 1996). Many of these interactions, including fouling (Davis et al. 1989, Wahl 1989, Clare 1996), competition for space (de Nys et al. 1991), and recognition of food (Tamburri & Zimmer-Faust 1996), occur at the surfaces of the relevant organisms. Of these various interactions, fouling, or 'the process of adsorption, colonisation and development of living and non-living material on an immersed substratum' (Clare 1996), has perhaps received the most attention in the literature. Both micro- (bacteria and diatoms) and macro- (algae and invertebrates) fouling can have a wide range of deleterious effects on host organisms (Sand-Jensen 1977, Dixon et al. 1981, D'Antonio 1985, Williams & Seed 1992, Littler & Littler 1995, Wahl & Hay 1995, Kushmaro et al. 1996).

That sessile marine organisms (sponges, ascidians, seaweeds, etc.) inhibit fouling of their surfaces via the production of chemical inhibitors has been a theme in the literature for many years (e.g. Sieburth & Conover 1965, Davis et al. 1989, Clare 1996 and references therein). And indeed, crude chemical extracts of marine organisms, or specific metabolites, which deter or kill fouling organisms in laboratory assays have been isolated from a broad range of marine bacteria, algae, seagrasses, and invertebrates (reviewed by Holmström & Kjelleberg 1994, Clare 1996). However, as previous authors have highlighted (Davis et al.

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1989, Hay 1996), one major difficulty with interpreting the vast majority of these studies is that rarely is any infomation provided on the concentration of metabolites at or near the surface of the host organisms. Although an extract or metabolite may be active at low concentrations, unless it can be demonstrated that those concentrations are present *in situ* at or near the surface of the host, an antifouling function cannot be inferred. Only a handful of studies have addressed this issue by attempting to measure ecologically relevant concentrations of metabolites, and then testing those concentrations against ecologically relevant fouling organisms. For example, Schmitt et al. (1995) found that the lipid soluble extract of surface swabs of the brown alga *Dictyota menstrualis*, which contained the diterpenes pachydictyol A and dictyol E, inhibited the settlement of larvae of the common epibiont *Bugula neretina*. Jennings & Steinberg (1997) showed that realistic concentrations of mixed phlorotannin fractions from *Ecklonia radiata* had no effect on settlement and germination of propagules of the green alga *Ulva lactuca*, an epiphyte of these kelps. However, even in these studies there was no quantitative measurement of individual, characterized secondary metabolites at or near the surface of the host.

In order to further advance our understanding of how marine natural products mediate interactions at surfaces we need methods for quantifying these compounds at or near the relevant surfaces. Such methods would be applicable not only to inhibition of fouling, but also to allelopathy (de Nys et al. 1991), induction of larval settlement (Pawlik 1992), recognition of food (Tamburri & Zimmer-Faust 1996), and many others.

Here we describe such a methodology for the extraction and quantification of non-polar secondary metabolites from the surfaces of marine algae. The method is based on similar studies from terrestrial chemical ecology, where quantitative analysis of furanocoumarins (Zobel & Brown 1988, 1990a, b), flavanoids (Stevens et al. 1995), flavonid aglycones (Wollenweber et al. 1987), and other metabolites (Tulloch 1987, Eigenbrode et al. 1991) has highlighted the importance of secondary metabolites on surfaces for ecological interactions (Chapman & Bernays 1989). We combined chemical and microscopic studies in order to optimise extraction and quantification of surface metabolites while minimising damage to cells. We first focused on 2 chemically rich red algae, *Delisea pulchra* and *Laurencia obtusa*, and then applied the technique in a non-quantitative way to an additional 8 species of algae in order to determine whether the technique was more broadly applicable to seaweeds. The ecological roles of secondary metabolites in *D. pulchra* and *L. obtusa* are discussed in regard to our results.

MATERIALS AND METHODS

Study organisms. The red algae *Delisea pulchra* (Greville) Montagne and *Laurencia obtusa* Lamouroux are common subtidal species in the Sydney, NSW, Australia, region. *D. pulchra* is abundant year round; *L. obtusa* is common during the warmer months (December to May). Both species contain biologically active non-polar secondary metabolites. *D. pulchra* produces a range of halogenated furanones and enones (Fig. 1, 1 to 4) (Kazlaukas et al. 1977, de Nys et al. 1992, 1993). *L. obtusa* from this location contains a range of halogenated sesquiterpenes and diterpenes with palisadin A (5), aplysistatin (6), brasilenol (7), and palisadin B (8) (Fig. 1) the most abundant (Battista 1995).

Other marine algae used in this study included 5 species of brown algae, *Dictyopteris acrostichoides* (J. Agardh) Boergesen, *Dictyota dichotoma* (Hudson) Lamouroux, *Zonaria diesingiana* J. Agardh, and *Dilophus marginatus* J. Agardh, *Sargassum linearifolium* (Turner) C. Agardh; 2 species of red algae, *Laurencia rigida* Agardh and *Pterocladia capillacea* (S.G. Gmelin) Bornet; and 1 green alga, *Ulva* sp. All species were collected from the sublittoral zone at Nielsen Park, Port Jackson (33° 51' 04" S, 151° 16' 12" E) or Cape Banks, NSW (34° 00' S, 151° 14' 43" E) and were identified using appropriate taxonomic keys (Womersley 1984, 1987, 1996, Farrant & King 1989).

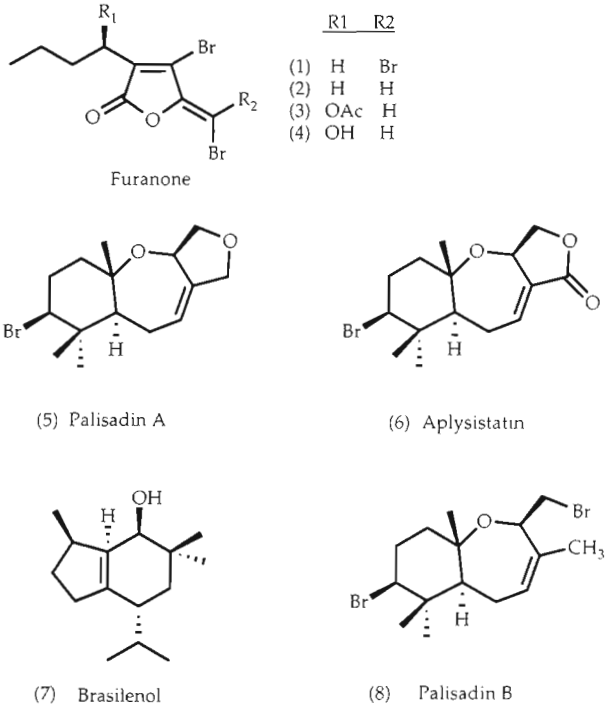

Fig. 1. Structure of the major secondary metabolites of *Delisea pulchra* (1 to 4) and *Laurencia obtusa* (5 to 8)

Whole plant chemistry of *Delisea pulchra* and *Laurencia obtusa*. In order to confirm the presence of secondary metabolites in the algae used here, and to compare levels of surface chemistry with those of whole plants, the concentration of secondary metabolites was measured for whole plants of *D. pulchra* (see also de Nys et al. 1996) and *L. obtusa*. Ten plants of each species were collected at the same time and locations as plants used for surface extraction. Plants were weighed, frozen, freeze dried and reweighed prior to the extraction. The freeze dried algae were homogenised and 200 mg of tissue extracted in dichloromethane (as per de Nys et al. 1996). The extract was taken to dryness and the resultant extract prepared for analysis by gc-ms (de Nys et al. 1996, and below).

Gas chromatography-mass spectrometry (gc-ms). Extracts for gc-ms were dissolved in ethyl acetate (EtOAc) containing napthalene as an internal standard at a concentration of 10 µg ml^{-1}. Surface extracts for gc-ms were made up in 400 µl of EtOAc (+internal standard). Whole plant extracts were made up in 5 ml of EtOAc (+internal standard). Standards used in gc-ms for both *Delisea pulchra* and *Laurencia obtusa* were isolated from dichloromethane extracts of the algae by established protocols (de Nys et al. 1993, Battista 1995). All standards were identified by comparison of ^{1}H and ^{13}C NMR (nuclear magnetic resonance) data with published data. Gas chromatography was performed using a Hewlett Packard (HP) 5980 series II gas chromatograph (GC) and a polyimide-coated fused-silica capillary column (BPX5, 12 m length, 0.15 mm i.d., 0.22 µm modified siloxane stationary phase; SGE Pty Ltd). All injections were performed in the splitless mode with an inlet pressure of 100 kPa. The injection port was held at 280°C and the interface at 300°C. For *D. pulchra* the GC was held at 50°C for 1.5 min and ramped at 20°C min^{-1} to 300°C (and held there for 10 min). For *L. obtusa* metabolites the GC was held at 50°C for 1.5 min and ramped at 20°C min^{-1} to 310°C (and held there for 10 min). Helium was used as the carrier gas.

The mass spectrometry was performed on a HP 5971 Mass Selective Detector (MSD). Ions characteristic of the internal standard, 4 metabolites for *D. pulchra* (compounds 1 to 4) and 4 metabolites for *L. obtusa* (5 to 8) were monitored in the selected ion monitoring (SIM) mode and were quantitatively analysed using purified standards. Quantification was performed by measuring peak areas for each compound and the internal standard. The ratio of peak areas (cmpd/internal standard) was calculated for each metabolite (cmpds 1 to 8) and converted to concentration by reference to standard curves. To determine the surface concentration of secondary metabolites the surface areas of pieces of *D. pulchra* and *L. obtusa* were calculated using a wet weight (mg) to surface area (cm^2) conversion factor. To calculate the conversion factor fresh pieces of *D. pulchra* and *L. obtusa* between 5 and 500 mg wet weight were dried and weighed before being placed on white paper. The algae were then immediately scanned and analysed for surface area using the NIH Imaging Program. For the planar *D. pulchra*, surface area was calculated to account for the area on both sides of the alga. For the cylindrical *L. obtusa*, the length and cross section of the alga were measured and the surface area of the cylinder calculated. The regression between wet weight and surface area for both species had associated R^2 values greater than 0.97 and probabilities less than 0.001.

Extraction procedure for surface metabolites. In an initial test to determine the appropriate solvent for extraction of algal surfaces, 5 organic solvents were assessed for their effects on the lysis of epithelial cells of *Delisea pulchra* and *Laurencia obtusa* using epifluorescence microscopy. The solvents, in order of increasing polarity, were hexane (least polar), dichloromethane, diethyl ether, ethyl acetate and methanol (most polar). All solvents were of analytical reagent grade. Twenty ml of each solvent was added to a 100 ml beaker and a ~1.5 g piece of either *D. pulchra* or *L. obtusa*, which had been dried in a salad spinner, was held in each of the solvents for times of 10, 20, 30 and 60 s and vortexed.

After each time period the piece was directly observed and qualitatively assessed for the presence of lysed cells using epifluorescence microscopy. The surface cells of whole axes were observed for fluorescence on a Leitz epifluorescent microscope with filter block D (BP 355–425, dichromic mirror 455, LP 460 nm). Pigments in chloroplasts of algae autofluoresce when excited under near UV to blue light, emitting a yellow to red fluorescence (O'Brien & McCully 1981). Healthy living cells have discrete chloroplasts which are identified by their fluorescence. When the plasmalemma of a cell is disrupted the chloroplasts of the cell lyse, filling the whole cell with pigment. This change is observed using epifluorescence microscopy. Using this technique surface cells of axes were qualitatively and quantitatively assessed for cell lysis.

The effects of extraction in hexane (our eventual solvent of choice) on cell lysis were measured quantitatively by counting the number of lysed versus intact cells for individual pieces of *Delisea pulchra* (n = 12) and *Laurencia obtusa* (n = 6) subjected to extraction in hexane (20 ml) as described above for 10, 20, 30, 40, 50 and 60 s. The effect of extraction time was directly assessed following extraction by counting the number of lysed cells per unit field (0.1 mm^2) for tissue from each extraction time.

Except for small plants, it is difficult to dip whole thalli. Therefore, an additional validation step for the procedure was performed to ensure that there was no effect of cutting the plants which were to be dipped. To assess the effects of cutting on surface extraction, samples (1 to 1.5 g wet weight) of *Delisea pulchra* were generated by a single cut to the thallus, and then extracted in 2 ways. In the first, the pieces of plant were held such that the cut end did not contact the hexane when vortexed for 30 s. In the second, the pieces of plant were immersed, including the cut end, and vortexed in hexane for 30 s. After vortexing, the algae were removed and the samples taken to dryness in air and the resultant extract prepared for analysis by gc-ms.

Quantitative analysis of secondary metabolite chemistry of *Delisea pulchra* and *Laurencia obtusa* following surface extraction. Levels of secondary metabolites for both *D. pulchra* and *L. obtusa* were measured following extraction by hexane. Ten plants collected from the field were dried in a salad spinner and single pieces (1.2 to 1.6 g wet weight) were cut from a plant for extraction at each time. Pieces were extracted for 10, 20, 30, 40, 50 or 60 s. Upon cutting each piece was immediately placed into hexane and vortexed for the required period. After vortexing the alga was removed and the hexane taken to dryness at room temperature and the resultant extract prepared for analysis by gc-ms.

Effects of solvent extraction on the epithelial cells of other algal species. To assess the broader application of the technique to other algal species the effect of surface extraction on cell lysis was examined for 5 species of brown algae, *Dictyopteris acrostichoides*, *Dictyota dichotoma*, *Zonaria diesingiana*, and *Dilophus marginatus*, *Sargassum linearifolium*; 2 other species of red algae, *Laurencia rigida* and *Pterocladia capillacea*; and 1 green alga, *Ulva* sp. Whole plants were collected from the field and after drying by spinning, samples were taken from the top portions of the thallus for surface extraction in hexane at 30 and 60 s, and in methanol at 30 s. Samples were extracted and immediately analysed for cell lysis using epifluorescence microscopy.

Statistical analyses. Metabolite levels and cell lysis were analysed using analysis of variance followed by Tukey's multiple range test (where appropriate). Homogeneity of variances was tested with Cochran's test. Concentrations of metabolites were analysed following arcsin $\sqrt{p}$ transformation (Sokal & Rohlf 1985). Analyses where more than 1 sample was taken from an individual plant were performed using plant as a blocked factor in ANOVA to partition the overall effect of variation among plants from the variation among individual parts of a plant (e.g. de Nys et al. 1996).

RESULTS

Whole plant chemistry of *Delisea pulchra* and *Laurencia obtusa*

Both *Delisea pulchra* and *Laurencia obtusa* are rich in non-polar secondary metabolites, and plants used in this study were no exception. Mean levels of secondary metabolites for *D. pulchra* were, in order of decreasing concentration, compound 3 (1.56 µg mg^{-1} dry weight), compound 4 (0.77 µg mg^{-1} dry weight), compound 1 (0.55 µg mg^{-1} dry weight) and compound 2 (0.53 µg mg^{-1} dry weight). Total concentration of furanones in *D. pulchra* averaged 3.41 µg mg^{-1} dry weight. Levels of metabolites in whole thalli of *L. obtusa* were, in order of decreasing concentration, palisadin A (3.70 µg mg^{-1} dry weight), aplysistatin (1.69 µg mg^{-1} dry weight), palisadin B (1.19 µg mg^{-1} dry weight) and brasilenol (0.43 µg mg^{-1} dry weight), with a total metabolite concentration of 7.01 µg mg^{-1} dry weight.

Effects of solvents and extraction time on cell viability

Of the 5 solvents tested for extraction of surface metabolites, all except hexane caused lysis of surface cells for both *Delisea pulchra* and *Laurencia obtusa* at all of the times tested (Table 1). Cells of *D. pulchra* dipped in hexane for 10, 20 and 30 s remained intact (Fig. 2a, b), but exposure for 60 s caused lysis of cells (Fig. 2c). *L. obtusa* was more robust with most cells remaining intact after dipping in hexane for 60 s. Lysis of cells (as percent of cells lysed) was then measured quantitatively at 10 s intervals following surface extraction in hexane. As suggested by Table 1, there was a significant effect of time of immersion in hexane on lysis of cells for *D. pulchra* (1-factor ANOVA, $p = 0.0001$; Fig. 3). No significant cell lysis occurred from 10 to 40 s (Fig. 2a, b), but it increased significantly from a mean of 1.55% at 40 s to greater than 20% when

Table 1 Effect of the time epithelial cells of *Delisea pulchra* (*D.p.*) and *Laurencia obtusa* (*L.o.*) were immersed in the solvents methanol, ethyl acetate, diethyl ether, dichloromethane and hexane as assessed by epifluorescence microscopy. (–) Lysed cells present; (+) cells intact

Solvent	Time (s)							
	10		20		30		60	
	D.p.	*L.o.*	*D.p.*	*L.o.*	*D.p.*	*L.o.*	*D.p.*	*L.o.*
Methanol	–	–	–	–	–	–	–	–
Ethyl acetate	–	–	–	–	–	–	–	–
Diethyl ether	–	–	–	–	–	–	–	–
Dichloromethane	–	–	–	–	–	–	–	–
Hexane	+	+	+	+	+	+	–	+

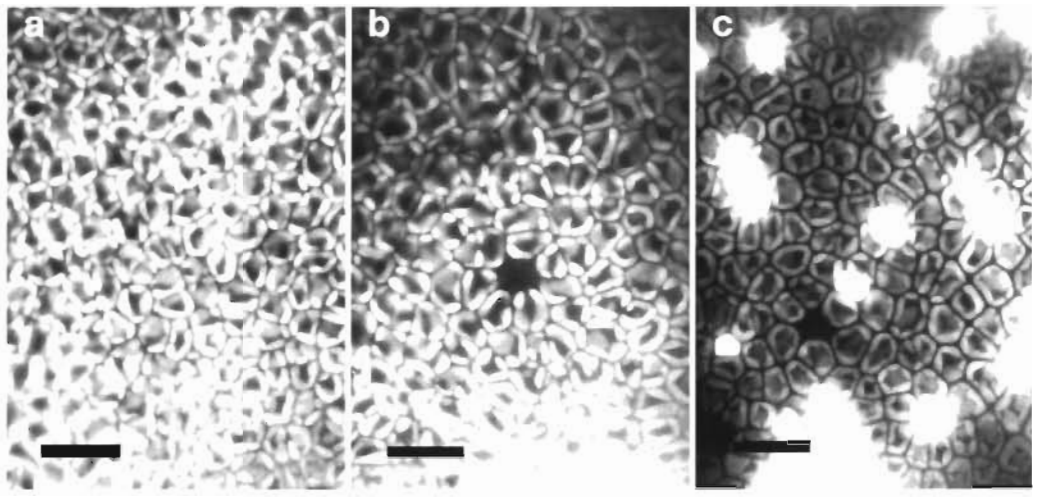

Fig. 2. *Delisea pulchra*. Fluorescence photomicrograph of the unstained surface which has been (a) untreated (b) extracted in hexane for 30 s and (c) extracted in hexane for 60 s. Scale bars = 10 µm

tissue was immersed for 50 or 60 s. Different immersion times had no significant effect on lysis of cells of *L. obtusa* at all immersion times (1-factor ANOVA, p = 0.508; Fig. 3), although the increase in percent lysed after 60 s suggests that cells of more susceptible thalli were beginning to lyse. No cells were lysed in plants prior to immersion in hexane, suggesting the process of collection from the field had a minimal effect on the algae.

Effect of cutting on surface chemistry

There was no significant difference in the levels of total metabolites measured between *Delisea pulchra* which were extracted with the cut surface immersed versus not immersed (1-factor ANOVA, $F_{1,8}$ = 0.001, p = 0.982). Concentrations of individual metabolites on the surface also did not vary between cut surface immersed and not immersed (2-factor ANOVA, analysis not shown).

Quantitative analysis of surface secondary metabolites

Delisea pulchra

Secondary metabolites were present on the surface of *Delisea pulchra* at concentrations greater than 100 ng cm^{-2}. Measured concentrations of furanones rapidly reached a plateau as a function of extraction time; concentrations of total metabolites extracted after 20, 30, or 40 s did not differ (Table 2; Tukey's test, α = 0.05; Fig. 4). There was a significant rise in measured levels of furanones for extraction times of more than 40 s (Table 2, Fig. 4), from approximately 250 ng cm^{-2} after 30 or 40 s to >600 ng cm^{-2} after 50 or 60 s of extraction. This corresponds to a significant rise in lysed cells between 40 and 50 s of extraction (Fig. 3). Changes in concentrations of individual metabolites were similar to each other, and to that of the total levels (note non-significant Metabolites × Time interaction term in Table 2). For each metabolite, there was no significant difference in metabolite concentrations for extraction times up to 40 s (Tukey's α = 0.05; Fig. 4). Metabolites 3 and 4 were present in the highest concentrations (122.4 and 113.3 ng cm^{-2} respectively) after extracting for 40 s. Levels of all metabolites increased with longer extraction times, again corresponding to the increase in the number of cells lysed.

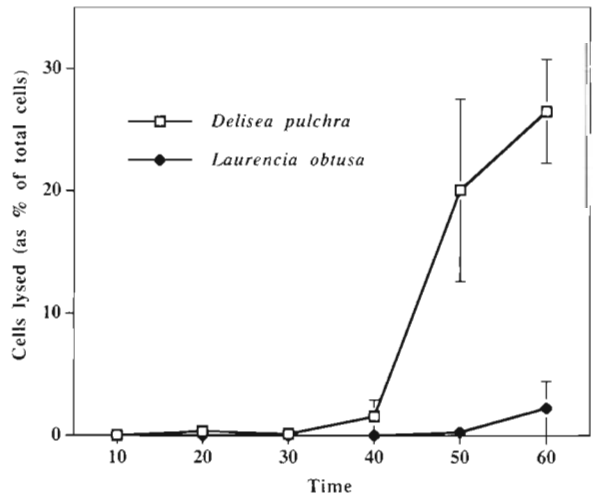

Fig. 3. *Delisea pulchra* and *Laurencia obtusa*. Percentage of cells lysed following extraction (immersion) in hexane at 10 s intervals from 0 to 60 s. Means ± SE, n = 12 for *D. pulchra*, n = 6 for *L. obtusa* at each time

Table 2. (a, c) Two-factor analysis of variance for arcsin √p transformed total concentration of metabolites extracted from the surface of (a) *Delisea pulchra* and (c) *Laurencia obtusa* plants at 10 s intervals from 10 to 60 s. (b, d) Three-factor analysis of variance for arcsin √p transformed concentration of metabolites extracted from the surface of (b) *D. pulchra* and (d) *L. obtusa* at 10 s intervals from 10 to 60 s

Factor	df	MS	*F*-test	p-value
(a) *Delisea pulchra* 2-factor ANOVA				
Block (= Plant)	8	0.212	6.427	0.0001
Time	5	0.233	7.074	0.0001
Error	40	0.033		
(b) *Delisea pulchra* 3-factor ANOVA				
Block (= Plant)	8	0.150	10.735	0.0001
Metabolites	3	0.942	67.306	0.0001
Time	5	0.163	11.633	0.0001
Metabolites × Time	15	0.021	1.512	0.105
Error	184	0.014		
(c) *Laurencia obtusa* 2-factor ANOVA				
Block (= Plant)	9	0.002	1.520	0.170
Time	5	0.012	11.702	0.0001
Error	45	0.001		
(d) *Laurencia obtusa* 3-factor ANOVA				
Block (= Plant)	9	0.002	3.060	0.003
Metabolites	1	0.002	3.146	0.079
Time	5	0.002	21.525	0.0001
Metabolites × Time	5	0.001	1.114	0.358
Error	99	0.001		

Laurencia obtusa

Surface concentrations of metabolites on *Laurencia obtusa* were very low, approximately 1 ng cm^{-2} or less for extraction times of less than 40 s. The level of total metabolites increased significantly with time (Table 2c, Fig. 5). The concentration of total metabolites for all times up to 50 s was not significantly different (Tukey's test, $\alpha = 0.05$; Fig. 5). There was a significant increase in the concentration of total metabolites between extraction times of 50 and 60 s. This corresponds to an increase in the mean number of cells lysed at 60 s (Fig. 3). Only 2 of the 4 major metabolites measured in the whole plant, palisadin A and aplysistatin, were detected from the surface of *L. obtusa*. The concentration of both metabolites increased significantly with time (Table 2d, Fig. 5). Mean levels of metabolites were significantly higher after 60 s extraction time than after all other times (Tukey's test, $\alpha = 0.05$; Fig. 5). For extraction times up to 40 s, where no measurable cell lysis occurred, concentrations of metabolites on the surface of *L. obtusa* were typically 2 orders of magnitude less than those on *Delisea pulchra*.

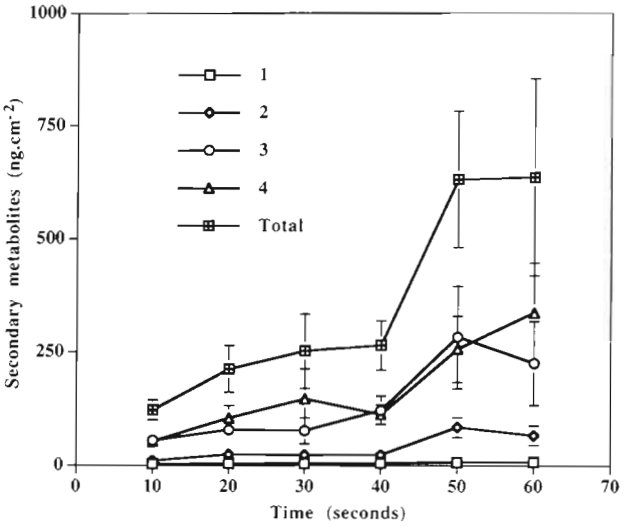

Fig. 4. *Delisea pulchra*. Concentration of furanones 1 to 4 and total metabolites extracted in hexane at 10 s intervals from 0 to 60 s. Data are mean concentrations ± SE, n = 10

Effects of solvent extraction on the epithelial cells of other algal species

The results of solvent extraction of a further 8 species of algae were similar to those in initial experiments of solvent and extraction time on cell lysis. Extraction in methanol caused widespread lysis of cells for all 8 algal species tested (Table 3). Cells of all species remained intact after extraction in hexane for 30 s (Table 3). Extraction in hexane for 60 s had no significant effect on cell lysis for all species except *Laurencia rigida*, where some lysis (comparable to that of *L. obtusa*) was observed. These results confirm that this technique is able to be applied to a broader range of marine algae including brown and green algal species.

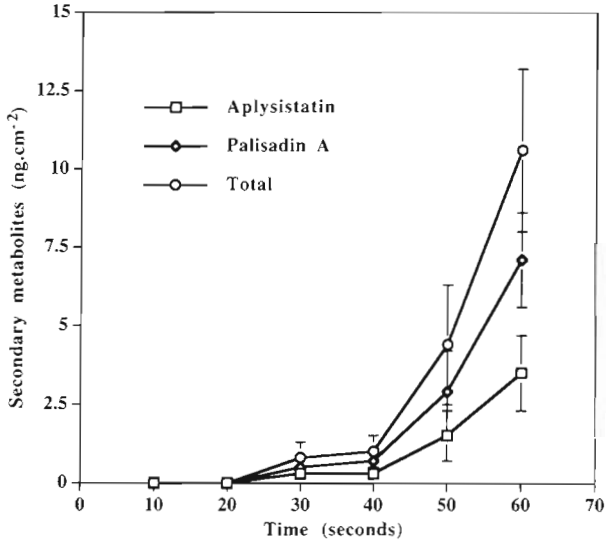

Fig. 5. Concentration of aplysistatin, palisadin A and total metabolites in *Laurencia obtusa* extracted in hexane at 10 s intervals from 0 to 60 s. Data are mean concentrations ± SE, n = 10

Table 3. Effect of immersion in methanol and hexane on epithelial cells of 8 algal species as assessed by epifluorescence microscopy. (–) Lysed cells present; (+) cells intact

Solvent	MeOH	Hexane	
Time (s):	30	30	60
Dictyopteris acrostichoides	–	+	+
Dictyota dichotoma	–	+	+
Zonaria diesingiana	–	+	+
Dilophus marginatus	–	+	+
Sargassum linearifolium	–	+	+
Laurencia rigida	–	+	–
Pterocladia capillacea	–	+	+
Ulva sp.	–	+	+

DISCUSSION

Understanding the quantitative distribution of natural products within and on terrestrial plants—and consequently the way in which they are presented to other organisms—has proven fundamental to understanding their ecological role (Zobel & Brown 1988, 1990a, b, Chapman & Bernays 1989). Such information is also clearly central to determining the ecological role of natural products for marine organisms (Hay 1996). For surface mediated ecological interactions among marine organisms, new techniques need to be developed to extract and quantify secondary metabolites associated with the surface of the producing organism. The method described here enables non-polar natural products from the surface of seaweeds to be extracted and quantified with minimal apparent cell damage, and minimising extraction of intracellular secondary metabolites. Although only metabolites from *Delisea pulchra* and *Laurencia obtusa* were quantified in this study, the method should be applicable for quantifying non-polar secondary metabolites on the surfaces of marine algae in general, since the extraction procedure did not cause obvious lysis to cells of 8 other species of red, green, or brown algae. While other common chemically rich organisms—sponges, ascidians, etc.—were not examined here, the technique may be useful for any other marine organisms with sufficiently robust cell surfaces (which can be determined via microscopy). However, the technique is limited to non-polar metabolites, and importantly the specific extraction solvent and immersion time need to be determined in each case to demonstrate a plateau in levels of secondary metabolites prior to cell lysis.

This hexane dip method has some advantages over swabbing the surface of an alga (Schmitt et al. 1995, Dworjanyn unpubl.), which is the only other published method used to assess surface concentrations or activities of natural products from algae. It is difficult to swab uniformly over the surface of an alga, and for foliose algae such as *Delisea pulchra* or *Laurencia obtusa* it is also difficult to ascertain exactly how much surface area has been swabbed. As well as using the dip procedure to quantify actual metabolites, it can also be used (like swabbing) as a means of harvesting realistic concentrations of extract from a surface for subsequent biological testing (e.g. Tamburri & Zimmer-Faust 1996). Here also it has some advantages over swabbing, as even supposedly 'clean' cotton swabs can contain contaminants which when extracted in organic solvents interfere with biological testing of target natural metabolites (de Nys pers. obs.).

The 2 algae studied in detail here provide a clear example of how whole plant chemistry can be misleading with regard to surface chemistry. Both *Delisea pulchra* (de Nys et al. 1993) and *Laurencia obtusa* (Erickson 1983, Battista 1995) are well studied, chemically rich algae. Whole plant levels of metabolites (7.01 µg mg^{-2} for *L. obtusa* and 3.41 µg mg^{-2} for *D. pulchra*) and levels of the most abundant metabolites (palisadin A is present at 3.7 µg mg^{-2} in *L. obtusa*, furanone 3 at 1.56 µg mg^{-2} in *D. pulchra*) in the 2 species are roughly similar. However, surface concentrations of metabolites of the species differed by more than 2 orders of magnitude, with metabolites barely present (e.g. ~1 ng cm^{-2} or less) on the surface of *L. obtusa*. Moreover, surface concentrations of furanones on specific parts of the thallus, or from samples taken at different times of the year from this study, can exceed 1.5 µg cm^{-2}. This is 3 orders of magnitude higher than levels of metabolites on the surface of *L. obtusa*.

Comparisons of the ultrastructure of *Delisea pulchra* and *Laurencia obtusa* are consistent with the difference in levels of surface metabolites between the 2 algae. Furanones in *D. pulchra* are contained in specialised vesicle cells which are present at the surface of the plant (Dworjanyn et al. unpubl.). Similar structures also occur in other genera in the chemically rich family Bonnemaisoniaceae (to which the genus *Delisea* belongs) (Womersley 1996), suggesting that other members of this family may also be able to use secondary metabolites to mediate surface interactions. In contrast, the terpenoid metabolites produced by *Laurencia* species are stored in membrane-bound vesicles—'corps en cerise'—which occur within the outer cortex of the plant (Young et al. 1980). These vesicles, and thus the metabolites contained within, are not typically released to the surface of the plant and the external environment (Howard 1978, in Hay et al. 1987, and this paper) except following damage to the plant. These observations suggest that even in those instances where the levels of secondary metabolites on the surface of a marine organism are not known, the ultrastructure of the organism may be very informative for inferring (or not) a surface mediating role for the metabolites.

Having quantified whole plant chemistry and surface chemistry of *Delisea pulchra* and *Laurencia obtusa*, we can compare ecological roles of secondary metabolites from the 2 plants. Herbivores consume both internal and exterior parts of the thallus, and therefore would be expected to come into contact with metabolites from both species. Consistent with this observation, metabolites or extracts from both species appear to play a role as deterrents of feeding by herbivores. *D. pulchra* is a very low preference food of the generalist herbivores *Tripneustes gratilla* (Echinodermata) and *Turbo undulatus* (Mollusca), and long-term growth rates of *T. gratilla* fed monospecific diets of *D. pulchra* are less than those of individuals which are starved (Steinberg & van Altena 1992). Both crude extract and furanones from *D. pulchra* incorporated into artificial diets strongly deter feeding by *T. gratilla* and *T. undulatus* (de Nys et al. unpubl.). Similarly, the few studies done to date on the ecological role of secondary metabolites from *L. obtusa* indicate a feeding deterrent role for at least some of the metabolites against some herbivores (Hay et al. 1987, Paul et al. 1988). Palisadin A (although not aplysistatin and palisadin B) deterred feeding by the herbivorous fish *Zebrasoma flavescens* in laboratory assays and by mixed assemblages of herbivorous fishes in field assays (Paul et al. 1988), although it had no effect on feeding by the herbivorous fish *Siganus doliatus* (Hay et al. 1988). Other structurally similar metabolites from *L. obtusa* from other locations and from other species of *Laurencia* have strong deterrent effects against herbivores (e.g. elatol; Hay et al. 1987).

Furanones and metabolites from *Laurencia* spp. also deter the settlement and growth of ecologically relevant marine fouling organisms in both laboratory (de Nys et al. 1995, 1996, Steinberg et al. 1998) and, for furanones, field studies (Maximilien 1995, Hodson et al. 1997). While the levels of secondary metabolites present at the surface of *Delisea pulchra* are sufficient to deter ecologically relevant fouling organisms, for *L. obtusa* they are not. For example, in laboratory assays the most abundant metabolite on the surface of *D. pulchra*, compound 3 (Fig. 4 in de Nys et al. 1995), strongly deterred the settlement and growth of spores of the locally common epiphyte *Ulva* sp. at concentrations (25 ng cm^{-2}) less than those measured on the surface of the plant. In similar assays with metabolites from *L. obtusa* (Table 1 in Steinberg et al. 1998, de Nys et al. unpubl.), palisadin A and 5β-hydroxyaplysistatin significantly deterred the settlement of the epiphytic green alga *Ulva* sp. and the bryozoan *Bugula neretina* at concentrations of 0.1 to 1 μg cm^{-2} respectively, while palisol deterred *Ulva* sp. at 1 μg cm^{-2} and *B. neretina* at 10 μg cm^{-2}. However, these concentrations are 2 to 4 orders of magnitude greater than the concentration of these metabolites present on the surface of the plant. These studies highlight the importance of understanding the quantitative distribution of these compounds in order to determine their ecological role(s).

We believe the methods presented here provide a procedure by which non-polar crude extracts or pure metabolites from the surface of seaweeds (and potentially other organisms) can both be quantified and used to 'calibrate' realistic tests of the effects of metabolites on epibiota. This method may also be a useful tool in addressing the role of surface chemistry in other surface mediated interactions such as the chemical defence of marine larvae (Lindquist & Hay 1996) and for comparing the effects of surface-bound versus water soluble chemical cues on the settlement of algal propagules and invertebrate larvae (e.g. Walters et al. 1996, Jennings & Steinberg 1997).

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