THEME SECTION

Marine chemical ecology

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ABSTRACT: Marine chemical ecology has emerged as a vibrant subdiscipline of marine ecology over the past 20 yr. The first symposium devoted specifically to marine chemical ecology was held, in conjunction with the 29th Annual Benthic Ecology Meeting, at the University of North Carolina at Wilmington, USA, from March 9 to 10, 2000, with over 70 symposium participants from around the world. Topics of oral presentations and posters ranged from chemical antipredatory defenses of algae and invertebrates to antifouling, allelopathy, sunscreening agents, antimicrobial defenses, and chemical cues for larval settlement and metamorphosis. Articles in this MEPS Theme Section were submitted as original research contributions to the symposium. Abstracts of all symposium presentations are available at the Benthic Ecology Meeting Web site: http://www.uncwil.edu/cmsr/bem2000.

INTRODUCTION

For the past 4 decades, marine organisms have provided natural products chemists with a remarkable source of novel secondary metabolites (Faulkner 2000, and reviews cited therein). Because these compounds are often structurally complex, or present at high concentrations, chemists generally assumed that secondary metabolites provided some ecological function, often without any observational or experimental evidence. At the same time, ecologists were observing patterns of the distributions of marine organisms, or direct interactions of predators and prey or of competitors, and often assuming that chemistry played a specific role. Beginning in the 1980s collaborations began between chemists and ecologists. They resulted in an increasing number of studies in which up-to-date techniques of chemical isolation and identification were paired with ecologically relevant laboratory and field experiments. Since then, marine chemical ecology has developed rapidly, as evidenced by the number of pertinent reviews (e.g. Bakus et al. 1986, Hay & Fenical 1988, 1996, Hay & Steinberg 1992, Paul 1992, Pawlik 1992, 1993, Fenical 1993, Hay 1996, McClintock & Baker 1997).

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Geographic variation in halogenated furanones from the red alga *Delisea pulchra* and associated herbivores and epiphytes

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ABSTRACT: We examine patterns of quantitative variation in halogenated furanones, secondary metabolites of *Delisea pulchra* (Rhodophyta; Bonnemaisoniales), and how this relates to variation in local abundance of herbivores and epiphytes. Fifteen populations of *D. pulchra* covering a distance of 650 km of temperate southeastern Australia were sampled. Concentrations of the 4 main furanones in *D. pulchra* (Compounds 1 to 4) showed large variability but there was no latitudinal trend to this variation. We found significant variation in the concentrations of both total and individual furanones among locations, between summer and winter, and between different life-history stages. Importantly, the range of total furanone concentration among plants within locations was large, often varying by an order of magnitude or more. A total of 5 species of herbivorous fish, **7** species of macroinvertebrate grazer and 6 groups of mesograzer (of varying taxonomic level) were found at the 15 locations. The abundance of both total macroinvertebrate grazers and total mesograzers known to consume *D. pulchra* varied significantly among locations. However, correlations between furanones and the abundance of macroinvertebrate grazers and mesograzers at the scale of location were all weak and non-significant, as was the correlation between furanones and the abundance of epiphytes on *D. pulchra*. The large variability in concentrations of furanones, and the absence of any positive relationships between furanones, herbivores and epiphytes, suggest that quantitative variation in furanones in *D. pulchra* is not driven by population-level selection or induction, but is more likely to be a result of small-scale variation in environmental factors such as nutrients and light, and genetic differences among individual plants.

KEY WORDS: Australia · Chemical ecology · *Delisea pulchra* · Epiphytes · Furanones · Herbivory · Macroinvertebrate grazers · Mesograzers · Red algae · Secondary metabolites

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INTRODUCTION

A large number of terrestrial and marine plants produce secondary metabolites that provide a defence against natural enemies (e.g. Rosenthal & Berenbaum 1992). For terrestrial plants, patterns of quantitative variation in secondary metabolites within species are well documented and have provided a basis for understanding the ecology and evolution of host-plant resistance (Fritz & Simms 1992 and references therein). For marine algae, patterns of quantitative variation in secondary metabolites within species are, in general, poorly described. Much of the work examining variation in algal secondary metabolites has focussed on differences in the concentration of brown algal phlorotannins, which are structurally uncharacterised compounds, and therefore measured as total phlorotannins (reviews by Steinberg 1992, Targett & Arnold 1998). Furthermore,

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differences in total phlorotannins are often examined among species at large geographic scales (Steinberg 1989, Van Alstyne & Paul 1990, Steinberg & Van Altena 1992, Targett et al. 1992, Van Alstyne et al. 1999). For example, total phlorotannins in temperate Australasian brown algae occur generally at higher concentrations than total phlorotannins in temperate North American brown algae. Herbivory may also be more intense in temperate Australasia, leading to models proposing herbivores as an important evolutionary force driving high phlorotannin concentrations in brown algae from that region (Steinberg 1989, Steinberg & Van Altena 1992, Steinberg et al. 1995). Studies of brown algae where variation within species has been examined show that total phlorotannins can differ among populations separated by as little as 5 km and between the same populations sampled at different times (Steinberg 1989, Steinberg & Van Altena 1992, Pavia & Aberg 1996, Van Alstyne et al. 1999). The limited data available for individual non-polar secondary metabolites from marine algae reveal that similar levels of variation can occur over the same spatial and temporal scales (Cronin & Hay 1996c, Puglisi & Paul 1997).

Descriptions of quantitative variation in algal secondary metabolites within species are important; this is the variation on which evolution acts (Hay & Steinberg 1992, Hay 1996). Concentrations of algal secondary metabolites can vary as a function of environmental factors such as nutrients, light and desiccation (Yates & Peckol 1993, Arnold et al. 1995, Cronin & Hay 1996a,b, Pavia et al. 1997) and induction by herbivores (Van Alstyne 1988, Cronin & Hay 1996c) but it is not known whether such variation could also be due to genetic factors. Quantitative variation has important consequences because chemical deterrence of marine herbivores and epiphytes is usually concentration dependent (e.g. Hay et al. 1987, Steinberg 1988, de Nys et al. 1995, Schmitt et al. 1995). Thus, studies describing patterns of variation in algal secondary metabolites, herbivores and epiphytes will provide important background information that will contribute to the current understanding of chemical resistance in marine algae.

In this paper we examine, at the population level, the relationship between quantitative variation in the secondary metabolites of the red alga *Delisea pulchra* and variation in the abundance of co-occurring herbivores and epiphytes. *D. pulchra* produces non-polar secondary metabolites (furanones; de Nys et al. 1992, 1993) that deter herbivores (Wright et al. unpubl.), deter settlement of epiphytes (de Nys et al. 1995) and reduce attachment of bacteria (Maximilien et al. 1998). Importantly, the activity of the furanones against epiphytes and bacteria varies both with compound and concentration (de Nys et al. 1995, Maximilien et al. 1998). In this study, we (1) document the extent of spatial and

temporal variation in concentrations of furanones in *D. pulchra* among 15 populations covering a distance of 650 km of temperate southeastern Australia, (2) document variation in the abundance of herbivores (macro- and mesograzers) and epiphytes in shallow (< 4 m) subtidal reefs where *D. pulchra* occurred at each of the 15 locations, (3) examine correlations between furanones and the abundance of herbivores and epiphytes, and (4) examine variation in the abundance of the different life-history stages of *D. pulchra* at each of the locations and determine whether concentrations of furanones differed between life-history stages.

MATERIALS AND METHODS

Natural history and secondary metabolites of *Delisea pulchra*. *Delisea pulchra* (Greville) Montagne (Bonnemaisoniales, Rhodophyta) is a medium-sized (typically 150 mm in height) shallow subtidal alga, occurring in waters throughout southern Australia, the Antarctic Peninsula and various Subantarctic Islands (Ricker 1987, Bonin & Hawkes 1988, Millar 1990). In southeastern Australia it is one of the more common foliose algae in the sublittoral zone (Millar 1990). It has been described as having a triphasic life history typical of algae in the Bonnemaisoniales, with free-living haploid (gametophytes) and diploid (tetrasporophyte) stages and a further diploid stage (carposporophyte) that remains attached to the female gametophyte (Bonin & Hawkes 1988, Womersley 1996). Gametophytes can be either monoecious or dioecious and are isomorphic with tetrasporophytes (Bonin & Hawkes 1988, Womersley 1996). *D. pulchra* contains a range of halogenated furanones, of which 4 make up approximately 95% of the total amount of furanones in the alga (Fig. 1; de Nys et al. 1992, 1993).

Study locations. *Delisea pulchra* on the southeastern Australian coast was sampled in August 1994 (hereafter winter 1994) and January/February 1995 (hereafter summer 1995). In winter 1994, we sampled *D. pulchra* at 9 locations from Nobby's Beach in the north (32° 55′ S, 151° 47′ E) to Kiama in the south (34° 40′ S, 150° 51′ E; Fig. 2). These initial surveys examined vari-

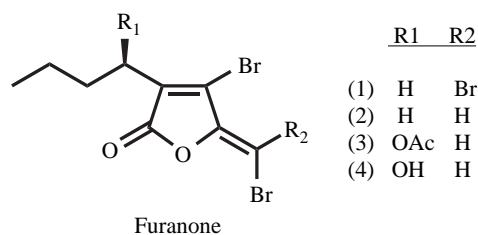|	R1	R2
(1)	H	Br
(2)	H	H
(3)	OAc	H
(4)	OH	H

Fig. 1. Structure of the major secondary metabolites of *Delisea pulchra* (Compounds 1 to 4)
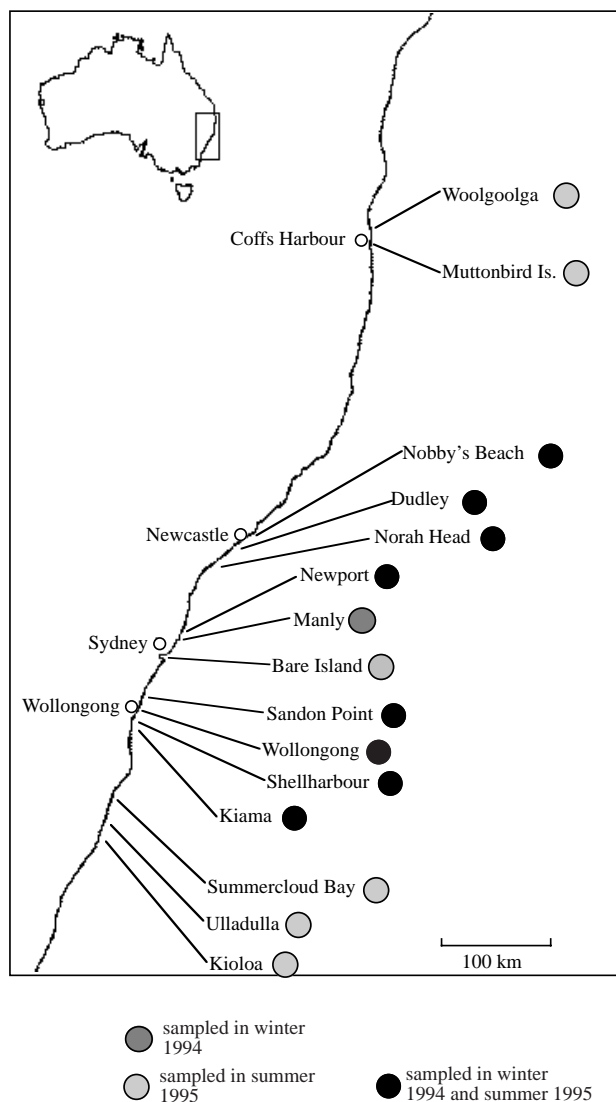
Fig. 2. Locations sampled for variation in life history, secondary metabolites, herbivores and epiphytes

ation among locations in (1) concentrations of the 4 main furanones and (2) the proportion of different life-history stages in each population. In summer 1995, we sampled those 9 locations again (except Manly, where no plants could be found) and also a further 6 locations, extending the distance covered in the survey to 650 km from Woolgoolga in the north (30° 07′ S, 153° 12′ E) to Kioloa in the south (35° 33′ S, 150° 22′ E). Although all the locations sampled occur within a single biogeo-graphical region (Womersley 1990), sea temperature over that distance decreases from north to south, rang-ing from a mean summer temperature of 25°C in the most northerly locations (Woolgoolga and Coffs Har-bour) to 21°C in the most southerly locations (Jeffrey et al. 1990). The relative temperature differences are sim-

ilar in winter and relate to the movement of the East Australian Current, which flows south down the east Australian coast until 33° S (near Sydney) where it moves away from the continent in a southeast direction (Jeffrey et al. 1990). The summer 1995 surveys asses-sed variation among locations in (1) concentrations of the 4 main furanones, (2) density of herbivores (fish, macroinvertebrates and mesograzers), (3) epiphyte loads on *D. pulchra*, and (4) the proportion of different life-history stages. There were differences among the loca-tions in terms of their orientation, exposure to waves etc. and also in the densities of *D. pulchra*, which ranged from 0.2 to 43.2 plants m^{-2}. However, there were no obvious morphological differences in *D. pulchra* at the different locations and sampling at all locations was done at approximately the same depth, 2 to 4 m.

Furanone extraction and gas chromatography-mass spectrometry. The concentrations of the 4 main fura-nones in *Delisea pulchra* was determined using gas chromatography-mass spectrometry (GC-MS) follow-ing the methods of de Nys et al. (1996). Eight plants in winter 1994 and 10 plants in summer 1995 were hap-hazardly collected from each location, placed into indi-vidual plastic bags and frozen at −20°C. These plants were then freeze dried, ground to a powder, and 100 mg (dry weight) of tissue from each plant extracted 5 times with 2 ml of dichloromethane. The 5 combined extracts were filtered (0.2 µm pore size), dried and redissolved in 1 ml ethyl acetate containing naphthalene (10 µg ml^{-1}) as an internal standard prior to analysis with GC-MS. Furanones are stable at room temperature under normal light conditions and thus our collection and handling was unlikely to have had any effect on their calculated concentrations.

Gas chromatography was performed with a Hewlett Packard (HP) 5890 Series II gas chromatograph (GC) and a polyamide-coated fused-silica capillary column (BP5, 12 m long, 0.22 mm i.d., 0.25 µm 5% phenyl [equiv.] polysilphenylene-siloxane stationary phase, SGE Pty Ltd). All injections were performed in the splitless mode with an inlet pressure of 3 psi. Glass wool (5 mm^{-2}) was inserted into the injection port liner. The injection port was held at 280°C and the interface at 300°C. The GC was held at 50°C for 1.5 min, ramped at 20°C min^{-1} to 250°C, then ramped at 50°C min^{-1} to 320°C, where it was held for 4 min. Helium was used as the carrier gas. Mass spectrometry was performed with a HP 5971 mass-selective detector (MSD). Ions characteristic of the in-ternal standard and Compounds 1 to 4 were monitored in the selective-ion monitoring mode. Standards used in the GC-MS analysis were isolated from dichloromethane extract of freeze-dried *Delisea pulchra*. Metabolites were identified by comparison of ^{1}H and ^{13}C nuclear magnetic resonance (NMR) data (de Nys et al. 1993). Compounds 1 to 4 were quantified by measuring the

peak areas for each compound and the internal standard. The ratio of peak areas (compound:internal standard) was calculated for each metabolite and converted to concentration by reference to standard curves.

Abundance of herbivores and epiphytes. The density of all herbivores (fishes, macroinvertebrates, mesograzers known to consume *Delisea pulchra*, and all other mesograzers) was determined at each of the 14 locations (except for Woolgoolga) in summer 1995 at the same time and place as *D. pulchra* was collected. Herbivorous fish were counted at each location by establishing three 20 m transects and recording all herbivorous fish seen during a swim along each transect. The accuracy of this method is dependent on water visibility and the chance that mobile species (e.g. *Girella tricuspudata*) are in the area at the time of the survey. As we cannot be sure that visibility was consistent at all locations, or that fish present at each location were always counted in the surveys, the data for fish are treated qualitatively. In southeastern Australia, the kelp specialist *Odax cyanomelas* can clear patches of *Ecklonia radiata* (Andrew & Jones 1990; see also Choat 1982, Choat & Clements 1992). To our knowledge, no fish have ever been recorded consuming *D. pulchra* and we too did not observe this. Macroinvertebrate herbivores (sea urchins and large gastropods) were counted at each location by setting up three 10×10 m sites and randomly sampling ten 0.5×0.5 m quadrats within each site. Although macrograzers do consume *D. pulchra*, it is a very low preference food for them (Steinberg & Van Altena 1992). Mesograzers are small invertebrate herbivores less than 2.5 cm in length, and can include juveniles of some larger species (Brawley 1992). The abundance of mesograzers on *D. pulchra* was examined on 10 haphazardly collected plants at each location. These plants were removed from the substratum and placed into individual plastic bags, which were immediately sealed. On shore, the entire contents of each bag were poured into a plastic jar containing 5% formaldehyde in seawater. In the laboratory, the contents of each jar were poured through a 300 µm sieve and the plants washed several times with fresh water to remove all animals. This technique removes 94 to 98% of all mesograzers (Poore & Steinberg 1999). Individual mesograzer species (echinoids, gastropods, isopods; see Table 5) or herbivorous taxa (polychaetes, amphipods, insects; Brawley 1992) were identified, counted, oven dried at 100°C for 24 h and weighed. Mesograzers known to consume *D. pulchra* had been previously determined by either feeding experiments (Rogers et al. 1995) or gut content analysis of field-collected animals (Wright unpubl.). We expressed the abundance of mesograzers on plants at each location in 2 ways: number of animals g^{-1} plant, and dry mass (mg) of animals g^{-1} plant.

Epiphyte load on *Delisea pulchra* was considered to be the total biomass of epiphytes per plant (mg g^{-1} dry weight) and was measured by removing epiphytes from plants with forceps, drying them at 100°C for 24 h, and weighing them. Ten plants were sampled at each location.

Proportion of life-history stages. The life-history stages of *Delisea pulchra* were identified visually by the presence of different reproductive structures. At most locations there were some small plants with no reproductive structures and these were designated as non-reproductive. To determine the proportion of the different life-history stages in winter 1994, 50 plants were haphazardly collected from each location (except Norah Head, where only 3 plants were found). In summer 1995, 30 to 50 plants were collected from the 6 locations that had not been sampled in winter 1994 (except Woolgoolga, where only 7 plants were found), and 9 to 10 plants were collected from locations that were sampled in winter 1994 (except Nobby's Beach and Kiama, where 49 and 44 plants were collected, respectively). Plants were removed from the substratum, placed into individual plastic bags and examined for reproductive structures back in the laboratory.

Statistical analyses. Statistical analyses were carried out using SYSTAT (Wilkinson 1997). Analyses of variance (ANOVAs) were checked for normality and heterogeneity of variance using frequency histograms of residuals and plots of residuals versus means, respectively. Logarithmic or arcsine transformations were performed as appropriate and are shown in the relevant table legends. Following ANOVAs, post-hoc tests (Tukey's multiple range) were performed where required.

Spatial patterns of quantitative variation in the 4 main furanones of *Delisea pulchra* were analysed in 3 ways. First, we compared the total concentration of furanones in plants between locations using ANOVA. Second, we compared the concentrations of the individual compounds using a 3-factor ANOVA with the factors location, compounds and plants (blocked factor). Plant was included as a blocked factor as it is possible that individual secondary metabolites within a plant are not independent of each other (Pennings & Paul 1993, de Nys et al. 1996). These 2 analyses were done separately for both winter 1994 and summer 1995 samples. Finally, for the 8 locations sampled in both winter 1994 and summer 1995, we performed analyses that included the additional factor of time and determined whether the concentration of total or individual furanones differed in plants from these locations collected at the 2 times.

To determine whether the concentrations of secondary metabolites differed between life-history stages (haploid vs diploid) and reproductive status (reproductive or non-reproductive), we determined the concentration of total

and individual furanones in reproductive diploid and haploid plants, and non-reproductive plants, from the one site where gametophytes occurred (Nobby's Beach). In winter 1994, we compared furanones in 8 tetrasporophytes and 5 female gametophytes. In summer 1995, we compared furanones in 5 tetrasporophytes, 5 female gametophytes and 5 non-reproductive plants. No male gametophytes were found at any locations in either season. The total concentration of furanones in the different life-history stages was analysed with an unpaired *t*-test in winter 1994 and a 1-factor ANOVA in summer 1995. Concentrations of the individual furanones in the different life-history stages were analysed at both times using a 3-factor ANOVA with the factors life-history stage, compounds and plants (blocked factor).

For herbivores, the main aim was to compare the relative abundance of macrograzers and mesograzers known to consume *Delisea pulchra* among locations, and use this as an estimate of grazing pressure. To examine variation in the abundance of macrograzers, we pooled all sea urchins and large herbivorous gastropods and determined whether there was variation in the total density of these species among locations using a nested ANOVA with the factors location and sites nested within location. Two estimates of mesograzer abundance were examined: the total number of mesograzers which consume *D. pulchra* per plant (standardised for plant dry weight) and the total biomass of these species per plant (mg mesograzers g^{-1} dry weight). For both of these measures, 1-factor ANOVAs were performed to test for differences among locations.

The total mass of epiphytes per plant (mg epiphytes g^{-1} dry weight) was analysed with a 1-factor ANOVA following an arcsine transformation of the data.

To examine relationships between furanones and the abundance of herbivores and epiphytes, correlations were performed between total furanones and the abundance of macroinvertebrates, the abundance and biomass of mesograzers, and the biomass of epiphytes.

RESULTS

Spatial patterns of variation in secondary metabolites

The total concentration of furanones differed significantly in plants from different locations in both winter 1994 (1-factor ANOVA; $F_{8, 58} = 4.945$, p < 0.001) and summer 1995 (1-factor ANOVA; $F_{13, 122} = 8.249$, p < 0.001; Fig. 3). In winter 1994, mean total concentrations ranged from 3.7 mg g^{-1} at Wollongong to 26.1 mg g^{-1} at Norah Head. In summer 1995, mean concentrations were generally much lower and ranged from 1.53 mg g^{-1} at Ulladulla to 8.19 mg g^{-1} at Dudley. For the 8 locations sampled in both times, plants collected in winter 1994 had a
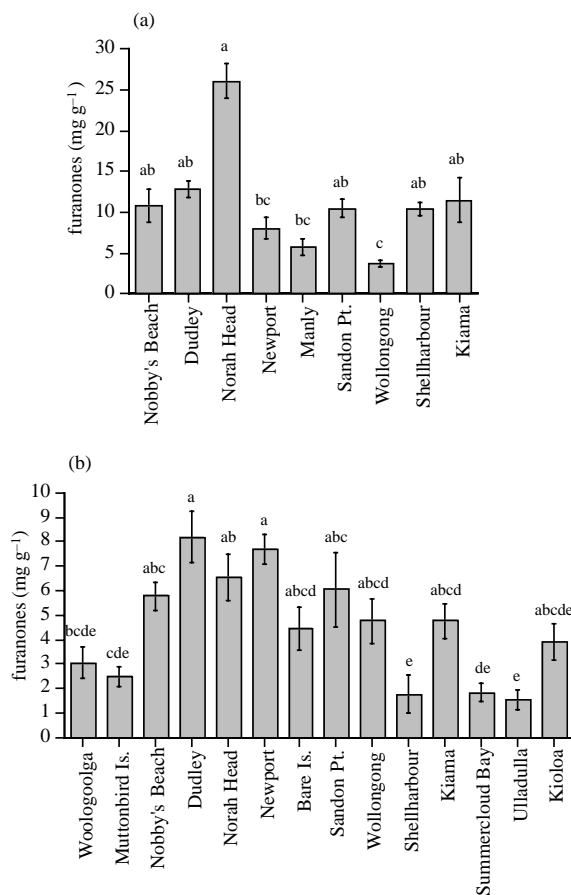

Fig. 3. Total concentration (mean ± 1 SE) of furanones in plants collected from each location in (a) winter 1994 (n = 8) and (b) summer 1995 (n = 10). Locations sharing the same letter do not differ at p = 0.05 (Tukey's analysis). In this and subsequent figures, sites are arranged from north (left) to south (right)

significantly higher concentration of total furanones compared to summer 1995 (Table 1). However, there was a significant time × location interaction (Table 1), indicating that this result was not consistent across all locations. Plants at Wollongong had higher total concentrations in summer 1995 than in winter 1994. There was no correlation between winter 1994 and summer 1995 in the mean total concentration of furanones for the 8 locations sampled at both times (r = 0.221, p = 0.599).

Table 1. ANOVAs examining the effect of time on total furanones for the 8 locations sampled in both summer 1994 and winter 1995. Data log(*x* + 1) transformed

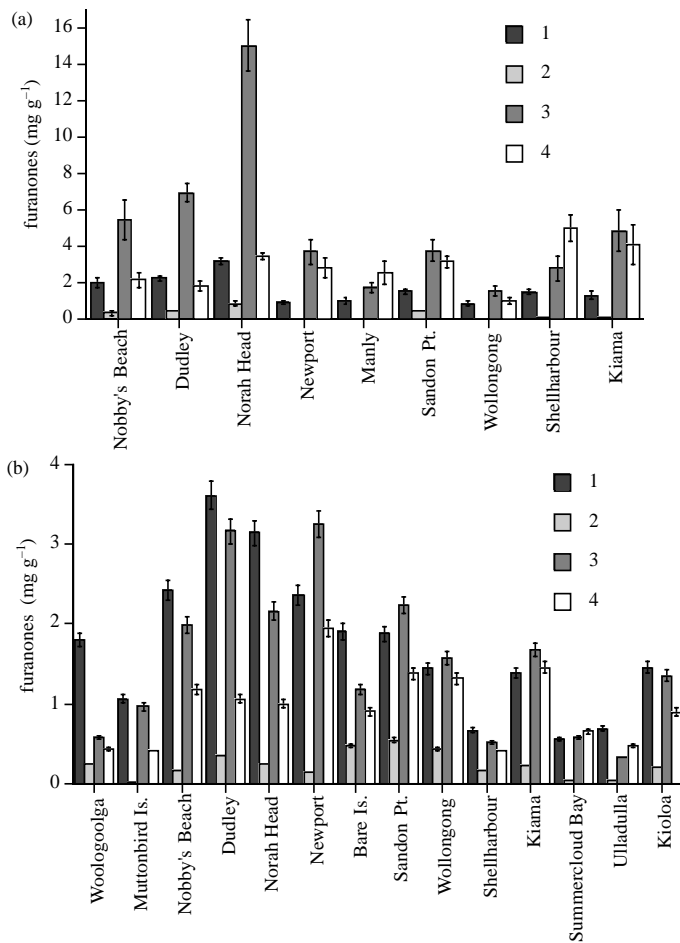| Source	df	MS	*F*	p
Location	7	1.900	1.044	0.478
Time	1	10.946	6.016	0.044
Location × Time	7	1.819	6.313	<0.001
Error	122	0.288		

(a)

(b)

Fig. 4. Concentrations of furanones 1 to 4 (mean ± 1 SE) in plants collected from each location in (a) winter 1994 (n = 8) and (b) summer 1995 (n = 10)

The range of concentrations of total furanones among plants within locations was large (Table 2). Often there was an order of magnitude difference between the plants having the lowest and highest concentrations. This was consistent across seasons, although it was more marked in summer 1995 than winter 1994.

Concentrations of the individual furanones also varied significantly among locations in both winter 1994 and summer 1995 (Fig. 4, Table 3). However, in both times, these differences among locations varied with compound (significant location × compound interactions for both seasons; Table 3). A posteriori tests conducted separately for each compound within each season revealed that there were significant differences in the concentrations of all 4 compounds among locations (Tukey's $\alpha < 0.05$). In general, the patterns of variation for individual furanones tracked the variation among locations for total furanones (Figs. 3 & 4). In winter 1994, Compound 3 had the highest concentration in plants at 7 out of 9 locations (Fig. 4a). At the other 2

locations (Manly and Shellharbour), Compound 4 had the highest concentration. In summer 1995, Compound 1 had the highest concentration in plants from 9 out of the 14 locations (Fig. 4b). At 4 other locations, Compound 3 was at the highest concentration while at one location (Summercloud Bay), Compound 4 was most abundant. In both seasons, Compound 2 generally occurred at lower concentrations than the other 3 compounds.

The analysis including the factor time for the 8 locations sampled in both winter 1994 and summer 1995 emphasised the complex patterns of variation in individual compounds (Table 4). The change in individual compounds between seasons was not consistent across compounds (significant compound × time interaction). Moreover, the response of individual compounds depended not only on season, but also on location (significant compound × location and time × location × compound interactions; Table 4). As with the total concentrations, there was also a significant location × time interaction.

Abundance of herbivores and epiphytes

Species of herbivorous fish recorded in beds of *Delisea pulchra* were *Parma microlepis*, *P. unifasciata*, *Girella tricuspudata*, *Crinodus lophodon*, *Odax cyanomelas* and also unidentified monocanthids (which were most likely omnivorous; Table 5).

Four species of sea urchin (*Heliocidaris erythrograma*, *H. tuberculata*, *Holopneustes purpurascens*, *Centrostephanus rodgersii*) were present in the surveys. All of these species were reasonably widespread, but only *H. erythrograma* (at Bare Island, Summer-

Table 2. Range of concentrations of total furanones (mg g^{-1} dry weight) in plants within each location in winter 1994 and summer 1995. n = number of plants; ns = no sample taken

Location	n	Winter 1994	n	Summer 1995
Woolgoolga	–	ns	8	0.52 – 5.17
Muttonbird Island	–	ns	10	0.99 – 4.20
Nobby's Beach	8	1.02 – 18.25	10	3.76 – 8.60
Dudley	8	8.69 – 15.86	10	1.97 – 13.41
Norah Head	3	22.78 – 30.16	10	3.37 – 13.09
Newport	7	2.62 – 11.76	10	5.33 – 10.43
Manly	8	2.27 – 10.41	–	ns
Bare Island	–	ns	9	0.80 – 8.18
Sandon Point	8	4.13 – 14.56	9	0.99 – 13.73
Wollongong	8	0.59 – 4.74	10	0.32 – 9.4
Shellharbour	8	6.51 – 13.23	10	0.05 – 7.13
Kiama	8	1.14 – 22.05	10	1.14 – 7.96
Summercloud Bay	–	ns	10	0.31 – 3.48
Ulladulla	–	ns	10	0.30 – 3.19
Kioloa	–	ns	10	0.76 – 8.19

Table 3. ANOVAs for individual furanones (Compounds 1 to 4) in winter 1994 and summer 1995. Data log(x + 1) transformed for both analyses

Source	df	MS	F	p
Winter 1994				
Location	8	1.656	15.249	<0.001
Compound	3	19.943	46.570	<0.001
Plant (block)	7	0.308	2.833	0.008
Location × Compound	24	0.428	3.944	<0.001
Error	221	0.109		
Summer 1995				
Location	13	2.069	23.000	<0.001
Compound	3	15.637	48.103	<0.001
Plant (block)	9	0.410	4.562	<0.001
Location × Compound	39	0.325	3.613	<0.001
Error	479	0.09		

Table 4. ANOVA examining the effect of time on individual furanones (Compounds 1 to 4) for the 8 locations sampled in both summer 1994 and winter 1995. Data log(x + 1) transformed

Source	df	MS	F	p
Location	7	2.074	1.224	0.398
Compound	3	27.703	5.928	0.073
Time	1	7.163	4.228	0.079
Plant (block)	9	0.403	3.768	<0.001
Location × Compound	21	0.583	2.320	0.030
Location × Time	7	1.694	15.820	<0.001
Compound × Time	3	4.341	17.274	<0.001
Location × Compound × Time	21	0.251	2.347	0.001
Error	475	0.107		

cloud Bay and Kioloa) and *H. purpurascens* (at Bare Island) reached densities above 5 ind. m^{-2} (Fig. 5). Three species of herbivorous gastropods (*Turbo torquatus*, *T. undulatus*, *Astralium tentoriformis*) were recorded in *D. pulchra* beds. Although *T. torquata* was widespread, occurring at 11 locations, it was generally in low densities (~1 ind. m^{-2}; Fig. 5). Only 1 *T. undulatus* was found at Dudley.

The total density of macroinvertebrate herbivores (*Astralium tentoriformis* was excluded as it is not known to consume macroalgae) differed significantly among locations ($F_{12, 24}$ = 10.943, p < 0.001). Some locations, such as Bare Island and Kioloa, had very high densities, while 2 locations, Muttonbird Island and Ulladulla, had no macroinvertebrates (Fig. 5). Bare Island, Kioloa and Summercloud Bay had significantly higher densities of macroinvertebrates than all other locations (Tukey's α < 0.05) and, to a large extent, this was due to high densities of *Heliocidaris erythrograma*. The nested ANOVA also revealed significant variation among sites within locations ($F_{24, 332}$ = 4.009, p < 0.001), indicating small-scale variation in macroinvertebrate densities.

Table 5. Herbivores co-occurring with *Delisea pulchra* at 13 locations on the southeastern Australian coast

Taxon/species	No. of locations present
Fishes	
Pomacentridae	
Parma spp. (*P. microlepis* and *P. unifasciata*)	8
Girellidae	
Girella tricuspudata	4
Aplodactylidae	
Crinodus lophodon	12
Odacidae	
Odax cyanomelas	8
Monocanthidae	3
Macro-invertebrates	
Echinoidea	
Heliocidaris erythrograma	7
Heliocidaris tuberculata	6
Holopneustes purpurascens	8
Centrostephanus rodgersii	9
Gastropoda	
Turbo torquatus	11
Turbo undulatus	1
Astralium tentoriformis	10
Mesograzers	
D. pulchra feeders	
Echinoidea	
Holopneustes purpurascens	5
Gastropoda	
Phasianotrochus eximius	13
Aplysia parvula	8
Polychaeta: Nereidae	13
Non *D. pulchra* feeders	
Amphipoda	
Ampithoidae	13
Hyalidae	12
Aoridae	12
Eusiridae	12
Isopoda	
Paridotea spp. (*P. munda* and *P. collingei*)	4
Insecta	
Trichoptera	2

The most widespread mesograzers were the gastropod *Phasianotrochus eximius*, nereid polychaetes, and ampithoid amphipods, which were recorded at all 13 locations (Table 5). Three other families of known herbivorous amphipods; Hyalidae, Aoridae and Eusiridae were also widespread (Table 5) and were often abundant (Figs. 6 & 7). Other mesograzers that were common, although less widespread, were juvenile *Holopneustes purpurascens*, the sea hare *Aplysia parvula*, isopods *Paridotea munda* and *P. collingei*, and caddis fly larvae (Trichopterans). Although *H. purpurascens* was recorded as a macroinvertebrate, juveniles of this species also occurred as mesograzers (see definition of mesograzers in 'Materials and methods') on *Delisea pulchra* and were included in the counts of mesograzer abundance. However, juvenile *H. purpurascens* are
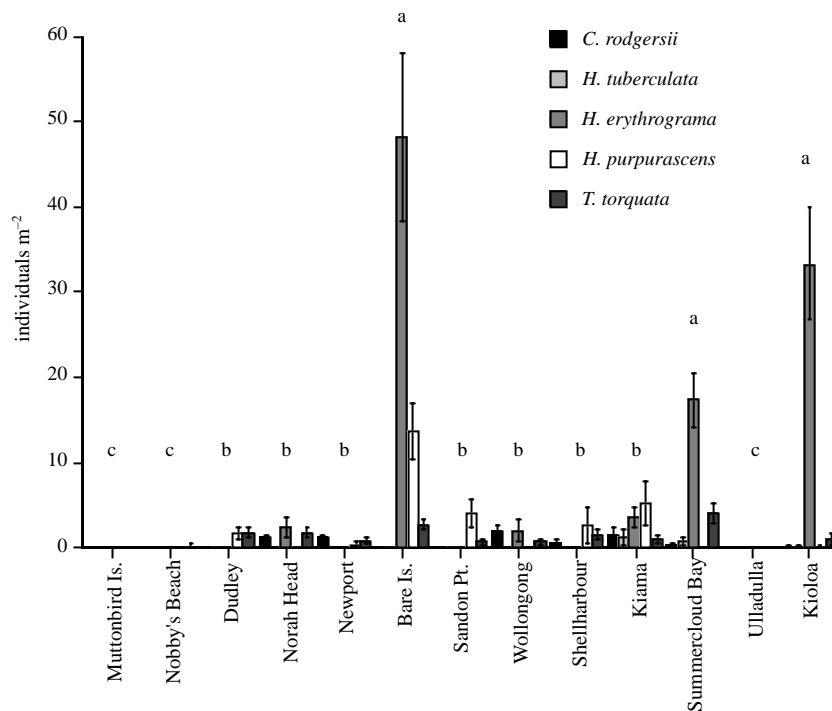
Fig. 5. Density of macroinvertebrate herbivores (mean ± 1 SE) at each location in summer 1995. One *Turbo undulatus* was found at Dudley (not shown). Locations sharing the same letter do not have a different total density of macroinvertebrates at p = 0.05 (Tukey's analysis)

much heavier than any other mesograzers, and including them in the biomass analysis greatly biased the results to those locations where they occurred. Therefore, they were excluded from the analysis comparing total biomass of mesograzers among locations.

The total number of mesograzers known to consume *Delisea pulchra* (Table 5) differed significantly among locations (1-factor ANOVA; $F_{12,116}$ = 6.241, p < 0.001) and ranged from 0.17 ind. g^{-1} of plant at Norah Head to 5.1 ind. g^{-1} of plant at Sandon Point (Fig. 6). Similarly, the total biomass of these mesograzers also differed significantly among locations (1-factor ANOVA; $F_{12,116}$ = 6.117, p < 0.001) and, in general, followed the same pattern as their total number (Fig. 7).

The most common epiphytes were foliose or filamentous species such as *Ulva* spp. and *Enteromorpha* spp., and *Colpomenia sinuosa*. The total epiphyte load differed significantly on plants from the different locations (1-factor ANOVA; $F_{12,114}$ = 2.179, p = 0.017; Fig. 8). Mean total mass of epiphytes ranged from zero at Muttonbird Island to 106 mg g^{-1} (10.6% of dry weight of plants) at Shellharbour.

Correlations between secondary metabolites, herbivores and epiphytes

The correlations between the mean total concentrations of furanones in plants from the different locations and the mean abundance of herbivores (macrograzers or mesograzers known to consume *Delisea pulchra*) or

epiphyte loads were all weak and non-significant (Fig. 9). Correlations between individual compounds and herbivores and epiphytes were all similarly weak and non-significant (r^2 ranged from 0 to 0.085).

Proportion of life-history stages

Populations of *Delisea pulchra*, with 1 exception, consisted entirely of tetrasporophytes or non-reproductive plants (Fig. 10). Often tetrasporophytes made up 100% of the plants present in the populations. Female gametophytes were found at only 1 of the 15 locations, Nobby's Beach, where they represented a small percentage of plants at both sampling times (10% in winter 1994 and 19% in summer 1995). No male gametophytes were found at any locations in either season.

Variation in secondary metabolites between life-history stages

In winter 1994, there was no difference in the concentrations of total furanones in tetrasporophyte and gametophyte plants from Nobby's Beach (Student's t_{11} = −0.345, p = 0.737; Fig 11a). There was also no difference between tetrasporophyte and gametophyte plants in the concentrations of individual furanones in winter 1994, although this was only marginally non-significant (Table 6). In summer 1995, tetrasporophytes had a sig-
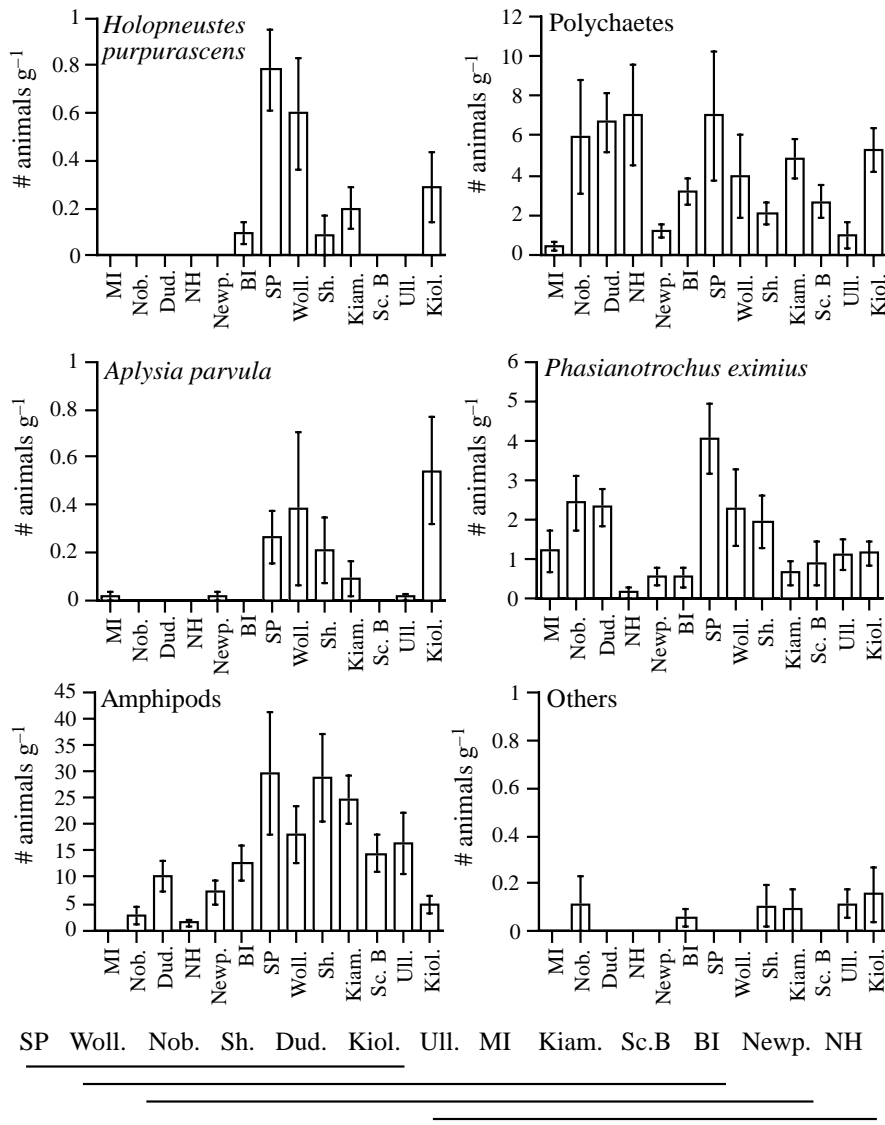
Fig. 6. Density of mesograzers (mean number of animals g^{-1} plant ± 1 SE) at each location in summer 1995. Results of Tukey's analysis on the total number of *Delisea pulchra* feeding mesograzers (see Table 4) are shown at the bottom. Locations that share a line do not differ at p = 0.05. Abbreviations: Muttonbird Island (MI), Nobby's Beach (Nob.), Dudley (Dud.), Norah Head (NH), Newport (Newp.), Bare Island (BI), Sandon Point (SP), Wollongong (Woll.), Shellharbour (Sh.), Kiama (Kiam.), Summercloud Bay (Sc B), Ulladulla (Ull.), and Kioloa (Kiol.)

nificantly higher concentration of both total and individual furanones than female gametophytes and non-reproductive plants (Tukey's α < 0.05; Table 7, Fig. 11b). Individual compounds also differed in concentration, but this pattern did not vary among the different life-history stages (non-significant life history × compound interaction; Table 7).

DISCUSSION

Patterns of variation in furanones, herbivores, epiphytes and life-history stages

Furanones produced by *Delisea pulchra* varied significantly with space (among locations), time (winter vs summer) and life-history stage (diploid vs haploid),

demonstrating a complex pattern of variation for the secondary metabolites of this alga. The range of furanones among plants within locations was large, often varying by an order of magnitude. Both the total and individual concentrations of furanones varied among locations in winter 1994 and summer 1995, but there was no latitudinal trend to this variation. This result for furanones in *D. pulchra* is similar to the absence of a latitudinal trend to the variation in phlorotannins in kelps and fucoids in the northeastern Pacific (Van Alstyne et al. 1999). A previous study of *D. pulchra* showed no significant difference in the concentrations of furanones in plants from 2 locations 10 km apart (de Nys et al. 1996). However, other macroalgae have shown among-site variation in concentrations of total phlorotannins (Steinberg 1989, Steinberg & Van Altena 1992, Pavia & Aberg 1996, Van Alstyne et al.
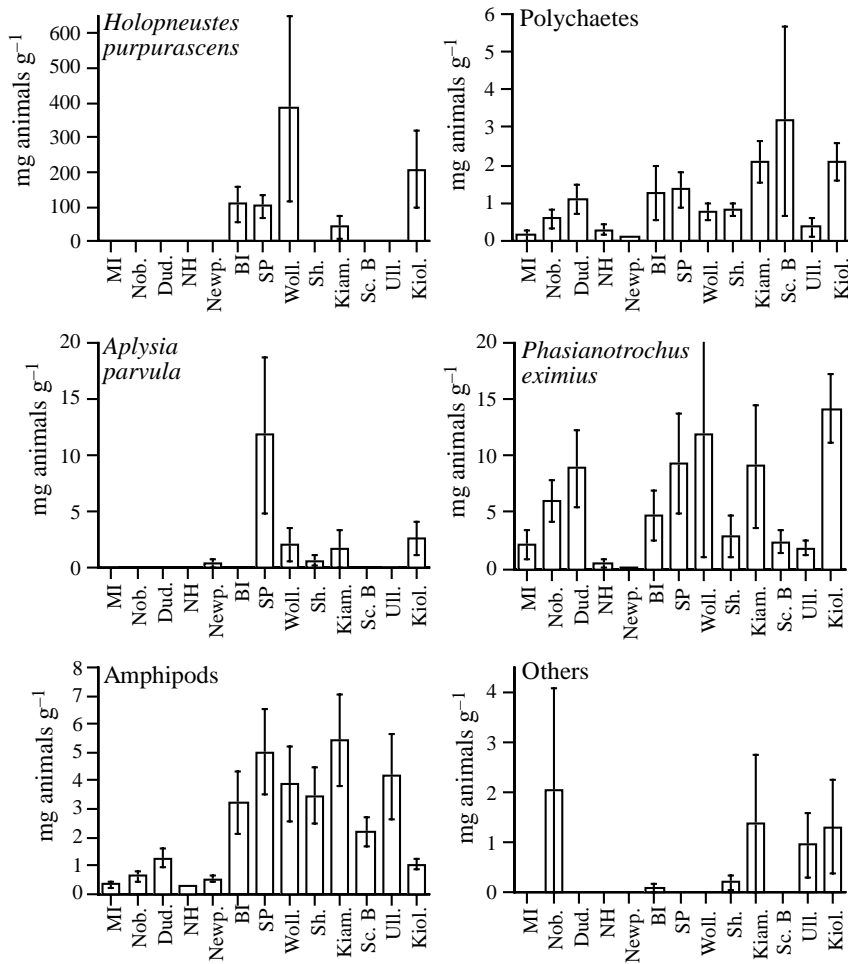
Fig. 7. Mass of mesograzers (mean mg mesograzers g⁻¹ plant ± 1 SE) at each location in summer 1995. Results of Tukey's analysis on the total mass of *Delisea pulchra* feeding mesograzers (Table 4) are shown at the bottom. Locations that share a line do not differ at p = 0.05. Location abbreviations as in Fig. 6
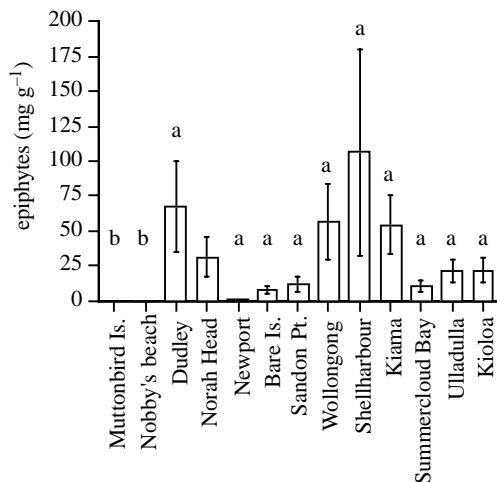

Fig. 8. Mass of epiphytes (mean mg g⁻¹ plant ± 1 SE) at each location in summer 1995. Locations sharing the same letter do not differ at p = 0.05 (Tukey's analysis)

1999) and non-polar secondary metabolites (Cronin & Hay 1996c, Puglisi & Paul 1997). The total concentration of furanones also varied with time, with levels generally being lower in summer, although this varied with location (Fig. 3). Temporal variation in secondary metabolites has also been observed in other macroalgae (Steinberg 1989, Steinberg & Van Altena 1992, Yates & Peckol 1993, Cronin & Hay 1996c, Peckol et al. 1996).

Concentrations of the 4 individual compounds differed within plants in both winter 1994 and summer 1995, but as with total furanones, these differences were not consistent across locations or time (see significant interactions between compound, location and time; Table 3). At different locations and/or times, Compounds 3, 4 and 1 were the most abundant. This complex and variable pattern of furanone production in *Delisea pulchra* suggests that variation may be a response to location- and time-specific factors. It also
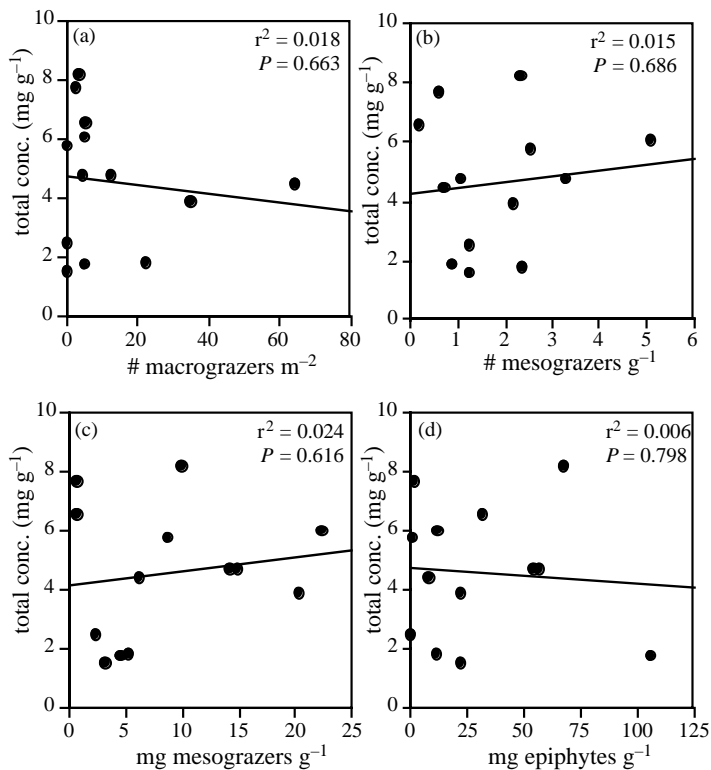
Fig. 9. Relationships between total furanones and (a) density of macroinvertebrate herbivores, (b) density of mesograzers known to consume *Delisea pulchra*, (c) biomass of mesograzers known to consume *D. pulchra*, and (d) epiphyte load at 13 locations in summer 1995

indicates that studies examining patterns of variation in algal secondary metabolites need to be adequately replicated in space and time to ensure that the sampling provides an accurate examination.

The abundance of macroinvertebrates, mesograzers and epiphytes differed significantly among locations, but also with no evidence of a latitudinal trend. The abundance of macrograzers also varied significantly among sites within locations, indicating small-scale patchiness in their abundance. Small-scale patchiness in the abundance of temperate marine herbivores is well documented (e.g. Underwood et al. 1991). The large sea urchin *Centrostephanus rodgersii* is generally considered the most important subtidal grazer in southeastern Australia (Fletcher 1987, Andrew 1993). However, it is found deeper than where *Delisea pulchra* is most abundant and was rare or absent at the locations sampled here. The 3 most common macrograzers that co-occurred with *D. pulchra* (*Heliocidaris erythrograma*, *Holopneustes purpurascens*, *Turbo torquatus*) consume *D. pulchra*; however, their impact on it is probably minimal as it is a low-preference food for all of them (Wright et al. unpubl.). *D. pulchra* is also a low-preference food for 2 other less common herbivores: the
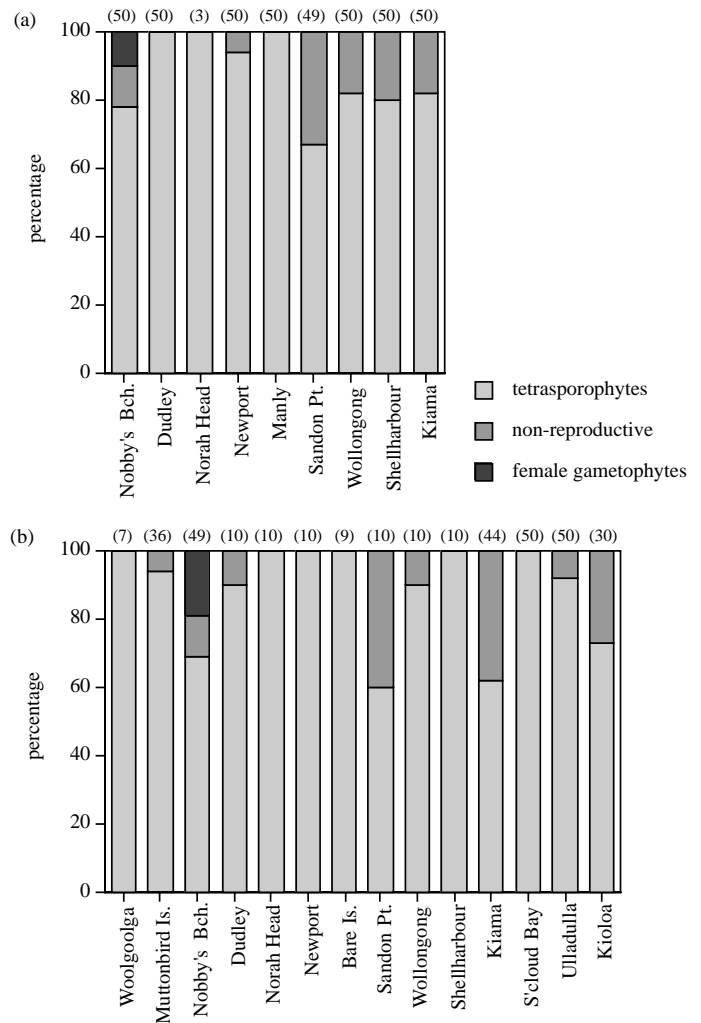

Fig. 10. Percentage of different life-history stages (tetrasporophytes, non-reproductive plants and female gametophytes) of *Delisea pulchra* found at each location in (a) winter 1994 and (b) summer 1995. The number of plants sampled at each location in each survey is shown in brackets

gastropod *Turbo undulatus* and the sea urchin *Tripneustes gratilla* (Steinberg & Van Altena 1992).

The most abundant mesograzers found in this study were amphipods (Fig. 6). No species of amphipod has been recorded as consuming *Delisea pulchra* in southeastern Australia (A. Poore pers. comm.) and those found were most likely consuming epiphytes. At least 1 species of nereid polychaete is known to consume *D. pulchra*. However, whilst at least 8 other polychaete families contain herbivorous species (Brawley 1992), we probably overestimated the number of herbivores by counting total polychaetes. The other common mesograzers (*Phasianotrochus eximius*, *Aplysia parvula*, *Holopneustes purpurascens*) consume *D. pulchra*. *P. eximius* and *A. parvula* prefer the chemically rich tips of *D. pulchra* (a relatively small proportion of the total
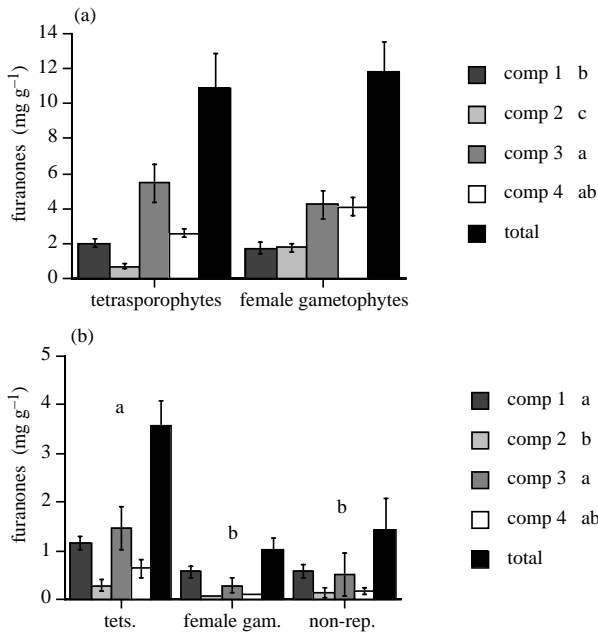
Fig. 11. Concentrations (mean ± 1 SE) of furanones in different life-history stages collected from Nobby's Beach. (a) Total and individual furanones (Compounds 1 to 4) in tetrasporophytes (n = 8) and female gametophytes (n = 5) in winter 1994. (b) Total and individual furanones (Compounds 1 to 4) in tetrasporophytes (tets.), female gametophytes (female gam.) and non-reproductive (non-rep.) plants in summer 1995 (n = 5 for all life-history stages). Life-history stages or compounds sharing the same letter do not differ at p = 0.05 (Tukey's analysis)

plant biomass) to other parts of the plant and are not deterred by crude extract at natural concentration (Rogers 2000, Wright et al. unpubl.).

Haploid plants of *Delisea pulchra* were found at only 1 of the 15 locations sampled, Nobby's Beach. All plants at the other locations were tetrasporophytes or juveniles. Significantly, this result was consistent over both winter and summer. *D. pulchra* is known to recycle tetrasporophytes via spores (Wright 2000) and clones have been described in *D. pulchra* populations (Wright et al. 2000). The results from this study indicate that asexual reproduction of tetrasporophytes is

Table 6. ANOVA comparing individual furanones (Compounds 1 to 4) in different life-history stages of *Delisea pulchra* (tetrasporophytes vs gametophytes) at Nobby's Beach in winter 1994. Data log(x + 1) transformed

Source	df	MS	F	p
Life history	1	0.075	3.862	0.057
Compound	3	0.152	7.873	<0.001
Plant (block)	7	0.054	2.777	0.020
Life history × Compound	3	0.031	1.595	0.207
Error	37	0.019		

Table 7. ANOVAs comparing total and individual furanones (Compounds 1 to 4) in different life-history stages of *Delisea pulchra* (tetrasporophytes, gametophytes and non-reproductive plants) at Nobby's Beach in summer 1995. Data log(x + 1) transformed

Source	df	MS	F	p
Total furanones				
Life history	2	0.980	7.695	0.007
Error	12	0.127		
Individual furanones				
Life history	2	0.812	13.944	<0.001
Compound	3	0.531	9.115	<0.001
Plant (block)	4	0.057	0.983	0.426
Life history × Compound	6	0.053	0.907	0.499
Error	44	0.058		

widespread in *D. pulchra* populations in southeastern Australia. Variation in the proportion of different life-history stages among populations of red algae is well documented (Hawkes 1990). Often this is dependent on latitude, suggesting that environmental factors such as photoperiod and temperature are important in determining specific patterns of life history at a given site (Hawkes 1990, Santelices 1990). Over the geographic range surveyed here, there was no evidence for a latitudinal trend in the proportion of different life-history stages of *D. pulchra*. However, in a different biogeographical region there is some evidence this may occur, as *D. pulchra* sampled from Macquarie Island by Ricker (1987) were all gametophytes. The reason for the lack of gametophytes in the populations in New South Wales is not clear.

Relationship between furanones, herbivores and epiphytes

At the level of population, correlations between total and individual furanones and the abundance of macro-grazers, mesograzers and epiphytes were weak and non-significant (Fig. 9). These results are in contrast to the correlation between high levels of phlorotannins and high levels of herbivory in temperate Australasian versus temperate North American brown algae (Steinberg 1989, 1992, Steinberg et al. 1995) and to the correlation between feeding deterrence of non-polar extracts and high herbivory in tropical versus temperate algae (Bolser & Hay 1996). The conclusions reached from those studies was that a historically high intensity of herbivory in Australasia and the tropics was important in driving the evolution of high concentrations of secondary metabolites in algae in those regions. There was no evidence that high densities of herbivores within populations have had a similar effect on the

concentrations of furanones in *D. pulchra*. Our results also show no evidence to indicate induction of furanones by herbivores.

One constraint on any ecological or evolutionary interpretation is the assumption that the densities of herbivores and epiphytes in each of the locations at the time of sampling were indicative of their densities over ecological and evolutionary time-scales may not be valid. Temporal variation in the abundance of marine herbivores is widely documented and the abundance of *Heliocidaris erythrograma* (Wright & Steinberg in press), *Holopneustes purpurascens* (Steinberg 1995), *Centrostephanus rodgersii* (Andrew 1991), and *Aplysia parvula* (Rogers 2000) show considerable variability in time at single locations. A second constraint on our conclusions is that given the large within-location variation in furanones, by examining the relationships between furanones, herbivores and epiphytes at population level, evidence for processes acting upon individuals may have been obscured. Examining those relationships more rigorously within populations will probably reveal more about the role of herbivores and epiphytes in the evolution of quantitative variation in algal secondary metabolites. A third constraint relates to our sampling. Given the distance covered in our surveys (650 km), it was not possible to control for environmental factors that may have varied between the different times the locations were sampled. For example, factors such as water temperature (via upwelling events), storms, UV light and nutrients may have varied among times and affected furanones, herbivores or epiphytes.

There are 2 further reasons that may explain the absence of a relationship between variation in furanones and variation in epiphytes. First, by measuring whole-plant chemistry, we were probably not measuring the levels of furanones encountered by epiphytes on the surface of the plants. Furanone levels of whole plants are, in general, not well correlated with surface furanone levels (Dworjanyn et al. 1999). Second, it is possible that by measuring epiphytes only on *Delisea pulchra*, we may not have adequately sampled the abundance of epiphytes, and thus their overall abundance, at each location. Differences in the degree of epiphytism on *D. pulchra* among locations could arise because of: (1) differences in the abundance of epiphytes at each location; (2) differences in chemical resistance of *D. pulchra* to epiphytes at each location.

Factors contributing to the quantitative variation in algal secondary metabolites fall into 2 broad categories: environmental (ecological) and genetic (evolutionary). Previous work in marine algae has focussed solely on the effects of environmental factors such as nutrients (carbon, nitrogen; Yates & Peckol 1993, Arnold et al. 1995, Cronin & Hay 1996a, Puglisi & Paul

1997), light (UV, visible; Cronin & Hay 1996a, Pavia et al. 1997, Puglisi & Paul 1997), desiccation (Cronin & Hay 1996b); or induction due to herbivory (Van Alstyne 1988, Cronin & Hay 1996a, Peckol et al. 1996). The low concentration of furanones in *Delisea pulchra* in summer 1995 (Figs. 3 & 4) may reflect the impact of high UV light in that season. However, as the concentration of furanones did not follow a latitudinal cline, environmental factors such as temperature and light that may vary over this geographic range do not appear to be the major cause of the differences among locations. The effect of nutrients in contributing to the differences in concentrations of furanones among locations is unclear.

Quantitative variation in total furanones and Compound 3 from *Delisea pulchra* show significant heritability (Wright 2000), indicating the potential for an evolutionary response. However, in general, feeding by herbivores does not vary with furanone concentration (Wright et al. unpubl.). In addition, surface concentrations of furanones are usually high enough to deter settlement of epiphytic algal propagules (de Nys et al. 1998, Dworjanyn et al. 1999). Thus, variation in the response of herbivores and epiphytes to different concentrations of furanones, which is necessary for them to impose selective pressure on quantitative variation of furanones, may be absent.

Finally, the large within-location variability in furanone concentration of *Delisea pulchra* supports the hypothesis of Karban et al. (1997) that variability itself can be the target of selection. This model proposes that variability can be favoured because as variability increases, benefits to herbivores decrease as it becomes more difficult for a herbivore to adapt to a defence. Concentrations of furanones not only vary among individual plants, but can also vary significantly among parts within individual plants (de Nys et al. 1996, Dworjanyn et al. 1999), further supporting this hypothesis.

Conclusions

The concentrations of secondary metabolites in *Delisea pulchra* varied among locations but did not vary in a predictable way with latitude or the abundance of herbivores and epiphytes. The lack of clinal variation in furanones suggests that environmental factors that vary with latitude (water temperature, light) are unlikely to be causing the variation observed among locations. The weak correlations between the abundance of herbivores, epiphytes and furanones suggest that quantitative variation in the secondary metabolites of *D. pulchra* is not driven by population-level selection or induction but by localised variation in environmental factors such as nutrients, or by genetic

differences among individual plants. Alternatively, it may be that looking for a correlation between secondary metabolites and herbivores or epiphytes at the scale of location obscures processes that are affecting individual plants. The large within-location variation in all of the factors examined, particularly concentrations of furanones, suggests that small-scale processes are more likely to be important in driving quantitative variation in the secondary metabolites of *D. pulchra* than large geographic-scale processes.

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Activated chemical defenses in tropical versus temperate seaweeds

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ABSTRACT: Chemical defenses that are rapidly activated in response to injury have been reported in numerous species of vascular plants, but activated chemical defense has been demonstrated for only 1 genus of seaweed. To investigate the frequency of potential activated chemical defenses in seaweeds and to determine if there are geographic differences in the frequency of theses, we conducted urchin and fish feeding assays using chemical extracts from 42 species of temperate or tropical seaweeds that were damaged immediately before extraction in organic solvents (= the potentially activated extract) or placed in organic solvents before they were damaged (= the non-activated extract). Seven species exhibited changes in palatability consistent with activated defenses while 4 species became more, rather than less, palatable if they were damaged 30 s before extraction. Frequency of activation did not vary geographically. Seventeen percent of tropical species (4 of 24) and 17% of temperate species (3 of 18) exhibited changes in palatability that were consistent with activation of chemical defenses. Thin-layer chromatography of lipid-soluble extracts indicated that damaging the thallus prior to extraction caused noticeable chemical changes in 70% of the species evaluated. Investigations of algal chemical defenses thus need to consider the effects of injury during herbivore attacks and the effects of extraction methodology on the types of, and concentrations of, metabolites discovered in seaweeds.

KEY WORDS: Activated chemical defenses · Fish · Marine · Plant-herbivore interaction · Sea urchin · Seaweed

INTRODUCTION

Herbivory strongly impacts algal distribution, abundance, and community structure in both temperate and tropical communities (e.g., Lubchenco & Gaines 1981, Carpenter 1986, Lewis 1986, Hay 1997). Sea urchins decimate temperate kelp beds (Lawrence 1975, Steinberg et al. 1995), and either fishes alone or urchins alone can remove 60 to 97% of total algal production on tropical reefs (Hatcher & Larkum 1983, Carpenter 1986), reducing competitive pressures on corals and facilitating the establishment of tropical reefs (Lewis 1986, Hughes 1994, Miller & Hay 1996).

Macroalgae persist by escaping, tolerating, or deterring herbivores (Lubchenco & Gaines 1981). One of the more extensively studied methods of deterring herbivores is via the production of chemical defenses (Hay & Fencial 1988, Paul 1992, Hay 1996). Chemical deterrents are widespread among seaweeds and have important direct and indirect effects on the evolution of seaweed-herbivore interactions and on benthic community structure in general (Hay & Fencial 1996, Hay 1997).

Plant chemical defenses may be either constitutive (constantly produced) or induced in response to changes in the environment (Karban & Baldwin 1997). Acti-

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vated chemical defenses are a special case of induced defenses that occur within seconds of damage. Like induced defenses, activated defenses are triggered by injury and result in increased resistance to herbivory. However, activated defenses are similar to constitutive defenses in that the plant has invested resources in the defensive precursors before the injury occurs (Karban & Baldwin 1997). Activation differs from normal induction in that (1) activated defenses convert a less deterrent metabolite to a more deterrent one, whereas induction involves increased production of an existing metabolite, and (2) activation occurs very rapidly (within seconds) (Paul & Van Alstyne 1992) whereas induction may take hours to months (Karban & Baldwin 1997). Although activation is initiated by damage, like an induced defense, it is more similar to constitutive defense because the stored chemical precursors are maintained at high concentrations and the response time to herbivory is very rapid (Paul & Van Alstyne 1992, Karban & Baldwin 1997). Activated defenses might be selected over constitutive defenses if the deterrent chemical is physiologically damaging to the plant (Baldwin & Callahan 1993, Wolfe et al. 1997) or if the defensive chemical attracts specialist herbivores (Carroll & Hoffman 1980, Giamoustaris & Mithen 1995).

Activation occurs in numerous terrestrial plants. As examples, several plant families activate hydrogen cyanide in response to injury (Conn 1979), crucifers hydrolyze glucosinolates to form thiocyanates, isothiocyanates, or isonitrils (Van Etten & Tookey 1979, Chew 1988), and, when damaged, *Populus balsamifera* rapidly converts phenolic glycosides into more potent feeding deterrents (Clausen et al. 1989, 1991). Activation of chemical defense also appears to occur in one of the few freshwater macrophytes in which specific chemical defenses have been demonstrated (Bolser et al. 1998).

Activation has important methodological as well as ecological implications. Most chemical extractions are done using uninjured plants, but this may not accurately represent the chemistry a herbivore encounters as it macerates a plant while feeding. Comparisons of recently injured versus uninjured plants may show differences in chemistry or palatability that would otherwise go unnoticed, suggesting an enzymatic conversion of precursors to more potent defensive molecules.

Marine examples of activated defenses appear to be rare, but few investigations have looked for them. Tropical green algae in the genus *Halimeda* convert the less potent halimedatetraacetate into the more deterrent halimedatrial within seconds of being damaged (Paul & Van Alstyne 1992) and a similar conversion of udoteal to the more deterrent petiodial has been suggested in *Udotea flabellum* (Paul 1992). The planktonic microalga *Emiliania huxleyi* converts dimethylsulphoniopropionate (DMSP) to dimethylsulfide (DMS) when

cells are lysed, deterring grazing by protozoa herbivores (Wolfe et al. 1997). Our study focused on determining whether rapid changes in the palatability of seaweed extracts following damage was uncommon in marine systems, or simply overlooked.

In this investigation, we determined the frequency of possible activated chemical defenses among seaweeds by testing the deterrence of extracts from undamaged seaweeds versus extracts from plants damaged for 30 s prior to extraction. Because seaweed chemical defenses appear better developed in tropical than in temperate seaweeds (Bolser & Hay 1996), we also compared the frequency of activated deterrence for temperate seaweeds from North Carolina versus tropical seaweeds from the Florida Keys.

METHODS

Study sites and organisms. Tropical algae were collected and field experiments conducted near Key Largo, Florida, USA (24° 59′ N, 80° 24′ W) during August to October 1997. Collection locations were in Florida Bay (*Acetabularia calyculus*, *Caulerpa mexicana*, *C. prolifera*), seagrass beds south and east of Rodriguez Key (*Avrainvillea nigricans*, *Udotea flabellum*, *Rhipocephalus phoenix*, *Penicillus dumatosis*, *Halimeda incrassata*, *Dictyosphaeria cavernosa*, *Ceramium nitens*, *Digenia simplex*, *Laurencia papillosa*, *L. intricata*, *L. poiteaui*), in spur and groove zones on Pickles Reef at depths of 6 to 20 m (*Lobophora variegata*, *Stypopodium zonale*, *Dictyota dentata*, *Amphiroa rigida*, *Halimeda goreaui*, *H. opuntia*, *H. tuna*), from Pine Key reef (*Sargassum pteropleuron*), and from surface waters off-shore of Key Largo (*Sargassum polyceratium*).

Temperate algae were collected near Morehead City, North Carolina, USA, during October 1997 and January to August 1998. Collection locations were at 10 Fathom Rock at a depth of 17 m (*Zonaria turnuefortii*, *Dictyopteris hoytii*, *Solieria filiformis*, *Halymenia trigona*, *Amphiroa beauvoissi*), at Radio Island's rock jetty at a depth of 0.5 to 4.0 m (*Calinitophyllum medium*, *Chondria* sp., *Dasya baillouviana*, *Rhodomenia divaricata*, *Sargassum filipendula*, *Scytosiphon lomentaria*, *Spatoglossum schroederi*, *Petalonia fascia*, *Padina gymnospora*, *Dictyota ciliolate*, *D. menstrualis*), from oyster reefs on the mudflat at Mitchell's Village (*Ectocarpus* sp.), and from intertidal rubble in the Beaufort channel (*Fucus vesiculosus*).

To determine if chemical defenses were activated by damage, we ground plants in a blender for 30 s before exposing them to the organic solvents used for extraction (a 2:1 blend of dichloromethane:methanol [= DCM:MeOH]), or we placed intact plants into these solvents prior to blending. This procedure follows that

used by Paul & Van Alstyne (1992) in their initial study of activated chemical defenses in the green seaweed *Halimeda*. Blending for 30 s chopped most seaweeds into small portions, probably rupturing many individual cells. Given that the pharengil mills of tropical parrotfishes and the aristotle's lantern of sea urchins both crush most of the algal cells consumed, this grinding should be a reasonable mimic of the physical processes occurring as these species feed. The temperate fish we used in our assays does not have these types of mouth parts and tends to bite off sections of plant and swallow these with considerably less cell damage before the seaweed enters the gut. Our grinding procedure may not adequately mimic damage done by herbivores that feed in this manner. The effects of seaweed extracts on herbivore feeding were tested in the field and laboratory using sympatric generalist fishes (tropical parrotfishes, predominantly *Sparisoma aurofrenatum* and *S. viride*, and the temperate sparid *Diplodus holbrooki*) and the sea urchin *Lytechinus variegata*, which occurred in both our temperate and tropical sites. Assays with tropical fishes were conducted in the field where many fishes would have access to our assays, but previous studies at this site (D. Malone pers. comm.) had shown that all feeding on our assay food (the seagrass *Thalassia testudinum*) was due to parrotfishes and that 94 % of all bites on this food were by the single species *S. aurofrenatum*. Extensive videotaping of transplanted macrophytes and of natural vegetation showed this species to be the most active grazer on these reefs, thus making it an especially appropriate assay organism for the macrophytes. It is the major consumer of common macrophytes such as species of *Halimeda*, *Sargassum* and *Lobophora* (D. Malone & M. E. Hay pers. obs., based on bite rates from approximately 200 h of video on these reefs). In temperate fish feeding assays, we used the spottail pinfish *Diplodus holbrooki* because it is the most common species of plant-eating fish on reefs in the South Atlantic Bight (Hay & Sutherland 1988) and because its feeding can strongly affect benthic community structure (Hay 1986, Miller & Hay 1996, Duffy & Hay 2000).

The sea urchin *Lytechinus variegatus* is common in both North Carolina and tropical habitats. We collected this sea urchin in North Carolina and used it in feeding choice assays with extracts from both temperate and tropical seaweeds.

Chemical methods. Because previous examples of activated chemical defenses in both seaweeds and freshwater macrophytes were lipid-soluble (Paul & Van Alstyne 1992, Bolser et al. 1998) and because geographic differences in chemical defenses between seaweeds from the regions we investigated were lipid-soluble rather than water-soluble (Bolser & Hay 1996), we focused exclusively on the effects of lipid-soluble extracts.

To test whether extracts from injured algae were more, or less, deterrent to herbivores than extracts from intact algae, we extracted injured versus uninjured plants using methods similar to those used by Paul & Van Alstyne (1992) in their initial study documenting activation of chemical defenses in the genus *Halimeda*. Algae were collected, spun in a salad spinner to remove excess water, and individual plants divided in half. For species in which plant tissues would be damaged appreciably by this division, individual plants were randomly selected for each treatment. Numerous individual plants were pooled to create each treatment group. The preinjured treatment was submerged in a measured amount of seawater (as determined for each species based on quantity and physical characteristics) and ground for 30 s in a blender. This damaged algal tissue was then rapidly immersed in 2:1 DCM:MeOH, within about 30 s after blending. The the non-preinjured treatment (= control) was submerged in the same amount of 2:1 DCM:MeOH, and these solvents were allowed to soak into algal tissue for about 30 s before blending. Seawater, equal in volume to that added to the preinjured treatment, was then added, and this mixture was blended for 30 s. It appears that enzymes in damaged plants act within seconds to convert less active precursors to more active defensive metabolites, and that this enzymatic conversion is prevented by the presence of organic solvents (Paul & Van Alstyne 1992, Bolser et al. 1998).

Lipophilic metabolites were extracted from both treatments of each algal species by adding and removing 2:1 DCM:MeOH to and from the blended algae 3 times. The pooled extracts for each treatment were then removed via rotary evaporation, and the lipid-soluble extract was separated from more polar components by partitioning with 1:1 DCM:deionized water. The crude lipid extract was obtained via rotary evaporation of the DCM-soluble material. The remaining algal tissue was dried to a constant mass at 60°C and the dry weight of the algal sample was determined through the addition of the extract dry weight to the dry algal mass.

There is a possibility that the types of, or concentrations of, compounds retrieved from particular seaweeds could be affected by the solvents chosen for extraction. However, for initial exploratory research assessing a large number of species and a large range of potential types of metabolites, one needs to choose 1 solvent system and use it across all species investigated. We chose DCM:MeOH because it is the most commonly used solvent mixture for extracting secondary metabolites from fresh seaweeds (e.g., see review of methods by Hay et al. 1998), and because this solvent mixture had been used in the only other investigation of activated seaweed defenses (Paul & Van Alstyne 1992).

We evaluated chemical differences between extracts of preinjured and non-preinjured seaweeds by diluting the extracts to equal concentrations in ether and visualizing them using thin-layer chromatography (TLC). Separate TLC plates spotted with each extract type were run in each of 3 solvent mixtures (1:1 ether:hexane, 100% ether, and 19:1 ether:methanol) to visualize algal metabolites across a range of polarities. After allowing the solvent to migrate up two-thirds of the TLC plate, the plate was removed, the solvent front marked, and the compounds visualized with the aid of UV fluorescence and acid charring. Compounds differing between treatments were noted as a function of R_f value (distance moved relative to the height of the solvent front), color, method of observation and solvent mixture.

Bioassay methods. We tested for preinjury effects on each algal species by comparing the rate at which herbivores consumed foods treated with the extract from the preinjured treatment versus extract from the non-preinjured treatment. For assays with tropical fishes, 6 cm length blades of the palatable seagrass *Thalassia testudinum* were coated with a solution of the lipophilic extract in diethyl ether so that the extract concentration on the blade following evaporation of the ether approximated the concentration (% dry mass) extracted from the algae being tested. Specific procedures for coating the assay food followed methods described in Hay et al. (1987, 1998). These procedures had worked well on other tropical reefs in the Caribbean and South Pacific.

Previous work indicated that 88 to 100% of similar extracts (i.e., non-polar lipids) coated onto the surface of palatable seaweeds could be recovered after 2 to 3 h in seawater (McConnell et al. 1982) and that 96% of a specific lipid-soluble metabolite could be recovered after 24 h in seawater (Hay & Fenical 1988). A length of 6 cm was chosen to duplicate the experimental protocol used in previous investigations (Hay 1984, Hay et al. 1987).

Four coated blades from the preinjured or non-preinjured treatments were woven between the strands of a 50 cm length of 3-strand rope so that 5 cm of each blade was exposed and available to be consumed. Treatment blades (= extract form preinjured plants) of *Thalassia testudinum* on one rope were paired with control blades (= extract from non-preinjured plants) on a separate rope by placing the ropes within ~0.5 m of each other at a depth of about 6 m on Pickles Reef. Thirty pairs of ropes were used in tests with each algal species against reef fishes. Each replicate pair was separated from the others by ~2 m. All assays were completed within 1 h of initiation.

When either rope in a replicate pair was observed to have >50% of the total *Thalassia testudinum* blade area removed, the amount removed per blade was

determined to the nearest 0.5 cm of length and the replicate pair was collected. In cases where blades were grazed along the sides instead of from the top down, we cut upper portions of the blades to fill in the grazing scars along the margins and then estimated the length missing.

Temperate fish assays were conducted at the University of North Carolina's Institute of Marine Science, Morehead City, North Carolina, USA. Individual *Diplodus holbrooki* (spottail pinfish) were housed in each half of a 38 l aquarium divided by a partition of plastic mesh. A total of 40 fish were used, with sizes ranging from 10 to 15 cm standard length. Within an assay, no fish was used more than once (i.e., replicates were independent). However, each fish was used to test multiple species of seaweeds. All fish for these assays were collected from a nearshore wreck and allowed to adjust to the indoor environment for 48 h prior to being used in assays.

For our preference assays with temperate fish, 6 cm segments of the palatable alga *Gracilaria tikvahiae* were coated with extract as in the assay with tropical fishes. Each alga was attached to a weighted clothespin, and 1 activated and 1 control alga were paired and placed in each half of each aquarium so that each alga was equally apparent. When either the treatment or control segment in a replicate was observed to have >50% of its length removed, the replicate pair was collected and the amount removed per segment was determined to the nearest 0.5 cm.

Because the sea urchin *Lytechinus variegatus* occurs in both temperate North Carolina and throughout the tropical Caribbean, we used it in bioassays with extracts from both temperate and tropical seaweeds. Activated extracts or control extracts were coated onto freeze-dried and powdered *Ulva* spp. at natural concentrations. This was achieved by placing the powdered *Ulva* spp. in a flask, dissolving the desired amount of extract in ether, and pouring this over the *Ulva* spp. This mixture was rotary-evaporated to dryness, leaving the extact coated onto the particles of *Ulva* spp. The powdered *Ulva* spp. (treated with the extract from preinjured or non-preinjured plants) was incorporated into agar and poured into a mold lying over a piece of window screen (see diagrams in Hay et al. 1994, 1998). After the agar solidified, the mold was removed, leaving 2 strips (one with activated extract, the other with the control extract) of artificial food adhering to the screen. This method basically makes an artificial seaweed with a graph-paper type matrix (i.e., the squares of the window screen) imbedded inside. The food strips were cut into individual replicates (containing equal amounts of each food type), offered to urchins, and removed when approximately one-half of either choice had been consumed, or at the end of the

6 h experiment. Consumption was measured by counting the number of window screen squares completely revealed by grazing. Extraction and food preparation were done so the extract from 2 g algal tissue (dry weight) was incorporated into 2 g dry mass of *Ulva* spp.

Statistical analyses. All bioassay results were analyzed by a 2-tailed, paired-sample *t*-test after excluding pairs in which all of both treatments, or neither of either treatment, had been consumed. This is standard procedure for paired-sample tests, justifiable because no consumption or total consumption do not give any data on the relative palatability of the 2 treatments (Glantz 1992). This resulted in a sample size of 15 to 38 replicates per assay.

The majority of species tested showed no significant difference in the effect of the activated versus the control extract. *A posteriori* power analyses were run in order to determine the likelihood that there was no treatment effect versus the likelihood that the experiment simply had inadequate power to detect such an effect. For these analyses, the minimal detectable effect, δ, was chosen to be 50% because, on average, there was a 54% difference in consumption between treatments in the 11 cases that showed significant changes due to activation.

Chi-square analyses were used to determine whether activation was more frequent as a function of geographic region (North Carolina vs Florida) or phylogenetic affinity (red, green, or brown seaweeds). Given the large number of assays we conducted (77) and an $\alpha = 0.05$, we would expect about 4 assays to be significant due to chance alone. We used a chi-square test to determine if the number of significant differences we documented was greater than what would be predicted from chance.

RESULTS

TLC demonstrated that damaging plants for about 30 s prior to extraction qualitatively altered the chemical composition of 70% (28 of 40) of the species examined by TLC (Appendix 1). However, changes in extract palatability to one or both of the herbivores used in our bioassays were much less frequent than changes in chemical composition, occurring in only 26% (11 of 42) of the species examined, with 7 species (16.7%) becoming significantly less palatable and 4 species (9.5%) becoming significantly more palatable (Figs. 1 to 5). For the 7 species in which damage created more deterrent extracts (i.e., those suggesting an activated chemical defense, Figs. 1, 2 & 4), TLC of the extracts showed unique compounds in the control extracts of 3 species (*Dictyota* sp., *Halimeda incrassata*, and *Zonaria tournefortii*) and unique compounds in the acti-

vated extracts of 4 species (*H. tuna*, *Rhipocephalus phoenix*, *D. menstrualis*, and *Scytosiphon lomentaria*) (Appendix 1). For the 4 species in which damage resulted in extracts that were more, rather than less, palatable (Figs. 2 to 4), 2 showed unique compounds in the activated extract (*Laurencia poiteaui* and *Petalonia fascia*), 1 showed a unique compound in the control extract (*Acetabularia calyculus*), and 1 showed no detectable difference between extracts (*D. ciliolata*). Given this variance in how damage changed chemistry versus palatability of the lipid-soluble extracts, tracking the chemistry involved in altered palatability will clearly require bioassay-guided fractionation and isolation of the specific chemicals, or concentrations of chemicals, altering palatability. The TLC results (Appendix 1) coupled with the bioassay results (Figs. 1 to 5) suggest that some of the important chemical changes will be quantitative (not well evaluated by TLC) rather than simply qualitative.

Although we evaluated more tropical species than temperate species, the proportion demonstrating activation was equivalent between these geographic regions. Four of 24 tropical species (17%; Figs. 1 to 3) and 3 of 18 temperate species (17%; Figs. 4 & 5) had activated extracts that were significantly less palatable than control extracts to at least one of our herbivores. In addition to the significant alterations in palatability that are discussed above, 3 tropical species (*Sargassum polyceratium*, *Halimeda opuntia*, and *Digenia simplex*) showed non-significant trends (e.g., p = 0.069 to 0.083) suggestive of activation (Figs. 1 to 3).

The proportion of species whose extracts differed in palatability (becoming either more deterrent or more attractive) as a result of damage did not differ between temperate and tropical regions (tropical = 4 deterrent, 2 attractive, and 18 showing no change; temperate = 3 deterrent, 2 attractive, and 13 showing no change [chi-square p-value = 0.156]). If we made this contrast only within the brown algae or only with the red algae (the lack of temperate green algae in our assays prevented such an analysis for this group), we also found no significant difference with latitude (chi-square p-value = 0.231 and 0.182, respectively).

The 2 herbivores used in our bioassays of algal extracts did not always show similar patterns in feeding. The preinjured extracts from *Dictyota* sp. (Fig. 1) and *D. menstrualis* (Fig. 4) were consistently deterrent to both the fish and the urchin, and the preinjured extract from *Petalonia fascia* (Fig. 4) was more palatable to both herbivores. However, preinjured versus non-preinjured extracts from *Acetabularia calyculus*, *Halimeda incrassata*, *H. tuna*, *Rhipocephalus phoenix* (Fig. 2), *Laurencia poiteaui* (Fig. 3), *D. ciliolata*, *Scytosiphon lomentaria*, and *Zonaria tournefortii* (Fig. 4) all differed significantly for one herbivore, but not for the other.
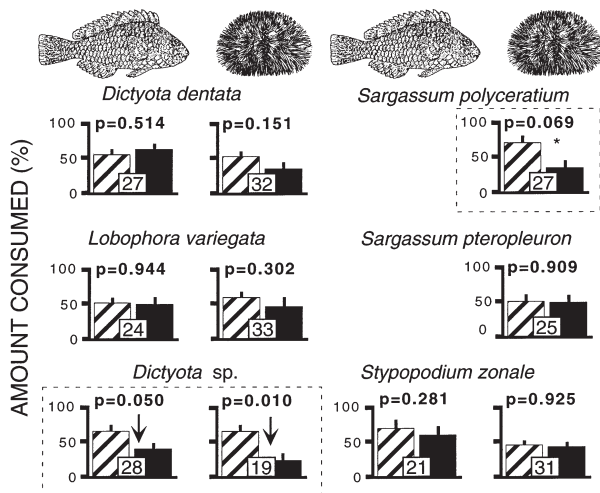
Fig. 1. Assays with extracts from tropical brown algae. Hatched bars show feeding on food treated with extracts from seaweeds that were not damaged before exposure to organic solvents (= controls); solid bars indicate feeding on food treated with extracts from preinjured seaweeds (= activated extracts). Graphs below fish indicate parrotfish assays. Graphs below urchins indicate urchin assays. Sample size is given at the base of each histogram pair. Error bars indicate 1 SE. p-values are from a 2-tailed, paired-sample *t*-test. Dashed lines around a graph highlight significant (p ≤ 0.05), or nearly significant (0.05 < p < 0.10) differences. Down-pointing arrows indicate an activated defense; *Nearly significant trends
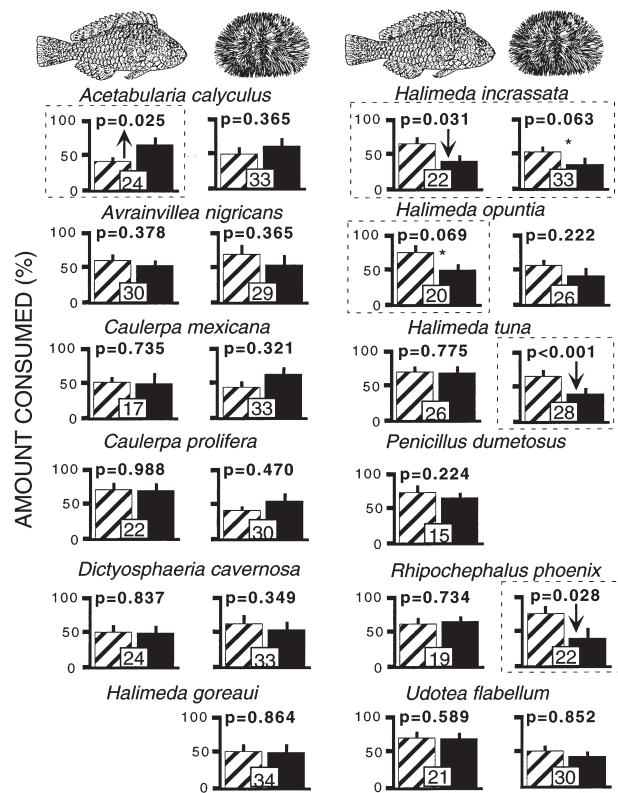

Fig. 2. Assays with extracts from tropical green algae. Up-pointing arrows indicate species in which damage increased, rather than decreased, palatability. Symbols and analyses as in Fig. 1
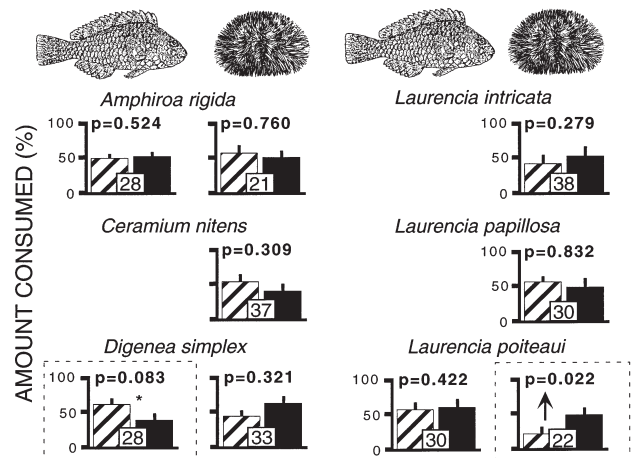

Fig. 3. Assays with extracts from tropical red algae. Symbols and analyses as in Figs. 1 & 2

The degree of confidence that one may have in several of the above contrasts will depend upon the power of our assays to detect an altered palatability of the extract. Of the 78 feeding assays performed, a significant difference in the palatability of preinjured versus non-preinjured extracts was detected in 14. These contrasts are irrelevant to *a posteriori* power analysis (Peterman 1990). Of particular interest for questions involving power are those assays with large differences between treatment means and p-values that are near 0.05. For the 7 instances that met these criteria (e.g., p < 0.20), power analyses indicated that we had a ≥75% chance of detecting a 50% change in feeding, indicating that the power to detect a significant difference between treatments was reasonable. The remaining 56 assays had smaller differences between treatments and larger p-values (p > 0.20). For 10 of these assays, power to detect a 50% change in feeding was <75% (ranging from 74.2 to 57.5%). All assays with low power involved urchins; power was relatively high in all fish assays.

With 77 separate assays and an α-value set at 0.05, chance alone would be predicted to produce 4 significant differences (i.e., 0.05 × 77 = 3.85). The 13 significant differences that we observed significantly exceed the frequency of differences that would be expected by chance (p = 0.021, chi-square test).

DISCUSSION

Although 70% of the species examined using TLC showed qualitative differences in chemistry between the extracts from preinjured and non-preinjured plants, extracts from preinjured plants were more deterrent
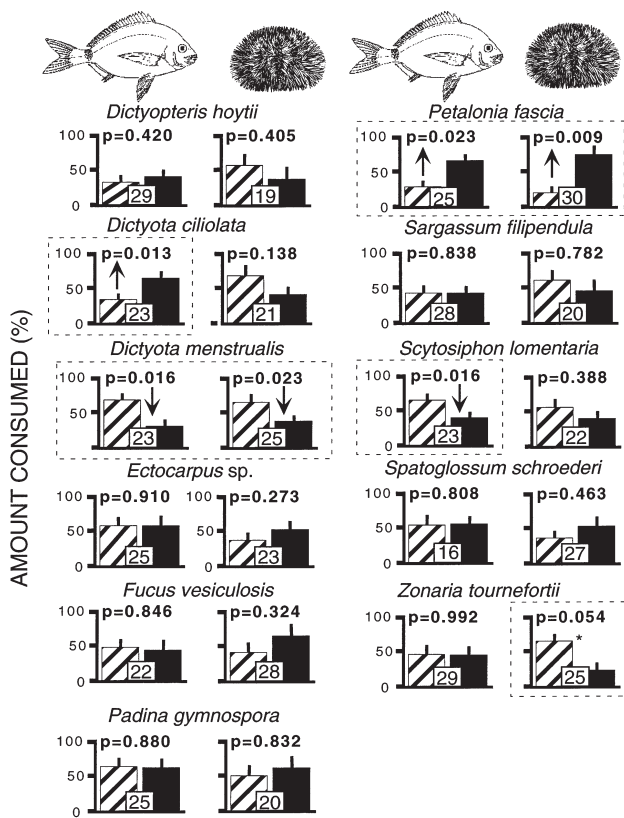
Fig. 4. Assays with extracts from temperate brown algae. Graphs below fish indicate assays with spot-tail pinfish. Graphs below urchins indicate urchin assays. Symbols and analyses as in Figs. 1 & 2
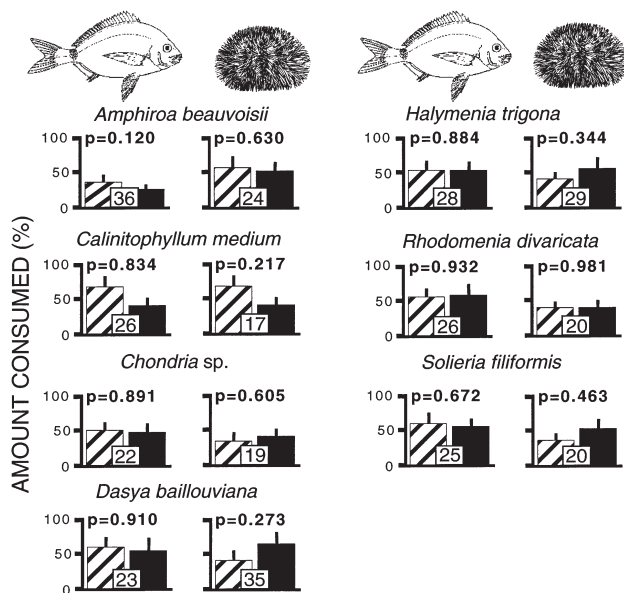

Fig. 5. Assays with extracts from temperate red algae. Symbols and analyses as in Figs. 1 & 2

to at least one herbivore for only 17 % of the species tested. In contrast to expectations based on the activation of chemical defenses, extracts from preinjured plants were significantly more palatable than extracts from non-preinjured plants for about 10 % of the species investigated. Thus, extract palatability immediately changed in response to damage in about 26 % of the species tested, with 7 of 42 species showing decreased palatability and 4 of 42 species exhibiting increased palatability as a result of damage.

Despite the evidence that intense herbivory has selected for increased chemical defenses among tropical compared to temperate plants in both terrestrial and marine systems (Coley & Aide 1990, Bolser & Hay 1996), our study found no evidence that the frequency of activated chemical defenses varied between the 2 geographic localities we investigated. However, tropical areas are not uniform in their intensity of herbivory, but instead contain a mosaic of spatial and temporal refuges (Hay 1984, 1985, 1997). Over half of the tropical species we tested were collected from seagrass habitats and from non-reef habitats in Florida Bay. Macrophytes in these types of habitats experience less selection for antiherbivore defenses (Hay 1984, 1991, Lewis 1985). Despite this, 2 of the 4 tropical species showing evidence for activated defenses (*Halimeda incrassata* and *Rhipocephalus phoenix*) were collected in seagrass habitats. These species, however, also occur in reef-slope habitats where herbivory can be intense.

Our results suggest that activation of deterrence may be most common among Dictyotalean brown algae and calcified green algae in the Halimedaceae. Alterations in palatability that would suggest activated chemical defenses did not occur in any of the red seaweeds we investigated, despite many red algae being rich in bioactive secondary metabolites (Hay & Fenical 1988, Faulkner 1998), several of which have been shown to deter feeding by reef herbivores (Hay et al. 1987, 1988a, Hay 1991, Paul 1992). The only effect of damage that we found for red seaweeds was that the extract from the preinjured treatment of *Laurencia poiteaui* was preferred over the non-preinjured treatment in urchin preference assays (Fig. 3).

The results of this study combined with Paul & Van Alstyne's (1992) initial investigation of activated chemical defenses indicate that of the 7 species showing activated defenses, 3 are calcified greens in the family Halimedaceae and 3 are brown algae in the family Dictyotaceae. However, activated chemical defenses are not universal in these families, or even within genera where the types of defensive metabolites produced are relatively similar. Numerous Dictyotalean algae failed to show activation following damage (e.g., *Dictyota dentata*, *D. hoytii*, *Lobophora variegata*, *Stypopodium zonale*, *Padina gymnospora*, *Spatoglossum schroderi*),

and the extract of 1 species (*D. ciliolata*) became more palatable following damage. Similarly, *Rhipocephalus phoenix* and many *Halimeda* spp. showed significant changes in palatability, or strong trends, indicating an activated defense (Fig. 2), but *H. goreaui*, *Udotea flabellum*, *Avrainvillea nigricans*, and *Penicillus dumetosus* all failed to show evidence for activation, despite being in the family Halimedaceae. It is interesting that all cases of altered palatabilities that are consistent with changes due to activated chemical defenses occurred among species that are known to be chemically defended from some common herbivores (see reviews by Hay 1991, Paul 1992). This suggests that most of these cases of increased deterrence may have been due to activation of chemical defenses rather than due to degradation of feeding stimulants, which could also be caused by damage. The specific chemical changes occurring in activated extracts were not investigated in our study, but bioassay-guided chemical investigations would be a logical next step toward understanding the chemical mechanisms producing the changes in palatability we documented. This mechanistic understanding would allow an evaluation of which alterations in palatabilities were due to changes in chemical defenses and which were due to changes in feeding stimulants, rather than defenses. Damage-induced changes in chemical defenses have been studied in the green seaweed *Halimeda*. Species in the family Halimedaceae produce several structurally similar diterpenoid metabolites. Most *Halimeda* spp. produce halimedatetraacetate and the epimers halimedatrial and epihalimedatrial (Paul & Fenical 1986). Levels of halimedatetraacetate and halimedatrial differ among plants and plant parts depending upon tissue age and intensity of herbivory in the habitat (Hay et al. 1988b, Paul & Van Alstyne 1988), and Paul & Van Alstyne (1992) demonstrated that some species of *Halimeda* produce increased levels of halimedatrial within seconds of being damaged. Other green algae, including species in the genera *Penicillus*, *Udotea*, *Rhipocephalus*, and *Caulerpa*, have been shown to produce higher concentrations or different types of secondary compounds in areas with intense herbivory versus areas with low levels of herbivory (Paul & Fenical 1986). However, the ability to activate chemical defenses as a function of herbivore feeding at particular sites has not been evaluated.

Like terpenoid defenses produced by species of *Halimeda*, diterpenoid defenses of brown algae in the genus *Dictyota* have been investigated extensively (e.g., Hay et al. 1987, Duffy & Hay 1994, Cronin & Hay 1996a,b, Stachowicz & Hay 1999). However, activation of defenses (Figs. 1 & 4), or of susceptibility (Fig. 4), was unknown for this genus, or for any brown seaweed, prior to this investigation. We tested 2 tropical and 2 temperate species of *Dictyota* and found evidence consistent with activated defenses against both fish and urchins in the tropical *Dictyota* sp. (Fig. 1) and the temperate *D. menstrualis* (Fig. 4). In contrast, *D. dentata* from Florida showed no effect of activation (Fig. 1), and *D. ciliolata* from North Carolina became more susceptible to fish grazing following our activation treatment (Fig. 4); susceptibility to urchins tended to decline, but this change was not significant (p = 0.138). Given the very similar diterpene alcohols that defend *D. ciliolata* and *D. menstrualis* against local herbivores (Cronin & Hay 1996a,b,c), it would be especially instructive to determine the mechanistic level chemical changes that produce the very different changes in palatability that occur in response to damage of these seaweeds.

While the chemistry of secondary metabolites is described for many algae, it is unclear how some seaweeds sequester bioactive precursor compounds away from activating enzymes. Examples of activation in terrestrial plants suggest that some plants may compartmentalize precursor compounds into vacuoles and physically separate them from activating enzymes (Conn 1979). Certain red algae store terpenoids in vesicles (Young et al. 1980) and brown algae localize phlorotannins in subcellular bodies termed physodes (Ragan & Glombitza 1986). Siphonous green algae contain protein bodies that migrate to sites of injury, plug these wounds, and prevent further loss of protoplasm (Bold & Wynne 1978). It is possible that these bodies could also contain defensive precursors or enzymes to activate these precursors, but this has not been investigated.

Two tropical and 3 temperate species' bioassay results indicate that injury may result in increased palatability, an outcome contrary to the notion of activated defenses. Although the cause of this difference is not known, several possibilities exist. Injury could release deterrent compounds that react or degrade vary rapidly, and immersing the species in solvent prior to injury may have preserved such compounds. Alternatively, the activation treatment could have released stimulatory compounds that were not released in the control treatment, or damage we inflicted prior to extraction could have caused degradation of feeding stimulants.

Rapid qualitative changes in algal chemistry in response to injury appear to be common in marine algae. We detected such changes in 70% of the 40 species we evaluated by TLC. However, activated changes in chemical defenses against herbivores appear to be less common. Damage before extraction produced more deterrent lipid extracts for only 17% of the 42 species we evaluated. In contrast to expectations based on activated chemical defenses, we found that about 10% of the species we investigated produced more

palatable extracts if they were damaged prior to extraction.

These significant changes in extract palatability caused by damage that occurred for about 30 s prior to extraction raise several questions regarding studies of prey chemical defenses. Our findings suggest that (1) modest variations in how prey are treated immediately prior to extraction (e.g., are they placed in solvent and then ground, or first cut into small pieces that will fit into the small vials sometimes used for initial extractions) could introduce considerable methodological variance into studies of prey chemical defenses, and (2) for studies of chemical defense against herbivores like urchins and fishes that crush plants as they feed, it may be more ecologically realistic to crush plants for short periods before extraction than it is to carefully protect them from stress until they are placed into solvents.

Additionally, we did not evaluate the effects of particular solvents in producing any of the patterns we documented. However, it is possible that some of the patterns in extract palatability (Figs. 1 to 5) or chemistry (Appendix 1) could be solvent-specific, and it is clear that neither fishes nor sea urchins will be using organic solvents such as DCM/MeOH to extract plants. As a final caution, we also did not evaluate changes in chemistry or extract palatability as a function of damage done for shorter or longer periods before extraction in solvents. Timing of damage, extent of damage, gut traits of the herbivores, or any number of other conditions could impact the effects that plant chemical defenses will have on herbivores. Our present understanding of the variance produced by such considerations is too poorly developed to suggest that particular procedures should be employed under all circumstances. However, as investigations of aquatic chemical ecology continue to mature, these types of questions will need additional attention.

Appendix 1. R_f values for spots present in only 1 treatment

	Activated	Control	Traits	Solvent regime
Tropical species				
Acetabularia calyculus		0.73	UV activity	19:1 ether:methanol
Amphiroa rigida	0.37		UV activity	1:1 ether:hexane
Amphiroa rigida	0.68		UV activity	1:1 ether:hexane
Avrainvillea nigricans		0.63	Chars pink	1:1 ether:hexane
Avrainvillea nigricans		0.65	Chars pink	1:1 ether:hexane
Caulerpa mexicana		0.64	UV activity	1:1 ether:hexane
Caulerpa mexicana		0.6	UV activity	1:1 ether:hexane
Caulerpa mexicana		0.36	UV activity	1:1 ether:hexane
Caulerpa prolifera	0.40	0.27	Chars pink	1:1 ether:hexane
Ceramium nitens		0.22	Brown	1:1 ether:hexane
Dictyosphaeria cavernosa		0.70	UV activity	1:1 ether:hexane
Dictyota dentata	No difference			
Dictyota sp.		0.05	Orange	100% ether
Digenia simplex	No difference			
Halimeda goreaui	No TLC			
Halimeda incrassata		0.71	Chars red	1:1 ether:hexane
Halimeda incrassata		0.77	Yellow	1:1 ether:hexane
Halimeda incrassata		0.55	UV activity	1:1 ether:hexane
Halimeda opuntia	0.58		Pink	1:1 ether:hexane
Halimeda opuntia	0.32		Grey	1:1 ether:hexane
Halimeda opuntia	0.18		Grey	1:1 ether:hexane
Halimeda opuntia	0.05		Yellow	1:1 ether:hexane
Halimeda opuntia		0.6	UV activity	100% ether
Halimeda tuna	0.52		UV activity	100% ether
Halimeda tuna	0.44		UV activity	100% ether
Laurencia intricata	No TLC			
Laurencia poiteaui	0.35		UV activity	1:1 ether:hexane
Laurencia poiteaui	0.39		UV activity	1:1 ether:hexane
Laurencia papillosa		0.16	UV activity	1:1 ether:hexane
Lobophora variegata	0.56		Chars pink	100% ether
Lobophora variegata	0.63		UV activity	100% ether
Penicillus dumatosis	0.71		Yellow	1:1 ether:hexane
Rhipocephalus phoenix	0.71		Chars yellow	1:1 ether:hexane
Sargassum polyceratium		0.4	UV activity	1:1 ether:hexane
Sargassum pteropleuron		0.4	UV activity	1:1 ether:hexane
Stypopodium zonale	0.47		UV activity	1:1 ether:hexane
Udotea flabellum	0.56		UV activity	1:1 ether:hexane
Udotea flabellum	0.19		UV activity	1:1 ether:hexane

Appendix 1 (continued)

	Activated	Control	Traits	Solvent regime
Temperate species				
Amphiroa beauvoissi	0.32		Chars brown	1:1 ether:hexane
Calinitophyllum medium	No difference			
Chondria sp.	No difference			
Dasya baillouviana	No difference			
Dictyopteris hoytii	No difference			
Dictyota ciliolata	No difference			
Dictyota menstrualis	0.6		Chars pink	100% ether
Dictyota menstrualis	0.72		Chars blue	100% ether
Ectocarpus sp.	0.72		Yellow	1:1 ether:hexane
Ectocarpus sp.	0.31		Green	1:1 ether:hexane
Ectocarpus sp.	0.22		Green	1:1 ether:hexane
Ectocarpus sp.	0.04		Chars orange	1:1 ether:hexane
Fucus vesiculosis	No difference			
Halymenia trigona	No difference			
Padina gymnospora	No difference			
Petalonia fascia	0.28		Green	1:1 ether:hexane
Rhodomenia divaricata	No difference			
Sargassum filipendula		0.23	Green	1:1 ether:hexane
Scytosiphon lomentaria	0.45		UV activity	100% ether
Solieria filiformis		0.26	Chars orange	1:1 ether:hexane
Spatoglossum schroederi	No difference			
Zonaria tournefortii		0.60	UV activity	100% ether

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Chemical defenses of the Caribbean sponges *Agelas wiedenmayeri* and *Agelas conifera*

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ABSTRACT: Previous studies have determined that Caribbean reef sponges of the genus *Agelas* are chemically defended from fish predation by brominated pyrrole alkaloids, and that the compounds responsible for this defense have been elucidated for 1 species, *A. clathrodes*. In this study, we expand our understanding of chemical defense in this common sponge genus to include the characterization of defensive metabolites in the tissues of *A. wiedenmayeri* and *A. conifera*. Bioassay-directed isolation of defensive metabolites was undertaken using fish feeding assays carried out in laboratory aquaria and in the field. *A. wiedenmayeri* contained the same 2 major metabolites as *A. clathrodes*, 4,5-dibromopyrrole-2-carboxylic acid (**1**), and oroidin (**2**), in addition to a small amount of bromoageliferin (**7**). The 2 major metabolites were present at higher concentrations in samples of *A. wiedenmayeri* than in *A. clathrodes*, and their relative concentrations were reversed, with *A. wiedenmayeri* on average containing more 4,5-dibromopyrrole-2-carboxylic acid (**1**) (2.0 mg ml^{-1}) than oroidin (**2**) (0.8 mg ml^{-1}). *A. conifera* contained a mixture of dimeric bromopyrrole alkaloids dominated by sceptrin (**3**), with <10% each of dibromosceptrin (**5**), bromoageliferin (**7**), dibromoageliferin (**8**), ageliferin (**6**), and bromosceptrin (**4**). Mean concentration of sceptrin (**3**) in sponge tissue was 5.3 mg ml^{-1}; this compound deterred feeding of reef fish in aquarium assays at 1.0 mg ml^{-1}, the lowest concentration assayed. Sceptrin (**3**) concentrations were higher in sponges collected in the southern Bahama Islands than in those collected in the middle Bahamas, but the reasons for this variation remain unclear. The structure-activity relationship of the pyrrole group was investigated by assaying derivatives of the active metabolites. Feeding deterrent activity of the molecule was enhanced by the addition of bromine to the pyrrole group, but not affected by exchange of the heteroatom from N to O or S. Combining an understanding of the structure-activity relationship of *Agelas* metabolites with an understanding of the variation in these metabolites across the genus may provide insight into the evolution of defensive chemistry in this highly successful taxa of pan-tropical sponges.

KEY WORDS: Chemical defense · Sponge · *Agelas* · Caribbean · Alkaloids · Structure-activity relationship

INTRODUCTION

Sponges of the genus *Agelas* (Family Agelasidae) are important components of Caribbean coral-reef communities because they are abundant in a variety of habi-

tats from shallow back-reef to deep-reef slope. Chemically, *Agelas* spp. are distinct because they contain primarily brominated pyrrole alkaloids as secondary metabolites, although other classes of compounds have been isolated from this genus (Braekman et al. 1992). The ecological roles of these compounds have only recently been examined. The antipredatory effects of crude organic extracts of 6 species of Caribbean *Agelas* sponges (*A. clathrodes*, *A. conifera*, *A. dispar*, *A. inaequalis*, *A. sceptrum*, *A. wiedenmayeri*) were sur-

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veyed using a common generalist fish, the bluehead wrasse *Thalassoma bifasciatum*, as an assay organism (Pawlik et al. 1995). For *A. clathrodes*, 2 previously described major metabolites, 4,5-dibromopyrrole-2-carboxylic acid (**1**), and oroidin (**2**; Fig. 1), were successfully identified as the deterrent metabolites (Chanas et al. 1996). Besides these 2 brominated alkaloids, a series

of dimeric bromopyrrole alkaloids, the sceptrins and ageliferins, have been isolated from *A. conifera*, *A. sceptrum* and *A.* sp. (Walker et al. 1981, Kobayashi et al. 1990, Keifer et al. 1991). Although it was surmised that the dimeric brominated alkaloids found in other species of *Agelas* from the Caribbean deterred feeding (Pawlik et al. 1995, Chanas et al. 1996), purified compounds were never tested.

We identified the brominated alkaloid metabolites in the feeding deterrent fraction of crude extracts of *Agelas wiedenmayeri* and *A. conifera*. Inter- and intraspecific variation in the concentrations of these metabolites in 5 specimens of *A. wiedenmayeri* and in 24 specimens of *A. conifera* were quantified by HPLC. Purified samples of dimeric bromopyrrole alkaloids isolated from *A. conifera* were tested at a range of concentrations in aquarium and field assays. Building on a recent study of the structure-activity relationship of synthetic oroidin-related alkaloids as feeding deterrents that focused on the 2-amino-imidazole-moiety (Lindel et al. 2000), the present study further investigated the importance of modification of the pyrrole part in altering compound activity.

MATERIALS AND METHODS

Sponge collection and identification. This study was performed over the course of 3 research expeditions: one at the National Undersea Research Center in Key Largo, Florida, USA, in May 1998, one on board the RV 'Edwin Link' in the Bahama Islands in September and October 1998, and one on-board the RV 'Seward Johnson' in the Bahama Islands in July and August 1999. Collection sites in the Bahamas are shown in Fig. 2.

Agelas wiedenmayeri (Alcolado) and *A. conifera* (Schmidt) were collected by SCUBA diving at all sites. Portions of sponges were collected by cutting tissue with a sharp knife, leaving the remaining sponge intact for re-growth. For each species, replicate collections were taken from distant sites (>10 km) to avoid collecting asexually produced clones. Tissue samples were immediately frozen and stored at –20°C until used for extractions. Sponges were identified on the basis of spicule and tissue preparations at the Zoölogisch Museum, Universiteit van Amsterdam, The Netherlands (Assmann & van Soest unpubl. results).

Isolation of deterrent metabolites. A freeze-dried sample of *Agelas conifera* (473 g ≅ 2400 ml sponge tissue), collected in May 1998 at Elbow Reef, Key Largo, Florida, USA, was extracted 3 times in methanol, twice in 1:1 methanol:dichloromethane, and once in dichloromethane. The organic extracts were combined and evaporated to dryness. The obtained crude extract was partitioned between *n*-hexane and methanol.
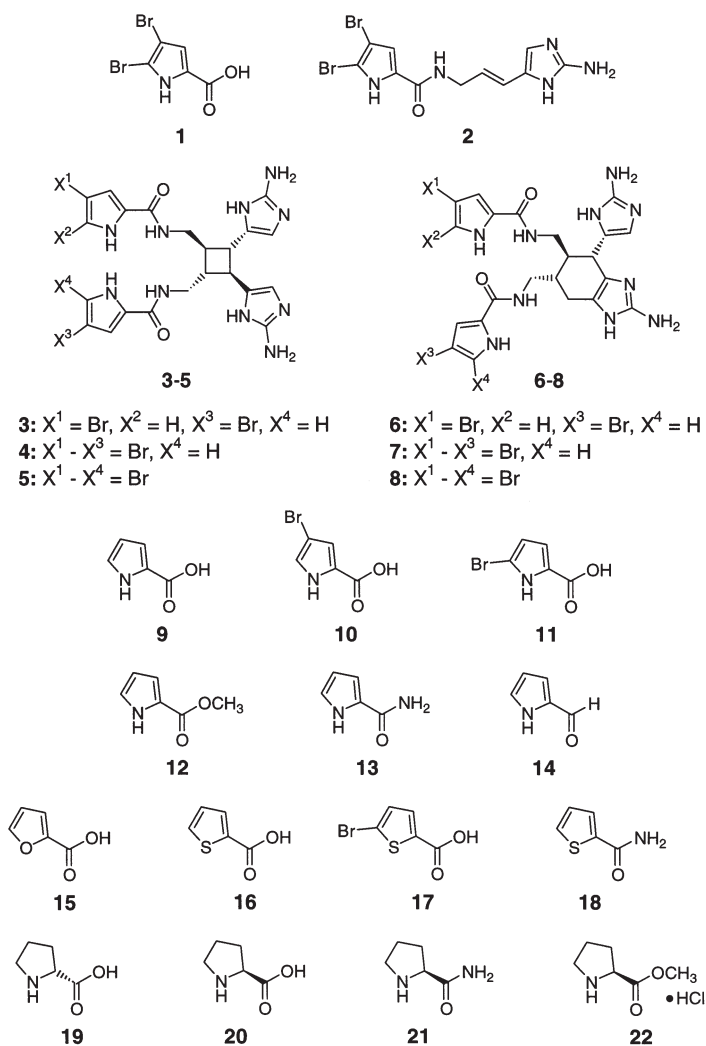

Fig. 1. Secondary metabolites isolated from *Agelas wiedenmayeri* (**1**, **2**, **7**) and *A. conifera* (**3–5**, **6–8**): 4,5-dibromopyrrole-2-carboxylic acid (**1**), oroidin (**2**), sceptrin (**3**), bromosceptrin (**4**), dibromosceptrin (**5**), ageliferin (**6**), bromoageliferin (**7**), dibromoageliferin (**8**). The remaining compounds were used to test structure-activity relationships, and were synthesized (**10–13**, **18**), or purchased (**9**, **14–17**, **19–22**): pyrrole-2-carboxylic acid (**9**), 4-bromopyrrole-2-carboxylic acid (**10**), 5-bromopyrrole-2-carboxylic acid (**11**), pyrrole-2-carboxylic acid methyl ester (**12**), pyrrole-2-carboxamide (**13**), pyrrole-2-carboxaldehyde (**14**), 2-furancarboxylic acid (**15**), thiophene-2-carboxylic acid (**16**), 5-bromothiophene-2-carboxylic acid (**17**), thiophene-2-carboxamide (**18**), D-proline (**19**), L-proline (**20**), L-prolinamide (**21**), L-proline methyl ester hydrochloride (**22**)

The methanol extract (18.9 g) was partitioned again between *n*-butanol and water. The *n*-butanol phase was concentrated and the residue (10.7 g) was purified by gel chromatography (Sephadex LH-20, methanol). A part of the fraction containing sceptrins and ageliferins (2.03 g) was further purified by preparative RP-HPLC to yield sufficient amounts of sceptrin (**3**), bromosceptrin (**4**), dibromosceptrin (**5**), ageliferin (**6**), bromoageliferin (**7**), and dibromoageliferin (**8**) for subsequent feeding assays. Isolation of brominated alkaloids from a specimen of *Agelas wiedenmayeri*, which had been collected in May 1998 at North Dry Rocks, Key Largo, was performed using a previously described procedure (Assmann et al. 1999). The isolated bromopyrrole alkaloids of both *A. conifera* and *A. wiedenmayeri* were identified by comparison of mass spectrometry and NMR data with those previously reported (Forenza et al. 1971, Garcia et al. 1973, Walker et al. 1981, Kobayashi et al. 1990, Keifer et al. 1991) as well as on the basis of 2D NMR data (COSY, HSQC, HMBC).

Extraction of crude extracts for quantification of metabolites. Sponge volume was determined by displacement of water or solvent with frozen material. Frozen tissue (*Agelas conifera*), or freeze-dried tissue (approximately 1.9 g freeze-dried material corresponds to 10 ml frozen sponge tissue of *A. wiedenmayeri*), from individual sponge specimens was chopped into small pieces and added to 40 ml of a 1:1 mixture of dichloromethane:methanol in a graduated centrifuge tube to a final volume of 50 ml. Capped tubes were inverted, agitated repeatedly, and shaken at room temperature during 24 h extraction time. After extraction, both phases were filtered and the solvent mixture was evaporated to dryness on a rotary evaporator using low heat (40°C). The remaining tissue was ex-tracted by shaking a second time with methanol for 24 h at room temperature, and the resulting extract was filtered. The organic extracts (methanol extract and dichloromethane:methanol extract) were combined and evaporated. The obtained residue was dissolved in a mixture of 15 ml methanol and 15 ml methanol:dichloromethane and transferred into 50 ml graduated centrifuge tubes. From this mixture a volume of 1.5 ml was kept for subsequent HPLC quantification. The remaining solvent (28.5 ml) was removed by Speed-Vac vacuum concentration and finally dried under vacuum. Three replicate crude extracts of each specimen were prepared in the same way and quantified by HPLC.

Quantification of secondary metabolites by HPLC. From the 1.5 ml volume of each crude extract saved for HPLC quantification, 200 µl were transferred to a vial and the solvent removed by Speed-Vac vacuum concentration. The obtained residue was dissolved in 500 µl acetonitrile:water 1:1 + 0.5% trifluoroacetic acid and 10 µl injected by auto-sampling into a HPLC
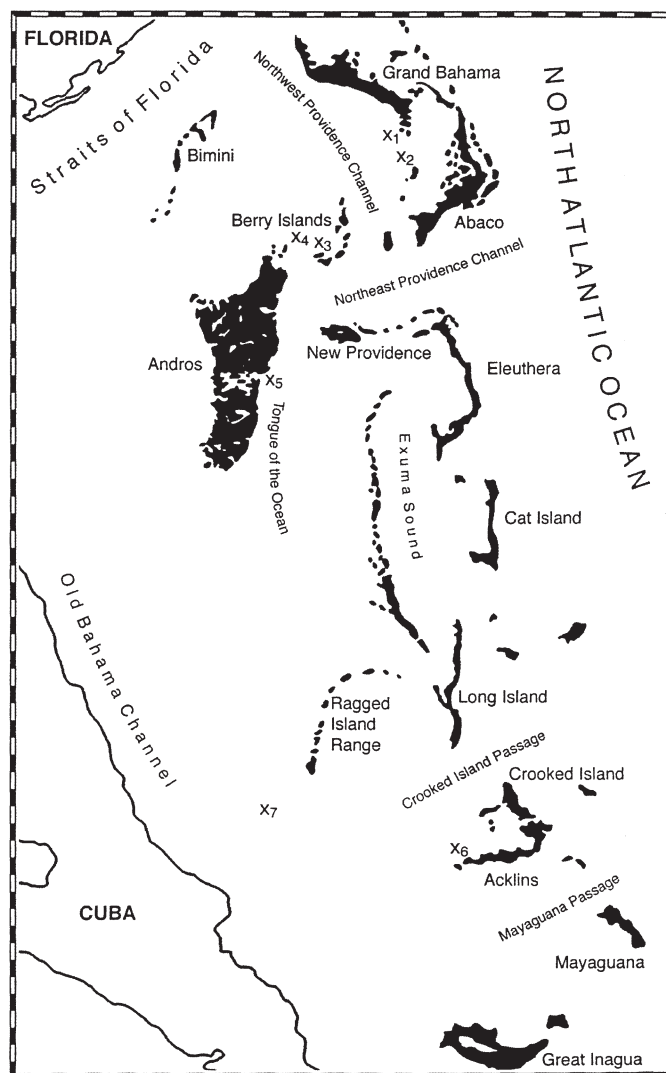

Fig. 2. Map of the Bahama Islands indicating collection sites of *Agelas conifera*. x_1: Sweetings Cay; x_2: Black Rock; x_3: Chub Cay; x_4: 33 km west of Chub Cay; x_5: Behring Point; x_6: Acklins; x_7: Cay Santo Domingo

system equipped with a photodiode-array detector (JASCO). Routine UV detection was at 280 nm. The separation column (analytical: 4.6×250 mm, 5 µm) was pre-filled with Kromasil RP-18 (Knauer GmbH) (gradient: 20 to 60% acetonitrile:water + 0.1% trifluoroacetic acid in 40 min; flow rate: 1 ml min^{-1}). Each quantitative analysis based on peak area calibration was achieved using purified sceptrin (**3**) (*Agelas conifera*) or oroidin (**2**) and 4,5-dibromopyrrole-2-carboxylic acid (**1**) (*A. wiedenmayeri*) as an external standard.

Synthesis of pyrrole-2-carboxylic acid derivatives. For studying the structure-activity relationship of the pyrrole part of bromopyrrole alkaloids, brominated pyrrole-2-carboxylic acid derivatives were either purchased commercially (**9**, **14**–**17**, **19**–**22**) or synthesized

(**10–13**, **18**) following procedures previously described by Bailey et al. (1971) and Anderson & Lee (1965). Structures of the compounds were determined by mass spectrometry and NMR spectroscopy.

Aquarium feeding assays. Purified natural or synthetic compounds were dissolved in a minimal volume of methanol and mixed with 1 ml of alginate-based food matrix (Pawlik et al. 1987, 1995) until all organic and water-soluble components were distributed uniformly throughout the paste. The alginate food matrix was then dispensed with a 1 ml syringe into a 0.25 M calcium chloride solution forming a strand that was allowed to harden for 2 min. The hardened strand was rinsed with filtered seawater and cut into 3 mm pellets with a scalpel. Control pellets were prepared identically but without the addition of natural or synthetic compounds. Feeding assays were performed with fish (1 terminal phase and 2 females in each of 10 compartments) in aquaria on board the RV 'Seward Johnson' or at the University of North Carolina at Wilmington using a common predatory reef fish, the bluehead wrasse *Thalassoma bifasciatum*. Rationale for the choice of this assay fish, and an explanation of the methods for scoring the assay and the statistical analysis, is well described in Pawlik et al. (1995). Each replicate assay was performed on a separate group of fish.

Field feeding assay. For field assays, a purified mixture containing sceptrins (**3–5**) and ageliferins (**6–8**) of *Agelas conifera* from a 60 ml volume sponge tissue was dissolved in a minimal volume of methanol and combined with 60 ml of preheated carrageenan-based food (Chanas & Pawlik 1995). Food dyes were added to both treated and control foods to make them the same color. The mixture was then poured into molds crossed by lengths of cotton string and allowed to harden. After hardening, 20 string-embedded strips were cut from the molds. Control strips were prepared identically, but without the addition of bromopyrrole alkaloids. Field assays were conducted on shallow water reefs (<15 m) off the Bahamas (South Bimini, North Turtle Rock) using previously described methods regarding deployment, retrieval and statistical analyses (Chanas & Pawlik 1995).

RESULTS

It had previously been established that the feeding deterrent activity of crude organic extracts of Caribbean *Agelas* spp. was localized in the brominated pyrrole alkaloid fraction of the extract (Pawlik et al. 1995, Chanas et al. 1996). For *A. wiedenmayeri*, the alkaloid fraction contained 4,5-dibromopyrrole-2-carboxylic acid (**1**), oroidin (**2**), and bromoageliferin (**7**), in the approximate mixture of 63, 28 and 9%, respectively. For *A. conifera*, the fraction contained dimeric alkaloids in the approximate proportion of 68% sceptrin (**3**), 9% dibromosceptrin (**5**), 8% bromoageliferin (**7**), 6% dibromoageliferin (**8**), 5% ageliferin (**6**), and 4% bromosceptrin (**4**).

The major metabolites in the alkaloid mixtures for both sponges were quantified by HPLC for 5 specimens of *Agelas wiedenmayeri* and for 24 specimens of *A. conifera* (Tables 1 & 2). Mean concentrations of 4,5-dibromopyrrole-2-carboxylic acid (**1**) and oroidin (**2**) in the 2 samples of *A. wiedenmayeri* were 2.00 and 0.82 mg ml^{-1}, respectively. Concentrations of oroidin in the 2 sponge samples from Cay Sal Bank (Bahamas) were much higher than those from Key Largo, Florida, but the low sample number precluded statistical analysis.

The mean concentration of sceptrin (**3**) in all samples of *A. conifera* was 5.3 mg ml^{-1} (Table 2). When the data were subdivided into 3 sets of samples collected in the northern Bahamas, middle Bahamas, and southern Bahamas, a significant difference in mean sceptrin content was found between the middle and southern collections, but not between the northern and southern collections or the northern and middle collections (ANOVA, $F_{2,21} = 4.03$, p < 0.05; Tukey multiple comparison, $\alpha = 0.05$; Zar 1999).

The 4 major bromopyrrole alkaloids present in the feeding deterrent fraction from the crude organic ex-

Table 1. Concentration of 4,5-dibromopyrrole-2 carboxylic acid (**1**) and oroidin (**2**) in samples of *Agelas wiedenmayeri* from the Florida Keys* and the Bahamas**

Sample	Location	Date (d.mo.yr)	Depth (m)	4,5-dibromo-pyrrole-2-carboxylic acid (mg ml^{-1}) mean ± SD	Oroidin (mg ml^{-1}) mean ± SD
MAWIE1	North Dry Rocks*	26.05.1998	9	2.51 ± 0.14	0.10 ± 0.03
MAWIE2	Conch*	29.05.1998	16	1.65 ± 0.16	0.72 ± 0.01
MAWIE3	North Dry Rocks*	23.05.1998	6	2.42 ± 0.15	0.20 ± 0.06
MAB146	Cay Sal Bank**	08.08.1999	6	1.83 ± 0.20	1.86 ± 0.36
MAB147	Cay Sal Bank**	08.08.1999	6	1.61 ± 0.76	1.23 ± 0.95
Mean				2.00 ± 0.43	0.82 ± 0.75

Table 2. Concentration of sceptrin (**3**) in samples of *Agelas conifera* from the Bahamas

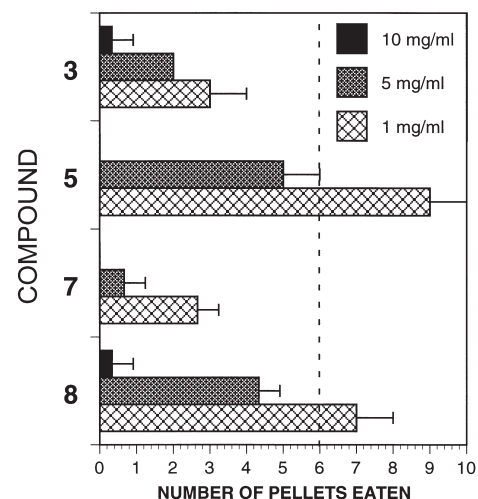| Sample	Location	Date (d.mo.yr)	Depth (m)	Sceptrin (mg ml^{-1}) mean ± SD
Northern Bahamas				
MAB23	Black Rock	18.09.1998	5	7.0 ± 4.6
MAB03	Sweetings Cay	16.09.1998	18	4.1 ± 2.0
MAB76	Sweetings Cay	29.07.1999	18	3.7 ± 3.4
MAB14	Sweetings Cay	17.09.1998	19	5.8 ± 0.2
Mean				5.15 ± 1.53
Middle Bahamas				
MAB35	Chub Cay	19.09.1998	4	3.3 ± 1.7
MAB38	Chub Cay	19.09.1998	11	5.2 ± 0.4
MAB85	Chub Cay	30.07.1999	16	3.3 ± 0.3
MAB87	Chub Cay	30.07.1999	17	7.7 ± 1.4
MAB32	Chub Cay	19.09.1998	18	3.3 ± 1.6
MAB86	Chub Cay	30.07.1999	18	5.1 ± 0.2
MAB29	Chub Cay	19.09.1998	20	2.9 ± 1.4
MAB84	Chub Cay	30.07.1999	20	3.5 ± 0.9
MAB26	Chub Cay	19.09.1998	21	3.1 ± 3.6
MAB28	Chub Cay	19.09.1998	21	5.2 ± 1.4
MAB25	Chub Cay	19.09.1998	22	4.0 ± 0.9
MAB93	Chub Cay	30.07.1999	24	8.9 ± 1.8
MAB43	33 km west of CC	21.09.1998	18	3.3 ± 1.0
MAB100	Behring Point	31.07.1999	18	6.6 ± 2.1
MAB102	Behring Point	31.07.1999	19	5.4 ± 0.2
MAB101	Behring Point	31.07.1999	22	4.8 ± 0.3
Mean				4.73 ± 1.77
Southern Bahamas				
MAB144	Cay Santo Domingo	07.08.1999	20	8.7 ± 0.7
MAB130	Acklins Island	05.08.1999	21	4.3 ± 0.5
MAB129	Acklins Island	05.08.1999	23	11.0 ± 0.1
MAB131	Acklins Island	05.08.1999	30	7.1 ± 1.1
Mean				7.78 ± 2.82
Grand mean				5.30 ± 2.16

Fig. 3. Aquarium assay results of feeding by *Thalassoma bifasciatum* on pellets treated with sceptrin (**3**), dibromosceptrin (**5**), bromoageliferin (**7**), and dibromoageliferin (**8**) isolated from *Agelas conifera* at concentrations of 1, 5, and 10 mg ml^{-1}. All control pellets were eaten in all assays. Three replicate assays were performed at each concentration. One SD above the mean number of food pellets eaten is indicated. For any individual assay, a treatment was considered deterrent if the number of pellets eaten was less than or equal to 6 (p < 0.043, Fisher exact test, 1-tailed) as indicated by the dashed line

tract of *Agelas conifera* were isolated, purified, and subjected to feeding assays at concentrations of 1, 5 and 10 mg ml^{-1} (Fig. 3). Sceptrin (**3**) and bromoageliferin (**8**) were deterrent at all 3 concentrations, while dibromosceptrin (**5**) and dibromoageliferin (**8**) deterred feeding in aquarium assays at 5 and 10 mg ml^{-1}, but not at 1 mg ml^{-1} (Fig. 3). Only the major metabolite sceptrin (**3**) deterred fish feeding within the range of natural concentrations (Table 2). Sceptrin (**3**) was subsequently tested at lower concentrations and was not significantly deterrent: 8 of 10 pellets eaten at 0.8 mg ml^{-1} and 10 of 10 pellets eaten at 0.5 mg ml^{-1}.

A reconstituted mixture of purified dimeric bromopyrrole alkaloids from *Agelas conifera* deterred feeding of a natural assemblage of reef fish in a field assay (Fig. 4). This assay was performed at a total natural compound concentration of 1.5 mg ml^{-1}, with the following compound proportions approximating those found in sponge tissue by HPLC quantification: sceptrin (**3**) (1.02 mg ml^{-1}), dibromosceptrin (**8**) (0.13 mg ml^{-1}), bromoageliferin (**7**) (0.12 mg ml^{-1}), dibromo-
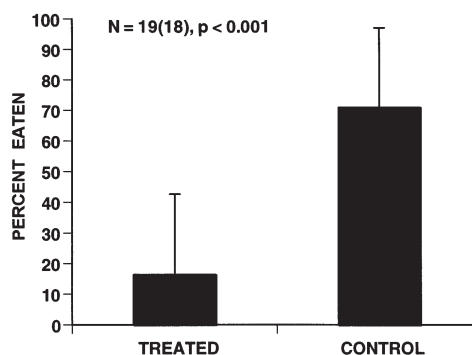

Fig. 4. Consumption by reef fishes of food strips treated with a purified mixture isolated from *Agelas conifera* containing only dimeric bromopyrrole alkaloids at natural concentrations and control food strips in a field assay. The tested mixture contained 68.3% sceptrin (**3**), 8.6% dibromosceptrin (**5**), 7.7% bromoageliferin (**7**), 6.1% dibromoageliferin (**8**), 4.7% ageliferin (**6**), and 3.5% bromosceptrin (**4**). Mean + 1 SD indicated. p-value computed using Wilcoxon paired-sample test (2-tailed); N = number of ropes retrieved out of 20 ropes deployed (number of ropes used in statistical analysis)
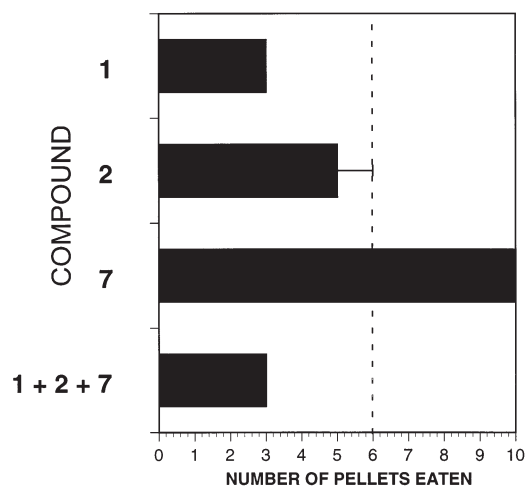
Fig. 5. Aquarium assay results of feeding by *Thalassoma bifasciatum* on pellets treated with purified bromopyrrole alkaloids isolated from *Agelas wiedenmayeri* at natural concentrations: 4,5-dibromopyrrole-2-carboxylic acid (**1**) at 1.6 mg ml^{-1}, oroidin (**2**) at 0.7 mg ml^{-1}, and bromoageliferin (**7**) at 0.24 mg ml^{-1}. All control pellets were eaten in all assays. Three replicate assays were performed at each concentration. Mean + 1 SD indicated. For any individual assay, a treatment was considered deterrent if the number of pellets eaten was less than or equal to 6 (p < 0.043, Fisher exact test, 1-tailed) as indicated by the dashed line

ageliferin (**8**) (0.09 mg ml^{-1}), ageliferin (**6**) (0.07 mg ml^{-1}), bromosceptrin (**4**) (0.05 mg ml^{-1}).

The purified bromopyrrole alkaloids from *Agelas wiedenmayeri* were subjected to aquarium assays separately and in combination (Fig. 5). At natural concentrations of 1.6 and 0.7 mg ml^{-1}, 4,5-dibromopyrrole-2-carboxylic acid (**1**) and oroidin (**2**) were deterrent when assayed separately, but bromoageliferin (**7**) was not deterrent at 0.24 mg ml^{-1} when assayed separately. The activity of all 3 metabolites combined was about the same as that for 4,5-dibromopyrrole-2-carboxylic acid (**1**) alone (Fig. 5).

Initial results of the structure-activity relationship of pyrrole-2-carboxylic acid derivatives were obtained. Activity of the molecule was enhanced with addition of bromine to the pyrrole group (compare **9**, **10**, **11**, **1**; Fig. 6). Compound activity was not affected by the exchange of the heteroatom from N to O or S (compare **9**, **15**, **16**, **11**, **17**; Fig. 6), whereas none of the proline derivates were active at 100 mg ml^{-1} (**9** compared to **19**, **20**; Fig. 6). Modification of the carboxy group did not lead to an unambiguous change in activity (**9** compared to **12**–**14**).

DISCUSSION

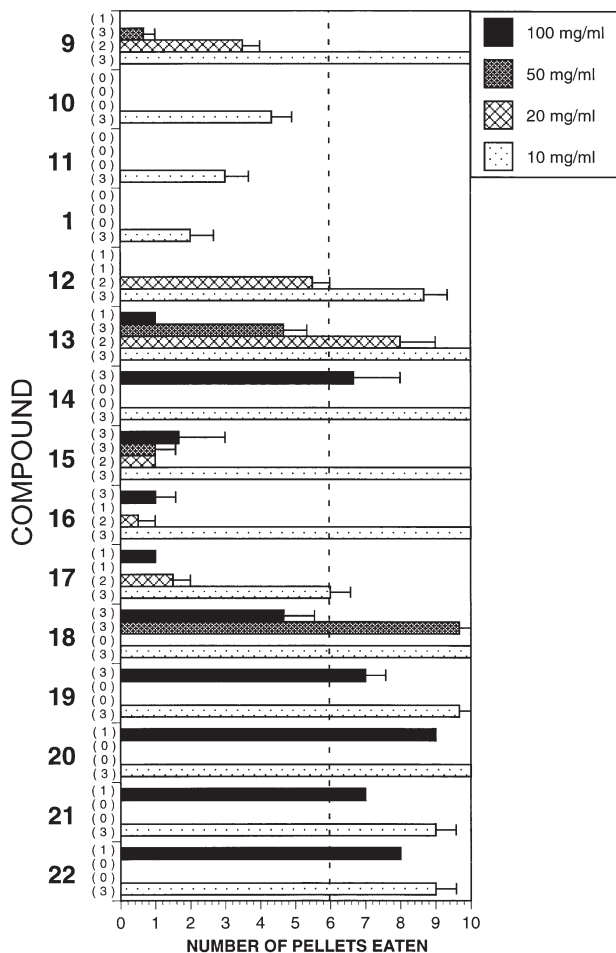Sponges of the genus *Agelas* appear to protect themselves from fish predators using chemical defenses,

Fig. 6. Aquarium assay results of feeding by *Thalassoma bifasciatum* on pellets treated with 1 purified compound from *Agelas wiedenmayeri*, and synthetic and purchased compounds used to assess structure-activity relationships (see Fig. 1) at concentrations of 10, 20, 50, and 100 mg ml^{-1}. All control pellets were eaten in all assays. The number of replicate assays is shown in parentheses to the right of each compound number. Mean + SE indicated. For any individual assay, a treatment was considered deterrent if the number of pellets eaten was less than or equal to 6 (p < 0.043, Fisher exact test, 1-tailed) as indicated by the dashed line

because structural defenses alone, in the form of spongin fibers and glass spicules, were ineffective feeding deterrents in aquarium and field assays (Chanas & Pawlik 1995, 1996). The present study expands on a previous report (Chanas et al. 1996) that identified 4,5-dibromopyrrole-2-carboxylic acid (**1**) and oroidin (**2**) as the defensive metabolites of *A. clathrodes* to demonstrate that the same metabolites protect *A. wiedenmayeri*, and that related, dimeric metabolites protect *A. conifera*.

Similarities in the secondary metabolites found in *Agelas clathrodes* and *A. wiedenmayeri* versus *A. conifera* parallel differences in the silicious spicules found

in the tissues of the 3 species of sponges (Wiedenmayer 1977, Assmann et al. 1999). Although shape, size, and color of all 3 *Agelas* species are distinctly different, *A. clathrodes* and *A. wiedenmayeri* appear more closely related based on similar spicule sizes: 95 to 140 × 4 to 8 µm, with 9 to 13 whorls of spines for *A. wiedenmayeri*, and 70 to 155 × 2.5 to 7.5 µm with 7 to 15 whorls of spines for *A. clathrodes*. For *A. conifera*, spicules are clearly longer and thicker (117 to 192 × 10 to 16 µm) and the whorls are more numerous (11 to 19) than in *A. clathrodes* and *A. wiedenmayeri*.

Although the same metabolites are found in *Agelas wiedenmayeri* as in *A. clathrodes*, the relative concentrations are different. Mean oroidin (**2**) concentrations in *A. clathrodes* were 1.4 mg ml^{-1}, with concentrations of 4,5-dibromopyrrole-2-carboxylic acid (**1**) that were estimated at 25% of those of oroidin (**2**) (Chanas et al. 1996). In contrast, for 4 of 5 samples of *A. wiedenmayeri*, concentrations of 4,5-dibromopyrrole-2-carboxylic acid (**1**) exceeded those of oroidin (**2**), and in the remaining sample, the concentrations were about the same (Table 1). The combined mean concentration of both of these metabolites was greater in samples of *A. wiedenmayeri* (~2.82 mg ml^{-1}) than in *A. clathrodes* (~1.75 mg ml^{-1}). Considering that 4,5-dibromopyrrole-2-carboxylic acid (**1**) and oroidin (**2**) were deterrent at 1.6 and 0.7 mg ml^{-1}, respectively (Fig. 5), the former compound was present in sufficient quantity in all samples of *A. wiedenmayeri* to inhibit feeding (Table 1); in combination with the latter compound, the effect is most likely additive, as a synergistic response to the mixture was not evident (Fig. 5). There was no evidence of synergy when combinations of brominated pyrrole alkaloids were tested in a previous study (Lindel et al. 2000). Although bromoageliferin (**7**) was present in samples of *A. wiedenmayeri*, its concentration was very low (<10% of the bromopyrrole alkaloid fraction), and its impact of the feeding deterrent effect of the mixture was negligible (Fig. 5).

Unlike *Agelas clathrodes* and *A. wiedenmayeri*, *A. conifera* is chemically defended by dimeric bromopyrrole alkaloids (Fig. 1). This suite of compounds (**3**–**8**) appear to be more potent feeding deterrents than either 4,5-dibromopyrrole-2-carboxylic acid (**1**) or oroidin (**2**) (compare Figs. 3, 5 & 6), perhaps because each dimeric molecule contains 2 brominated pyrrole groups rather than just one. With more potent chemical defenses on a per molecule basis, it might be expected that *A. conifera* would have lower concentrations of these compounds than *A. clathrodes* or *A. wiedenmayeri*; on the contrary, sceptrin (**3**) concentrations alone in *A. conifera* are about twice those of 4,5-dibromopyrrole-2-carboxylic acid (**1**) and oroidin (**2**) in *A. wiedenmayeri* (Tables 1 & 2). Moreover, there were higher levels of sceptrin (**3**) in samples of *A. conifera* taken from the

southern Bahamas than those taken from sites around the 'Tongue of the Ocean', a deep body of water that is mostly enclosed by land and the Great Bahama Bank in the middle Bahamas. Fluctuations in sceptrin (**3**) concentrations were not compensated by changes in the concentrations of the other dimeric compounds identified from this species to yield equivalent total concentrations; HPLC analysis revealed that the proportion of the minor compounds fluctuated proportionally to sceptrin (**3**). It is unclear whether differences in sceptrin (**3**) concentration reflect differences in predation pressure, or perhaps differences in water quality or flow associated with geography.

The first systematic investigation of the structure-activity relationship between marine natural products and fish feeding deterrents was performed for brominated pyrrole alkaloids from *Agelas* spp. (Lindel et al. 2000). The present study expands on the importance of the pyrrole moiety for fish feeding inhibition by demonstrating the importance of the unsaturated 5-membered ring (pyrrole). Substitution of the heteroatom in the ring did not greatly affect compound activity, but the loss of unsaturation resulted in the loss of activity (Fig. 6). The importance of bromination was clearly demonstrated, as compound palatability increased with each loss of a bromine atom from the pyrrole (Fig. 6). Dimerization of oroidin (**2**) does not appear to alter compound activity; in fact, the brominated pyrrole groups appear to retain their independent activity to increase the potency of the dimerized compounds (**3**–**8**). Comparing the relative feeding deterrent activities of the major naturally occurring metabolites at 1 mg ml^{-1} concentrations, but expressed as mol ml^{-1}, a hierarchy of activity can be determined: bromoageliferin (**7**) = sceptrin (**3**) > dibromoageliferin (**8**) = dibromosceptrin (**5**) > oroidin (**2**) > 4,5-dibromopyrrole-2-carboxylic acid (**1**). Although dibromoageliferin (**8**), dibromosceptrin (**5**) and bromoageliferin (**7**) are potent feeding deterrents, they are present in only trace amounts in sponge tissue, at concentrations lower than would be required to deter feeding. Sceptrin (**3**) is the only dimeric compound present in sponge tissue at concentrations sufficient to defend *A. conifera*. The minor dimeric compounds may represent metabolic byproducts.

These results extend our understanding of the chemical basis of the ecological functions of bromopyrrole alkaloids in marine sponges and confirm the importance of the presence and arrangement of functional groups to compound activity.

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Biogeographic comparisons of chemical and structural defenses of the Pacific gorgonians *Annella mollis* and *A. reticulata*

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ABSTRACT: Compared to other areas of chemical ecology, biogeographic comparisons of the chemical defenses associated with benthic marine organisms are few. This study addresses geographic differences in the chemical and structural defenses of 2 Pacific gorgonians (*Annella mollis* and *A. reticulata*), found at 2 islands: Guam, Micronesia, and Lizard Island, Australia. Crude extracts and sclerites extracted from the mid-axis and tips of colonies were assayed against natural assemblages of reef fishes at Western Shoals, Guam, and Mermaid's Cove, Lizard Island. Reciprocal feeding assays clearly demonstrated that crude extracts from *Annella* spp. were unpalatable to natural assemblages of reef fishes at Western Shoals and Mermaid's Cove and sclerites have little or no role as generalist predator defenses. Sclerites from the tips of *A. reticulata* were only effective as feeding deterrents at high concentrations. Variation in the palatability of the mid-axis extracts of *A. mollis* suggested that chemical defenses are more concentrated at the tips of the colonies from Guam and Lizard Island. When assayed at concentrations similar to and higher than the tips the mid-axis extracts did not deter fish feeding at Mermaid's Cove.

KEY WORDS: Biogeography · Chemical defenses · Marine chemical ecology · *Annella mollis* · *Annella reticulata* · Indo-pacific · Gorgonian

INTRODUCTION

Biogeographic comparisons of defenses in marine plants and invertebrates have evaluated qualitative and quantitative differences in secondary metabolites (Steinberg 1989, 1992, Targett et al. 1992, Harvell et al. 1993, Pavia & Aberg 1996, Van Alstyne et al. 1999) or directly tested the responses of generalist consumers to organic extracts or plant materials (Van Alstyne & Paul 1990, Bolser & Hay 1996, Cronin et al. 1997, Pennings et al. in press). In comparisons between temperate and tropical species, higher levels of predator-deterrent compounds have been shown to be more common in tropical species (Bakus & Green 1974, Bakus 1981,

Bolser & Hay 1996), although there is evidence to suggest that this is not always the case (Steinberg 1992, Targett et al. 1992). The higher instance of defenses in marine organisms from tropical regions is proposed to be a consequence of increased predation and competition at lower latitudes (Bakus & Green 1974, Bolser & Hay 1996). This hypothesis is supported by comparisons made at the consumer level between temperate and tropical sea urchins and fish (Bolser & Hay 1996, Cronin et al. 1997). Overall, consumers from North Carolina and the Bahamas preferred temperate algal species in paired assays when offered side-by-side with their congeners from the tropics (Bolser & Hay 1996). In assays with sea urchins and fish from North Carolina and Guam, lower concentrations of pure metabolites from the brown alga *Dictyota acutiloba* were needed to deter the temperate herbivores com-

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pared to the tropical species (Cronin et al. 1997). Cronin et al. (1997) proposed that tropical herbivores (i.e. in Guam) are more resistant to seaweed chemical defenses.

Comparisons among species of brown algae from geographically different regions show that polyphenol (phlorotannins) concentrations can exhibit considerable latitudinal and local variation (Steinberg 1989, 1992, Steinberg & Van Altena 1992, Pavia & Aberg 1996, Van Alstyne et al. 1999). On the global scale, brown algae from Australia and New Zealand were shown to produce higher concentrations of polyphenols compared to their North American counterparts (Steinberg 1989, 1992). On the more local scale, Pavia & Aberg (1996) reported considerable variability in polyphenol concentrations between 2 areas in the North Atlantic separated by >1000 km. Site-to-site comparisons in kelp and rockweeds along the north Pacific coast of the United States showed that only 25% of the kelps and 3 of 4 rockweeds exhibited geographic variation in polyphenol concentrations, suggesting that localized selection or phenotypic plasticity may be phylogenetically constrained (Van Alstyne et al. 1999). More recently, Pennings et al. (in press) addressed variability in herbivore defenses of marsh plants along the Atlantic coast of the USA with a variety of consumers. Reciprocal feeding assays clearly demonstrated that fresh marsh plant material from Rhode Island and Maine was more palatable to consumers from both northern and southern regions than plants from Florida and Georgia. Local variation of herbivore communities within temperate regions is suggested to select for these latitudinal trends (Steinberg 1992, Pennings et al. in press). Pavia & Aberg (1996) point out the importance of local herbivores with a limited habitat range in explaining the differences in defenses between local communities. Early work on coral reefs suggested that almost all of the common, exposed coral reef invertebrates are chemically and/or structurally defended from predators (Bakus & Green 1974, Green 1977, Bakus 1981). Because chemical and structural defenses are prevalent in tropical benthic marine organisms (Hay 1996) few studies have addressed local variation in defenses between tropical regions (Harvell et al. 1993) compared to tropical versus temperate and temperate versus temperate comparisons.

Gorgonian corals are conspicuous members of coral reef communities (Kinzie 1970, Yoshioka & Yoshioka 1989). Except for a few specialist predators, coral reef fish and invertebrates do not readily consume sea fans and whips (Lasker 1985, Harvell & Suchanek 1987, Lasker & Coffroth 1988, Ruesink & Harvell 1990, Van Alstyne & Paul 1992, Vrolijk & Targett 1992, Cronin et al. 1995, Slattery 1999). Gorgonians produce chemical (i.e. secondary metabolites) and/or structural (i.e. scle-

rites) defenses against predation (Pawlik et al. 1987, Harvell et al. 1988, 1996, Fenical & Pawlik 1991, Pawlik & Fenical 1992, Van Alstyne & Paul 1992, West 1997, 1998). Some species exhibit intra-colony variation in secondary metabolite and sclerite concentrations suggesting that different parts of the colony rely more upon chemical defenses and others rely upon structural defenses (Harvell & Fenical 1989, Van Alstyne & Paul 1992). For example, the Caribbean gorgonians *Pseudopterogorgia* spp. have higher concentrations of metabolites at the tips of colonies and more sclerites in the base (Harvell et al. 1988, Harvell & Fenical 1989). In other species, crude extract and sclerite concentrations are uniform throughout the colony (Van Alstyne & Paul 1992, Slattery 1999). *Gorgonia ventalina*, also found in the Caribbean, exhibited no quantitative variation in crude extract and sclerite concentrations in the upper portions of the colony (Van Alstyne & Paul 1992). However, Kim et al. (2000) have shown that crude extracts from the tips of healthy *Gorgonia* spp. colonies are more resistant to the fungal pathogen *Aspergillus sydowii* than extracts from other parts of the colony.

For some species, a variety of different secondary metabolites have been reported from colonies collected from discrete regions (Faulkner 1999 and references cited within). A geographic comparison of colonies of the Caribbean gorgonian *Briareum asbestinum* from the Bahamas and St. Croix showed that the 2 populations produced different classes of diterpenes as chemical defenses (Harvell et al. 1993). Further, in shallow habitats where colonies are exposed to higher levels of predation, *B. asbestinum* produced smaller sclerites at higher densities as structural defenses (West et al. 1993).

Biogeographic comparisons are thought to be essential to the understanding of the evolution and ecology of coral reef communities (Hay 1996, Sammarco 1996). In this study we address quantitative differences in crude extract and sclerite concentrations within colonies (base, mid-axis, tips) of 2 Pacific gorgonians, *Annella mollis* and *A. reticulata*, and among sites at 2 islands, Guam (GU) and Lizard Island (LI). We assay crude extracts and sclerites from the mid-axes and tips of the colonies against natural assemblages of reef fishes at: (1) their island of origin and (2) the other island. Previous studies of gorgonian corals on Caribbean reefs suggest that there is considerable variability in the production of chemical and structural defenses among species (Pawlik et al. 1987, Harvell & Fenical 1989), making predictions of intra-colony variation in *Annella* spp. difficult. However, a broad survey of gorgonian crude extracts demonstrated that the extracts from species with small sclerites were usually unpalatable to fish (Pawlik et al. 1987, Harvell & Fenical 1989). Small, colorless sclerites are characteristic of

sea fans in the genus *Annella* (Chen & Chang 1991); therefore we expected the crude extracts to be unpalatable to natural assemblages of reef fish. GU and LI are tropical, and predation should be intense at both islands (Hay 1996), suggesting that populations of *Annella* spp. will be under similar or equal pressure to produce defenses against generalist predators. This hypothesis predicts no differences in chemical and/or structural defenses among sites or between islands. Alternatively, local predators associated with different collection sites can result in differences in defenses (Pavia & Aberg 1996).

METHODS AND MATERIALS

Study organisms. The *Annella* spp. (formerly *Subergorgia* spp., Family: Subergorgiidae) (Grassoff 1999) are azooxanthellate gorgonians common in the Indo-Pacific from the northern Red Sea to the central Pacific. They are the largest and most conspicuous sea fans on the reefs of GU, growing to 2–3 m at depths of >20 m. At LI, *Annella* spp. can be found on reefs as shallow as 5 m and at depths greater than 25 m. Sea fans in this genus have branches closely anastomosed as a network. The sclerites are small and colorless (Chen & Chang 1991). *A. mollis* (Nutting) is typically brown or orange with oblong cells. 'Double-wheel' sclerites characteristic of this species are found densely packed in the coenenchyme (Grassoff 1999). *A. reticulata* (Ellis & Solander) is pink to orange with small cells characterized by 'double-head' sclerites. The larger, oblong cells of *A. mollis* and small cells of *A. reticulata* are often found in the same colony. In addition, the 'double-heads' and 'double-wheels' can be found in the same colony (Phil Alderslade pers. comm.). Colonies used in this study were identified by sclerite analysis and limited to those specimens that contained either the 'double-heads' or the 'double-wheels'.

Collection. Sea fans were collected by SCUBA from sites around GU (144° 45′ E, 13° 30′ N) between March and July 1998 (Fig. 1) and sites around LI (145° 28′ E, 14° 41′ S) between January and February 1999 (Fig. 2). GU is a high island with fringing reefs. LI is a continental island on the Great Barrier Reef approximately 35 km off the coast of Queensland, Australia. *Annella mollis* was collected from Blue Hole, Cocos Wall and Hospital Point, GU, between 30 and 40 m. Collections at LI were made at Mac Gillivray's Reef, North Point and Pidgin Point between 10 and 20 m. *A. reticulata* was collected from the wall between the Blue Hole and Crevice and at Hospital Point, GU, between 30 and 40 m, and from Bird Islets, Mac Gillivray's Reef and Pidgin Point, LI, between 10 and 15 m. Voucher specimens were preserved in 70% ethanol or 10%
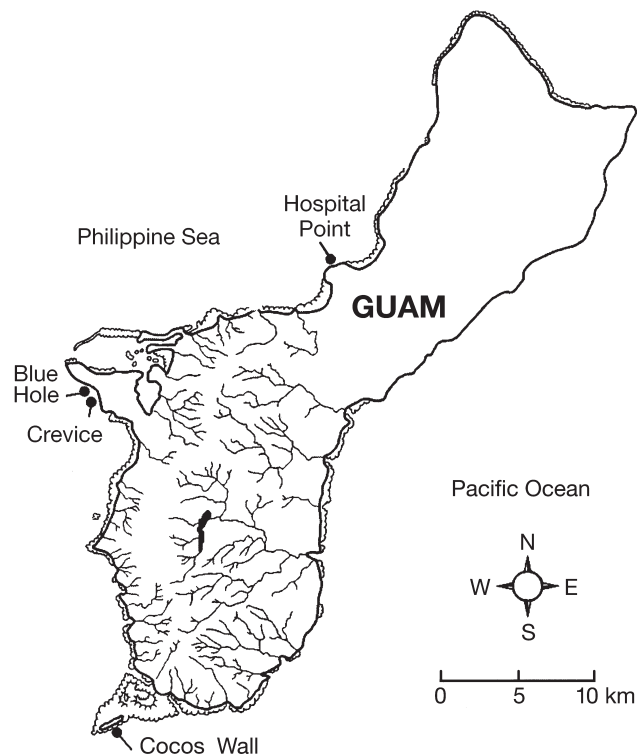

Fig. 1. Collection sites at Guam

formalin and air-dried depending upon what was available on site. A representative of each species was deposited in the collections at the University of Guam Marine Lab (UOGML), Museum and Art Gallery of the Northern Territories, Darwin, and/or Australian Museum of Natural History (all Australian samples), Sydney.

Between 2 and 6 individual colonies were removed from each site by cutting at the base of the sea fan with underwater shears. Colonies were separated into base (lowest 10 cm of colony), mid-axis (the center of the colony between the base and tip) and tip (top 5 cm of colony around the outer edges) sections (Harvell & Fenical 1989, Van Alstyne & Paul 1992). When possible, colonies were extracted immediately at the UOGML or LI Research Station (LIRS); otherwise, colonies were frozen at 0°C and freeze-dried for transport to the University of Mississippi, Oxford, or the University of New South Wales, Sydney, for extraction.

Crude extract, gorgonin and sclerite concentrations. A 3 × 3 cm square piece of fresh or freeze-dried animal tissue was weighed and exhaustively extracted in 1:1 dichloromethane/methanol or 1:1 ethanol/ethyl acetate over 72 h. The remaining tissue was dried in an oven for 24 h at 64°C. After determining the dry mass, samples were dissolved in 5.25% sodium hypochlorite (bleach) solution to obtain the sclerites and the gor-
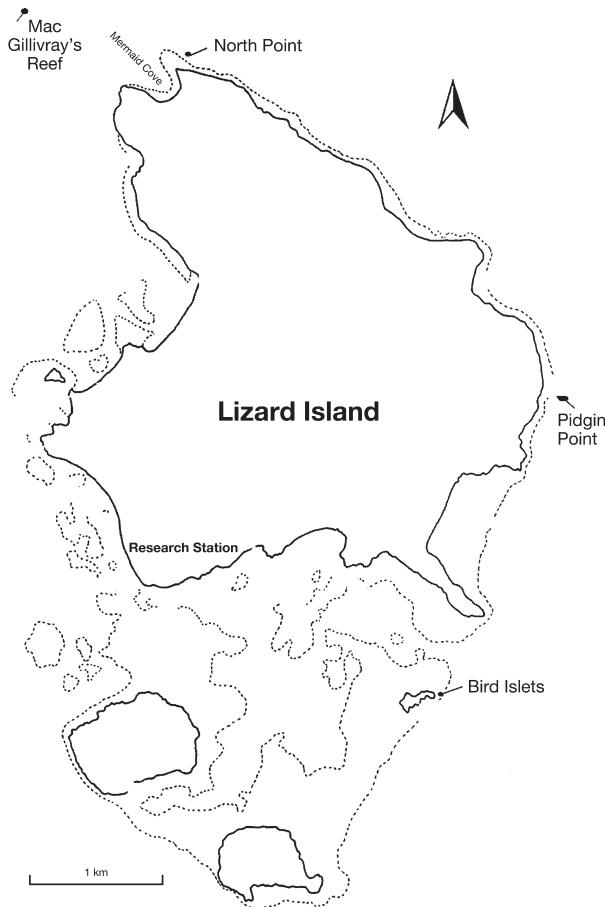
Fig. 2. Collection sites at Lizard Island

gonin skeleton. These were rinsed in fresh water and dried in an oven for 24 h at 64°C. Crude extracts were dried down under reduced pressure (when available) and then weighed. All extracts were stored at 0°C and transported frozen to study sites. The concentration of sclerites was determined as a proportion of the entire colony (dividing by the total dry mass and multiplying by 100) and as a proportion of the soft tissue as described below for the crude extracts.

$$\text{Yield (\%)} = \frac{\text{crude extract mass}}{(\text{dry mass} - \text{gorgonin skeleton mass})} \times 100$$

Data did not meet the requirements for parametric analysis. Therefore, differences in base, mid-axis and tip yields among sites and within individuals were calculated with a 2-way Kruskal-Wallis test (Sokal & Rohlf 1981). The factors were site and part of colony. Differences between the GU and LI populations were calculated by a Mann-Whitney U-test. Differences between sites were also calculated by a Mann-Whitney U-test with α adjusted for the number of

analyses. In addition, the soft tissue concentrations of sclerites and crude extracts were compared by a simple regression to determine if there was a relationship between these variables. All statistical analyses were generated with Statview 5.0 for Macintosh (Abacus Concepts Inc.).

Protein concentration. The protein concentrations of the base, mid-axis and tips were determined by a modified Bradford protein assay (Slattery et al. 1995, Karentz et al. 1997). Approximately 50 mg of tissue was removed from the gorgonin skeleton and digested for 12 h in 5 ml of 1 N NaOH. Five ml of 1 N HCl was added to neutralize the solution. Of each sample 200 µl was diluted with 5 ml of Bio-Rad protein solution and the absorbance (λ = 595 nm) was recorded on a DU-65 Beckman spectrophotometer. Protein concentrations were calculated with a calibration curve generated from standards with bovine serum albumin that were prepared following the same digestion procedure described above. Data did not meet the requirements for parametric analysis even after transformation. Differences in the protein concentration of base, mid-axis and tip among sites and within individuals were calculated with a 2-way Kruskal-Wallis test and post-hoc comparisons with a Mann-Whitney U-test as described above (Sokal & Rohlf 1981). Mean protein concentration did not differ significantly among parts (base: 26.43 ± 4.322; mid-axis: 37.30 ± 17.506; tips 32.27 ± 8.608); (p = 0.0696) or among sites (p = 0.6862) for colonies of *Annella mollis*. There was a significant difference between the mean protein concentration of the base (20.59 ± 1.435) and the mean protein concentration of the mid-axis (27.79 ± 3.557) and tips (27.02 ± 4.909) in colonies of *A. reticulata* (p = 0.0023) but none among sites (p = 0.3323). The artificial diets were prepared to approximate the mean protein concentration of the mid-axes and tips of the colonies.

Feeding assays. Feeding experiments were conducted at Mermaid Cove, LI, in May 1999 and Western Shoals, GU, in June and July 1999. Enough material was available to test the crude extracts and sclerites from the mid-axes and tips, but not from the bases. Extracts and sclerites from the *Annella mollis* colonies collected from Hospital Point, GU, and Pidgin Point, LI, were selected for the feeding assay studies. We used extracts and sclerites from *A. reticulata* colonies collected at Blue Hole, GU, and Mac Gillivray's Reef, LI. To eliminate possible biases, the sources of the extracts and sclerites were chosen at random without prior knowledge of secondary metabolite composition.

Because we were unable to obtain the same diet at GU and LI we used 2 products that report similar nutritional qualities. In feeding assays conducted at GU, the artificial diet was prepared with 5.0 g Kruses™ Brand

catfish food, 2.5 g carrageenan (Type 1) and 80 ml water. In assays at LI, 5.0 g Atlantic Salmon Starter Crumbles was substituted for the catfish food and 3.75 g of carrageenan was added to adjust the protein concentration. The carrageenan was stirred into a 250 ml beaker containing the water and microwaved on high until the mixture boiled, approximately 2 min. The fish food and extract (dissolved in ethyl acetate) or sclerites were stirred into the beaker after the carrageenan had cooled for approximately 30 s. One ml of ethyl acetate was added to the control cubes for the crude extract assays. Twenty-five 1×1 cm food cubes were prepared by pouring the mixture into a partitioned tray. At LI, the mixture was poured into a tray without partitions and 1×1 cm cubes were cut with a wire.

A snorkeler or diver offered 1 control cube paired with 1 treatment cube to natural assemblages of fishes on the reef. The first cube that was completely eaten and not regurgitated within 15 s was scored as eaten and the other cube was scored as uneaten. This was usually very clear because the less palatable food cubes accumulated on the reef at the assay site. Fish were randomly offered food prepared with the crude extract from one part of the colony (mid-axis or tips) collected at GU or LI paired with a control. By testing the GU and LI extracts in the same assay, we eliminated any effects (i.e. learned aversion) due to the prior exposure which might occur if the fishes were exposed to one set of extracts before the other. We followed the same procedure for the sclerite assays. Only 2 sets of assays were conducted per day at a site. Data from the feeding assays were analyzed by a chi-square analysis for a 2×2 contingency table to compare the number of eaten and uneaten food cubes (Sokal & Rohlf 1981).

An informal survey of the fish assemblages we encountered at Western Shoals, GU, and Mermaid's Cove, LI, suggests that there were considerable feeding guild differences. In feeding assays on GU we encountered schools of scissor-tail sergeant *Abudefduf sexfasciatus*, staghorn damsel *Amblyglyphidodon curacao*, juvenile parrotfishes *Scarus schlegeli* and *S. sordidus*, the occasional wrasses *Cheilinus fasciatus* and *Thalossoma hardwickii*, surgeonfishes *Naso vlamengii*, *N. literatus* and *Acanthurus triostegus*, and the butterflyfish *Chaetodon auriga*. At LI more species were present in fewer numbers during the assays. These included the butterflyfish *C. citrinellis*, *C. lunula*, *C. vagabundus* and *C. unimaculatus*, the angelfish *Pomacentrus imperator* and *Centropyge flavissimus* and the wrasses *Cheilinus fasciatus*, *C. trilobatus*, *Epibulus insidiator*, *Heliochoeres trimaculatus*, *Thalossoma lutescens*, *T. purpurea* and *T. quinquevittatum*. Also present were many species of damselfishes, goatfishes, and parrotfishes.

RESULTS

Crude extract, gorgonin, and sclerite concentrations

The gorgonin concentrations in colonies of *Annella mollis* and *A. reticulata* collected from sites around GU and LI were highest in the bases and decreased significantly (p < 0.001) approaching the tips (Table 1). Colonies of *A. mollis* did not exhibit differences in gorgonin concentrations among sites (p = 0.6019) or between islands (p = 0.4729). For *A. reticulata*, the overall gorgonin concentrations in the colonies collected from LI were significantly higher than from GU (p = 0.0166) but there were no significant differences among sites (p = 0.0618).

As a proportion of the whole colony dry mass, the sclerite concentrations (Table 1) were lowest in the bases and increased significantly (p < 0.001) approaching the tips in colonies of both species. There were no differences among sites (p = 0.1235) or between islands (p = 0.0648) in colonies of *Annella mollis*. Colonies of *A. reticulata* from GU had higher concentrations of sclerites than colonies from LI (p = 0.0166) but there were no significant differences among sites (p = 0.0618).

Overall the sclerite and crude extract concentrations in the soft tissue (coenenchyme and polyps) of *Annella mollis* (Table 1a) were significantly higher in colonies collected from GU (p < 0.001 and p = 0.0164, respectively), but there were no significant differences among parts (p = 0.2053 and p = 0.2813, respectively). Colonies from Cocos Wall and Hospital Point, GU, had higher sclerite concentrations than the other 4 sites (p = 0.0011), and colonies from Hospital Point, GU, and Mac Gillivray's Reef, LI, had lower extract concentrations compared to the other 3 sites (p < 0.001). There were no correlations between the soft tissue sclerite and extract concentrations for colonies from GU (r^2 = 0.008, p = 0.2711), LI (r^2 = 2.46×10^{-4}, p = 0.9288) or the combined data set (r^2 = 0.021, p = 0.1251).

Soft tissue sclerite concentrations did not differ significantly among parts of *Annella reticulata* colonies (p = 0.2254), collection sites (p = 0.2144) or between islands (p = 0.0783) (Table 1b). There were significant differences among sites (p = 0.0013) in crude extract concentration (Table 1b) but no overall significant difference among parts (p = 0.0779) or between the islands (p = 0.7102). Extract concentrations were higher at Hospital Point, GU, and Mac Gillivray's Reef, LI compared to the other 3 sites. This species also showed no correlation between the soft tissue sclerite and crude extract concentrations for colonies from GU (r^2 = 0.016, p = 0.5191), LI (r^2 = 0.021, p = 0.4246) or the combined data set (r^2 = 0.001, p = 0.7661).

Table 1. Mean gorgonin, coenenchyme sclerite *, whole animal sclerite ** and crude extract concentrations (%) with the standard error for the base, mid-axis and tips of (a) *Annella mollis* and (b) *A. reticulata* collected from Guam (GU) and Lizard Island (LI). n = number of colonies samples. P = part, B = base, M = mid-axis and T = tips

Site	n	P	Gorgonin concentration (%)	Sclerite concentration (%)*	Sclerite concentration (%)**	Crude extract concentration (%)
(a) *Annella mollis*						
GU						
Blue Hole	3	B	83.63 ± 2.67	59.39 ± 9.43	9.97 ± 2.92	12.48 ± 1.51
		M	70.63 ± 4.92	74.48 ± 0.47	21.83 ± 3.52	9.71 ± 0.19
		T	<1	56.40 ± 7.86	56.40 ± 7.86	20.05 ± 5.51
Cocos Wall	5	B	87.59 ± 1.03	66.31 ± 5.45	8.31 ± 1.15	15.59 ± 1.86
		M	74.14 ± 4.79	66.91 ± 8.64	17.38 ± 4.12	16.22 ± 0.79
		T	20.86 ± 3.78	75.49 ± 4.57	59.08 ± 0.78	31.80 ± 5.07
Hospital	3	B	71.40 ± 6.27	86.17 ± 6.89	23.75 ± 6.43	10.81 ± 4.92
Point		M	52.63 ± 12.90	70.09 ± 2.50	33.21 ± 9.08	7.28 ± 0.12
		T	18.04 ± 4.57	70.03 ± 5.66	57.26 ± 5.16	6.71 ± 1.08
LI						
Mac	5	B	85.74 ± 0.64	43.73 ± 5.95	7.11 ± 1.29	4.63 ± 0.41
Gillivray's		M	73.89 ± 3.21	69.52 ± 5.98	20.32 ± 5.44	7.86 ± 3.32
Reef		T	24.78 ± 7.17	72.41 ± 7.42	50.12 ± 3.88	5.23 ± 2.01
North Point	3	B	82.69 ± 1.79	58.45 ± 6.23	10.15 ± 1.46	8.81 ± 1.76
		M	77.96 ± 2.25	59.90 ± 16.11	17.81 ± 8.71	9.35 ± 1.34
		T	26.27 ± 2.51	89.38 ± 0.62	74.3 ± 3.10	15.77 ± 4.22
Pidgin	4	B	78.52 ± 4.55	26.23 ± 4.57	26.23 ± 4.57	11.21 ± 3.25
Point		M	77.56 ± 4.63	57.61 ± 8.38	13.22 ± 2.56	19.43 ± 1.13
		T	22.85 ± 3.91	33.41 ± 12.25	20.46 ± 10.69	13.72 ± 4.10
(b) *Annella reticulata*						
GU						
Blue Hole	5	B	87.52 ± 1.65	73.79 ± 2.96	9.23 ± 1.34	11.03 ± 1.32
		M	70.21 ± 5.22	71.26 ± 2.03	21.06 ± 3.52	10.24 ± 1.59
		T	6.38 ± 1.97	82.68 ± 2.20	78.18 ± 3.39	12.80 ± 1.46
Hospital	5	B	85.16 ± 3.06	88.55 ± 1.61	24.42 ± 3.36	5.70 ± 0.93
Point		M	36.26 ± 6.98	83.91 ± 3.88	61.36 ± 12.95	3.39 ± 0.21
		T	10.91 ± 2.49	71.46 ± 0.43	62.64 ± 8.06	9.4 ± 0.84
LI						
Bird Islets	6	B	85.66 ± 0.64	40.56 ± 2.04	5.53 ± 0.56	6.03 ± 1.09
		M	77.31 ± 4.09	54.86 ± 3.72	3.11 ± 3.30	13.52 ± 2.91
		T	21.04 ± 3.28	64.38 ± 2.20	50.63 ± 1.58	11.43 ± 2.61
Mac	2	B	88.22 ± 0.90	37.63 ± 0.78	4.42 ± 0.24	5.14 ± 0.40
Gillivray's		M	81.29 ± 1.33	51.52 ± 1.96	9.78 ± 1.07	3.67 ± 2.21
Reef		T	36.70 ± 1.52	48.94 ± 6.15	31.13 ± 4.65	8.64 ± 1.92
Pidgin	3	B	84.8 ± 2.51	32.30 ± 0.66	5.11 ± 0.71	10.61 ± 1.04
Point		M	74.94 ± 2.06	53.68 ± 2.93	13.56 ± 1.62	11.87 ± 0.87
		T	42.31 ± 17.52	45.59 ± 1.66	38.40 ± 4.34	14.53 ± 7.91

Feeding assays

The crude extracts from the mid-axes and tips of *Annella mollis* colonies deterred feeding by natural assemblages of fishes at Western Shoals and Mermaid's Cove ($p < 0.001$) with 2 exceptions (Fig. 3a,c). The mid-axis extracts from GU and LI were not unpalatable to fish at Mermaid's Cove ($p = 0.2037$ and $p = 0.4056$, respectively). Sclerites do not appear to serve as structural defenses for this species (Fig. 3b,d).

Crude extracts from the mid-axes and tips of *Annella reticulata* colonies deterred feeding by fish ($p < 0.001$) (Fig. 4a,c), and some evidence suggests that sclerites can deter predators at high concentrations ($p < 0.001$) (Fig. 4b,d). At Western Shoals, the LI mid-axis extract was palatable to reef fish ($p = 0.1524$) (Fig. 4a). Sclerites from the tips of *A. reticulata* from GU deterred feeding at Western Shoals ($p = 0.05$) and Mermaid's Cove ($p < 0.001$) when incorporated into the diet at 78.18% (Fig. 4d). Low concentrations of sclerites from the tips of

a) Mid-axis Extract

c) Tip Extract

b) Mid-axis Sclerites

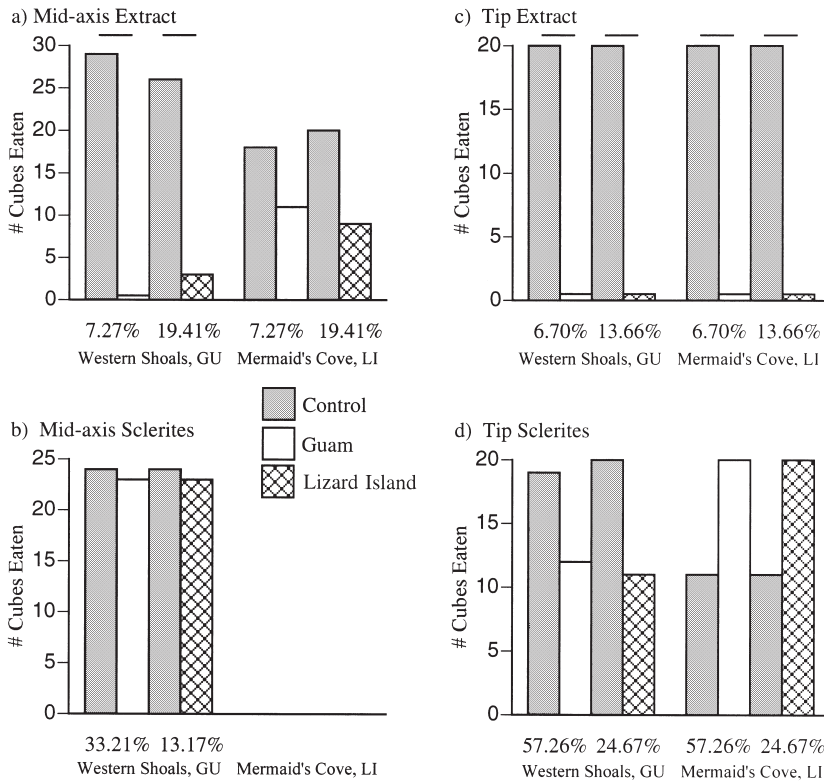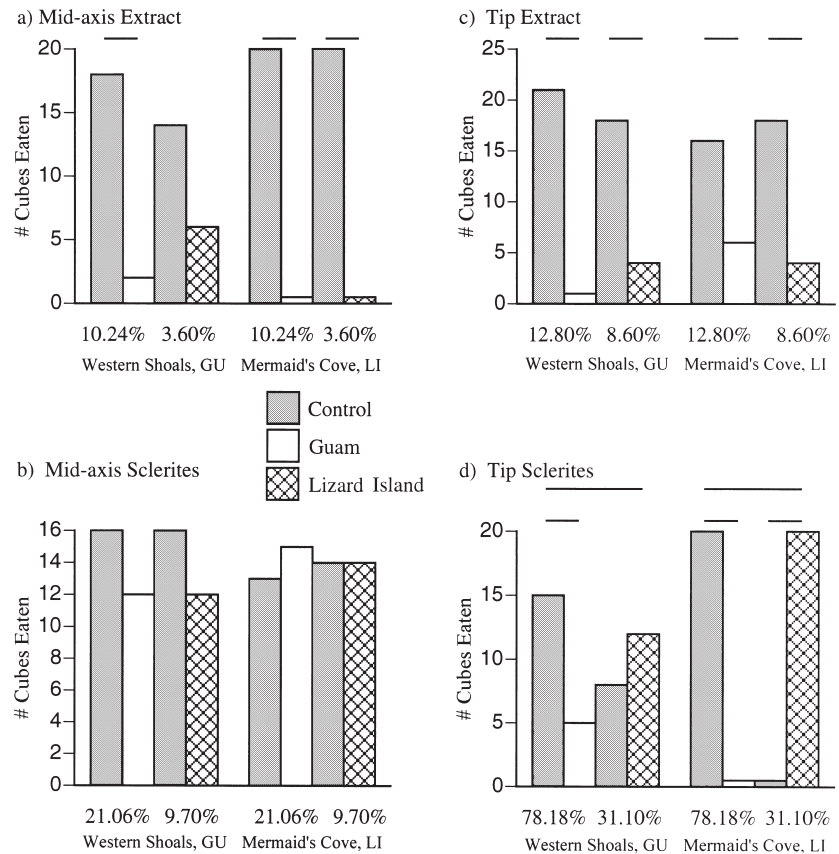d) Tip Sclerites

Control

Guam

Lizard Island

Fig. 3. Feeding assay results for (a) mid-axis crude extracts (n = 29) and (b) sclerites (n = 47), and (c) tip crude extracts (n = 20) and (d) sclerites (n = 31) from *Annella mollis* collected at Pidgin Point, Lizard Island (LI) and Hospital Point, Guam (GU). Crude extract and sclerite concentrations are indicated below each bar. A significant difference (p < 0.05) between the palatability of the control and treated cubes is indicated by a line drawn above the 2 corresponding bars. n = number of replicate pairs in each assay

a) Mid-axis Extract

c) Tip Extract

Control

Guam

Lizard Island

b) Mid-axis Sclerites

d) Tip Sclerites

Fig. 4. Feeding assay results for (a) mid-axis crude extracts (n = 20) and (b) sclerites (n = 28), and (c) tip crude extracts (n = 22) and (d) sclerites (n = 20) from *Annella reticulata* collected from Blue Hole, GU, and Mac Gillivray's Reef, LI. Crude extract and sclerite concentrations are indicated below each bar. A significant difference (p < 0.05) between the palatability of the control and treated cubes is indicated by a line drawn above the 2 corresponding bars. A line drawn above the 4 corresponding bars indicates a significant difference between the palatability of the GU and LI extracts or sclerites. n = number of replicate pairs in each assay

A. reticulata colonies collected at LI were preferred over the control cubes at Mermaid's Cove (p < 0.001).

DISCUSSION

The reciprocal feeding assays conducted in this study clearly show that the crude extracts from *Annella mollis* and *A. reticulata* are unpalatable to natural assemblages of reef fishes at Western Shoals and Mermaid's Cove (Figs. 3 & 4). Sclerites from some Caribbean species have been shown to be unpalatable to fish when incorporated into artificial diets at high concentrations (Harvell et al. 1988, Van Alstyne & Paul 1992, West 1998, Slattery 1999). We observed similar results for sclerites from the tips of *A. reticulata*. These were only effective feeding deterrents at Western Shoals and Mermaid's Cove when assayed at 78.18% (Fig. 4d). In fact, at 31.10%, sclerites from the tips of *A. reticulata* collected at LI were preferred over the control cubes by fish at Mermaid's Cove. While some species, such as *Gorgonia ventalina*, invest in the production of chemical and structural defenses (Van Alstyne & Paul 1992, Slattery 1999), gorgonians in the genus *Annella* specialize in the production of chemical defenses against generalist predators (Harvell & Fenical 1989).

Unlike the tip extracts, the mid-axis extracts from *Annella mollis* and *A. reticulata* did not always deter feeding by natural assemblages of fishes. The mid-axis extracts of *A. mollis* from GU and LI did not deter fish feeding at Mermaid's Cove when assayed at concentrations similar to and higher than the tip extracts. These results suggest that the chemical defenses of *A. mollis* are more concentrated at the tips of the colonies. Similar patterns of intracolony variation have been reported for the Caribbean gorgonians *Pseudopterogorgia rigida* (Harvell et al. 1988, Harvell & Fenical 1989) and *Gorgonia* spp. (Kim et al. 2000). Higher concentrations of predator-deterrent secondary metabolites were found at the tips of *P. rigida* colonies (Harvell et al. 1988, Harvell & Fenical 1989). Crude extracts from the colony edges of *Gorgonia* spp. exhibited greater fungal resistance against the pathogen *Aspergillus sydowii* (Kim et al. 2000). It is not surprising to find the tips of the colonies better defended against generalist predators. Optimal defense theory would predict that chemical defenses would be greatest in new growth. This pattern has been observed for many marine organisms (Paul & Van Alstyne 1988, Harvell & Fenical 1989, Van Alstyne et al. 1994, Becerro et al. 1998, Kim et al. 2000). During vegetative reproduction new polyps are added to the outer colony edges (Szmant-Froelich 1974).

In feeding assays at Mermaid's Cove, the mid-axis extracts of *Annella reticulata* from LI and GU were both unpalatable to reef fishes (Fig. 4a), but at Western

Shoals the LI extract did not deter fish feeding. The natural concentration of the mid-axis extract from GU (10.24%) was approximately 3-fold higher than the natural concentration of the LI mid-axis extract (3.60%). The results of these assays suggest that fishes in GU may have a higher tolerance to the mid-axis extracts at low concentrations. Since we used different diets in the feeding assays at Western Shoals and Mermaid's Cove we were not able to make direct comparisons between the results from the feeding assays at the 2 sites.

Differences in fish assemblages between the islands may account for some of the variability seen in the palatability of the mid-axis extracts for both *Annella mollis* and *A. reticulata* (Pavia & Aberg 1996). The Indo-Pacific, Indonesia and the Great Barrier Reef host a greater diversity of coral reef fishes (Thresher 1991). Informal surveys of the fish assemblages we encountered at Wester Shoals and Mermaid's Cove suggest that there were considerable feeding guild differences. On GU we mainly encountered schools of *Abudefduf sexfasciatus*, *Amblyglyphidodon curacao*, juvenile parrotfishes and the occasional wrasses, surgeonfishes and butterflyfishes. At LI more species were present in fewer numbers during the assays, including several species of butterflyfishes, angelfishes, wrasses, damselfishes, goatfishes, and parrotfishes.

Overall, the crude extract concentrations in the *Annella mollis* from GU were significantly higher than in colonies from LI (Table 1a). The natural concentrations of extracts collected at some sites around LI were approximately $^1/_2$ of those in the colonies from GU. And, crude extract concentrations did vary among sites at both islands for colonies of *A. reticulata* (Table 1b). However, palatability of the extracts does not appear to be highly correlated with crude extract concentration.

The *Annella* spp. had fairly consistent sclerite and crude extract concentrations in the soft tissue throughout the colony (Table 1). In this respect, *A. mollis* and *A. reticulata* are similar to the Caribbean gorgonian *Gorgonia ventalina* (Van Alstyne & Paul 1992, Cronin et al. 1995, Slattery 1999) but not the *Pseudopterogorgia* spp. (Harvell & Fenical 1989). Colonies of *Pseudopterogorgia* spp. exhibited an inverse relationship in crude extract and sclerite concentrations, where sclerite concentrations increased from tips to bases. We did not observe any correlation between the crude extract and sclerite concentrations for *A. mollis* or *A. reticulata*. Sclerites in cnidarians and spicules in other phyla, when densely packed, have been shown to increase rigidity and resist excessive deformation and tearing that could result from environmental stresses such as heavy currents (Koehl 1982, Lewis & Von Wallis 1991, West et al. 1993). In the basic architecture of *Annella* spp., the gorgonin skeleton com-

prised up to 90 % of the dry mass at the bases but less than 1 % of the total dry mass in the tips, while sclerites could comprise more than 70 % of the dry mass at the tips (Table 1). A major role of sclerites in *Annella* spp. is probably the support of new tissues at the tips before an internal gorgonin skeleton can be deposited (Szmant-Froelich 1974).

Compared to other areas of chemical ecology, biogeographic comparisons of the predator/herbivore defenses associated with benthic marine organisms are few, mainly addressing differences between tropical and temperate species (Hay 1996, Sammarco 1996). This is the first study to address geographic differences in the palatability of crude extracts and sclerites of gorgonians collected from 2 distant tropical islands in the Pacific Ocean. The feeding experiments clearly demonstrated that *Annella mollis* and *A. reticulata* from GU and LI are chemically rather than structurally defended against natural assemblages of coral reef fishes, thus supporting the hypothesis that extracts from species with small sclerites are usually unpalatable to fish (Pawlik et al. 1987, Harvell & Fenical 1989). With a few exceptions, the crude extracts from the mid-axes and tips of both species collected from GU and LI were unpalatable to fishes at both Western Shoals and Mermaid's Cove. The production of chemical defenses by *Annella* spp. appears to be ubiquitous over a broad range in the Pacific. In other geographic comparisons of predator defenses in octocorals, the production of chemical defenses was reported to be highly conserved (Coll & Sammarco 1988). Harvell et al. (1993) also demonstrated that, while colonies of *Briareum asbestinum* from the Bahamas and St. Croix are both chemically defended against predation (Pawlik et al. 1987), the 2 populations produced different classes of secondary metabolites. Further studies are needed to determine if *A. mollis* and *A. reticulata* from GU and LI produce similar predator-deterrent compounds.

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Allelopathic activities of sponge extracts

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ABSTRACT: Although there is anecdotal evidence for allelopathic interactions between benthic invertebrates engaged in spatial competition, only a few studies have established the involvement of chemical agents in field experiments. We have developed a technique in which crude organic extracts of marine sponges were incorporated into hard, stable gels that serve as a substratum for overgrowth by invertebrates in the field. Overgrowth organisms were secured to the center of assay plates and allowed to grow laterally over control gels and gels containing a crude organic sponge extract that was volumetrically equivalent to the concentration found in sponge tissues. Extracts of 20 species of Caribbean sponges were assayed in Florida using 3 overgrowth sponges: *Tedania ignis*, *Lissodendoryx isodictialis*, and *Haliclona hogarthi*. In addition, extracts of the sponge *Aplysilla longispina* were assayed in North Carolina using the tunicate *Diplosoma listerianum* as the overgrowth organism. The allelopathic effects varied between extracts of different sponge species, but were consistent across overgrowth organisms. Of the sponge extracts tested, 30% inhibited sponge growth (*Amphimedon compressa*, *Aplysilla longispina*, *Aplysina cauliformis*, *Dysidea etheria*, *Ectyoplasia ferox*, and *Phorbas amaranthus*), while 15% promoted overgrowth (*Agelas wiedenmeyeri*, *Geodia gibberosa*, and *Halichondria* sp.). The remaining 55% of sponge extracts had no effect on sponge growth. The technique described herein represents a more ecologically relevant method than past techniques for assaying the allelopathic properties of extracts of marine organisms. Moreover, this study provides evidence that sponge metabolites may act as allomones by preventing overgrowth of some sponge species, and as both allomones and kairomones by enhancing overgrowth of others.

KEY WORDS: Allelopathy · Caribbean sponges · Chemical defenses · Overgrowth · Spatial competition · Kairomones · Allomones

INTRODUCTION

Chemical interactions between organisms have been reviewed extensively (e.g., Whittaker & Feeny 1971, Kittredge et al. 1974, Rice 1985, Harborne 1988), and are divided into 2 classes that have either intraspecific or interspecific effects (Whittaker & Feeny 1971). While pheromones are secondary metabolites that are synthesized by one organism to affect other individuals of the same species, allelopathic compounds are chemical agents that are produced by one organism to affect the health, growth, behavior, or population biology of organisms of other species (Whittaker & Feeny 1971).

While anecdotal evidence of allelochemical interactions date back as early as 300 BC, the term allelopathy was coined in 1937 by Molisch to describe biochemical interactions between terrestrial plants (Rice 1985 and references therein). Kittredge et al. (1974) were among the first to review allelopathy with regard to marine invertebrates. Given that spatial competition among sessile invertebrates is intense (Connell 1961, Dayton 1971, Paine 1974, Jackson 1977), many studies investigated potential allelopathic interactions among benthic invertebrates, yet few studies documented both the chemical and ecological aspects of allelopathy (reviewed in Pawlik 1993, Hay et al. 1998).

Some of the earliest investigations of presumed allelopathy in spatial competition among sessile marine invertebrates were strictly observational. Jackson & Buss

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(1975) demonstrated that sponges were more likely to overgrow corals than vice versa, and that the sponge-coral interface commonly exhibited zones of necrosis on the coral. Bryozoan competitors often showed similar fates in competitive interactions with sponges (Jackson & Buss 1975). Allelopathic defense mechanisms have also been suggested between corals. Sheppard (1979) proposed that toxic exudates of hard corals such as *Ctenella chagius* are responsible for growth retardation in usually aggressive *Acropora* species, preventing adjacent growth up to 5 cm. Other studies used manipulative experiments to document growth retardation, tissue necrosis, and mortality of corals in competitive interactions (Sammarco et al. 1983, 1985, La Barre et al. 1986). While these observational results suggest the involvement of chemical compounds in spatial interference competition, they only provide circumstantial evidence for allelopathy.

To gain a better understanding of allelopathy in spatial interference competition, the focus of some studies shifted to the isolation of chemical compounds believed to be responsible. Coll et al. (1982) developed a submersible sampling apparatus for the isolation of toxic terpenes released *in situ* by 2 species of soft corals. Sullivan et al. (1983) isolated siphonodictidine, a sesquiterpene that inhibits coral growth, from the mucus secreted by a burrowing sponge of the genus *Siphonodictyon*. Secretion of mucus by sponges was suggested as a mechanism for the concentration of allelochemicals near the sponge, preventing excessive dilution of the metabolites due to water flow (Jackson & Buss 1975).

Thompson (1985) and Thompson et al. (1985) hypothesized that allelochemicals released from the sponge *Aplysina fistularis* protect it from overgrowth because the compounds cause behavioral modifications in laboratory assays conducted with adult and larval fouling organisms. Walker et al. (1985) measured the release rate of 2 potential allelochemicals from *A. fistularis* in the field, but it was unclear whether metabolite concentrations in moving seawater would ever reach the concentrations assayed in the lab (Thompson 1985, Thompson et al. 1985). Although these studies demonstrate a chemical basis for allelopathic interactions, the ecological functions of these compounds remain inconclusive without relevant field experiments.

To date, few studies have employed field experiments to demonstrate the involvement and direct effect of allelopathic chemicals. Porter & Targett (1988) observed the liver sponge *Plakortis halichondroides* overgrowing the sheet coral *Agaricia lamarcki* and creating a zone of necrotic coral tissue when the 2 species were in direct contact with each other. To determine if tissue necrosis in *A. lamarcki* was the result of allelochemical secretions by *P. halichondroides*, they

developed a field experiment in which extracts of the sponge were coated onto synthetic cellulose pads that were tied directly to the surface of the coral. Pads treated with extracts of *P. halichondroides* caused bleaching within 24 h while control pads produced no effect (Porter & Targett 1988). Manipulative field experiments were also conducted with live tissue and extracts of the red alga *Plocamium hamatum* to investigate the necrotic effect of the red alga on the soft coral *Sinularia cruciata* (de Nys et al. 1991). Tissue necrosis of *S. cruciata* was only observed when the soft corals were in direct contact with the alga or plastic plants treated with extracts of the alga. Turon et al. (1996) developed a field experiment to investigate potential allelopathic effects of the sponge *Crambe crambe* on the tissue regeneration of the sponge *Scapalina lophyropoda*. Circular patches that were previously cut on large individuals of the sponge *S. lophyropoda* were exposed to freshly cut tissue of *C. crambe* or *S. lophyropoda*, while other individuals were unexposed as a control group. Tissue regeneration in the control individuals occurred faster than in those treated with *S. lophyropoda*, while individuals treated with *C. crambe* tissue never healed (Turon et al. 1996). Most recently, Thacker et al. (1998) used a method similar to that of Porter & Targett (1988) to test the crude extract and purified 7-deacetoxyolepupuane from a *Dysidea* sp. in direct contact with a sponge of the genus *Cacospongia*. The extract and pure compound were incorporated at natural volumetric concentrations into agar that was poured onto strips of window screen. Treated and control strips were fastened around different branches of a single *Cacospongia* sp. colony. Thacker et al. (1998) found that both the crude extract as well as the pure secondary metabolite of *Dysidea* sp. caused necrosis of *Cacospongia* tissue within 7 d, with no evidence of necrosis under control strips.

In this study, we employed a newly developed field technique to survey allelopathic effects of sponge extracts on the overgrowth of 3 sponges and 1 colonial tunicate. The technique involves the incorporation of crude organic extracts of marine sponges into hard, stable gels that serve as a substratum for overgrowth organisms. We tested the rate of release of crude sponge extracts from gels over a 21 d period in the field. Next, we surveyed the allelopathic properties of extracts of 20 different sponge species by comparing lateral growth differences of overgrowth organisms over paired extract-treated and control gels.

MATERIALS AND METHODS

Preparation of crude organic extracts. The sponge *Aplysilla longispina* was collected from the floating

docks at Wrightsville Beach, North Carolina, USA. All other sponges were collected on shallow reefs and mangroves near Key Largo, Florida, USA. After collection, samples were taken back to the lab for immediate processing. For each sample, the volume of wet sponge tissue was measured by displacement in a 2 l, graduated cylinder filled with 1 l of seawater. Samples were then drained of excess seawater and placed into separate, air-tight, plastic freezer bags and frozen overnight. The frozen tissue of each sample was then cut into small cubes and lyophilized. Tissue samples were separately extracted in methanol for 24 h. The resulting solution was filtered into a round bottom flask and the methanol was removed by rotary evaporation. The same tissue sample was then extracted a second time in 1:1 methanol:dichloromethane. After filtration the solution was added to the flask with the previous methanol-derived extract. The remaining 1:1 was then removed by rotary evaporation. The resulting crude extracts were then transferred into scintillation vials so that each vial contained an 80 ml equivalent of the originally measured tissue. All extract samples were dried under high vacuum and stored under nitrogen in a freezer at –20°C until used in assays.

Preparation of assay plates. Acrylic plates were used to construct a square assay plate measuring $15 \times 15 \times 0.8$ cm and containing 4 gel wells in a 3 by 3 array (Fig. 1). Each well measured $5 \times 5 \times 0.8$ cm and had a volume of 20 ml. In order to completely enclose all 4 wells, a single acrylic plate measuring $5 \times 5 \times 0.8$ cm was placed in the center of the assay plate. Two opposing wells were used for extract-treated gels, while the remaining 2 were used for control gels. On each plate the overgrowth organism was allowed to overgrow paired extract-treated and control gels from the center plate.

Fig. 2. Percentage retention of extracts of the sponges *Aplysina cauliformis*, *Agelas wiedenmeyeri*, and *Niphates digitalis* in gels over 21 d in the field. For each species, 2 replicate gels were extracted at each time interval. Control gels were kept in plastic bags at 5°C for 21 d. Diffusion rates were similar across species, so the mean retention of all extracts was plotted (mean ± SD, n = 6)

Preparation of overgrowth organisms. The frog egg tunicate *Diplosoma listerianum* was chosen for assays at Wrightsville Beach, North Carolina. The acrylic center plates were mounted onto the underside of heavily fouled floating docks next to colonies of *D. listerianum*. After 2 to 4 d *D. listerianum* began overgrowing the acrylic plates and covered the plates within 2 to 3 wk.

Lobes of the sponges *Tedania ignis*, *Lissodendoryx isodictialis*, and *Haliclona hogarthi* were collected from mangrove roots near Dusenbury Creek or near Sexton Cove, Key Largo, Florida. Each lobe, which measured at least 5×5 cm, was secured to the center square of a prepared assay plate with cable ties, and sponge tissue that extended beyond the edge of the center square was removed with a sharp knife.

Preparation of gels. Treatment gels were prepared by adding 1.5 g of Phytagel™ to 80 ml of distilled H_2O and mixed thoroughly. The mixture was heated in a microwave oven for 1 min. An aliquot of extract, re-
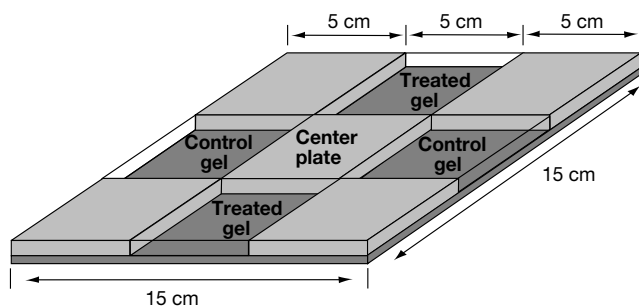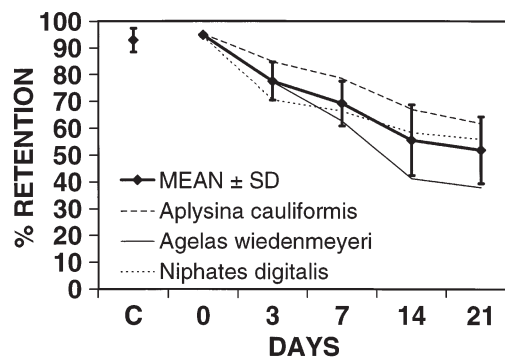

Fig. 1. Schematic diagram of assay plate containing four 20 ml wells for paired treatment and control gels. Treatment wells were filled with gels containing a natural volumetric concentration of crude organic sponge extract. Control wells were filled with gels containing only a volumetric equivalent of the solvent used to re-suspend the extract for treated gels. The overgrowth organism on the center plate was allowed to laterally grow over the adjacent gels for 21 d in the field
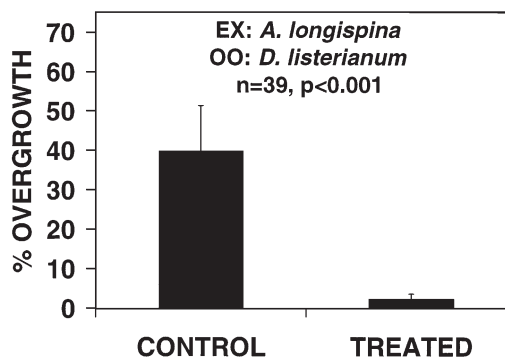

Fig. 3. Percentage overgrowth of the tunicate *Diplosoma listerianum* over paired control gels and gels treated with extracts of the sponge *Aplysilla longispina* (mean ± SD, n = 39, paired *t*-test on arcsine transformed data) (EX = extract; OO = overgrowth organism)

suspended in 10 ml of methanol, was stirred into the hot mixture until homogenous. The resulting mixture was then poured into 2 opposing 20 ml wells on each of 2 assay plates (Fig. 1). Control gels were prepared by adding 3 g of Phytagel™ to 80 ml of distilled H_2O. A higher concentration of the inert gelling agent was necessary in control gels to maintain equal gel hardness between treatment and control gels. Gel hardness was measured using a gel tester (Marine Colloids GT-2, Springfield, NJ, USA). For the control gels, the mixture was heated, 10 ml of methanol was added, and the gels were poured in the remaining two 20 ml wells, as before.

Assessing retention of extracts in gels. Extracts of the sponges *Agelas wiedenmeyeri*, *Aplysina cauliformis*, and *Niphates digitalis* were used to assess the percentage retention of extracts in the gels over 21 d in the field. Before pouring the gels, the extract mass of each sample was recorded. Six sets of 2 gels for each sponge extract were prepared as detailed above. Four of the 6 sets were hung from a floating dock, while 2 sets were retained as controls. The first control set was stored at 5°C in a plastic bag throughout the 21 d assay period and subsequently re-extracted. The second set of control gels was re-extracted immediately after being poured (Day 0). The remaining 4 sets were extracted after 3, 7, 14, and 21 d in the field and the mass of recovered extract was recorded. To compensate for salts and organics taken up by the gels in the field, the mass gained by paired control gels was subtracted from the mass recovered from treatment gels.

Running the assay. Assays with the tunicate *Diplosoma listerianum* as the overgrowth organism were conducted at the floating dock of the La Que Center for Corrosion Technology, Inc., Wrightsville Beach, North Carolina. Six assay plates were deployed at any one time, with the experiment repeated to give 39 replicates (some plates were lost). Center plates overgrown with *D. listerianum* were fixed to the center of each assay plate
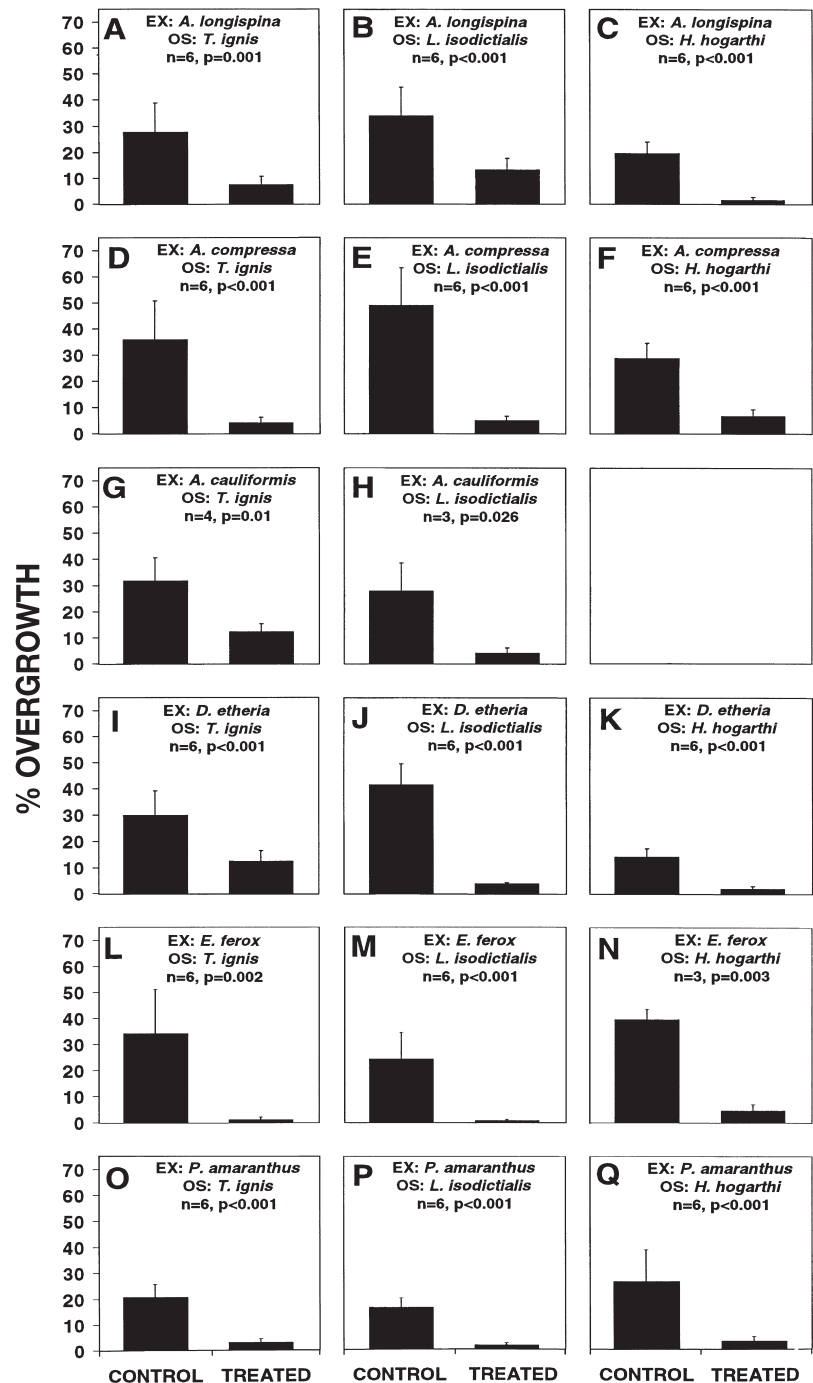

Fig. 4. Sponge extracts that inhibited the overgrowth of sponges. Percentage overgrowth of 3 species of sponges, *Tedania ignis*, *Lissodendoryx isodictialis*, and *Haliclona hogarthi*, over paired control and treatment gels. Treatment gels were treated with extracts of Caribbean sponges. Percentage overgrowth on paired control and extract-treated gels (EX = extract; OS = overgrowth sponge). See text for extract genera

with double-sided carpet tape. Each individual assay plate was hung with a monofilament line and swivel 30 cm below the water surface, allowing the plate to orient to water flow. All assay plates were left in the

water for 21 d. The lateral growth of *D. listerianum* over each of the 4 gels was scored by placing a 5 × 5 cm piece of window screen over each gel and counting the number of squares in which the overgrowth organism appeared.

Assays with sponges as the overgrowth organisms were conducted in Florida Bay, Key Largo, Florida. Six replicate plates were prepared for each extract. The plates were hung 50 cm below the low tide level on a rope that was horizontally stretched between 2 mangrove prop roots. Assays were conducted and scored as described above. Percentage overgrowth was calculated by dividing the number of growth squares by the total number of squares, and multiplying by 100. A paired *t*-test, performed on arcsine-transformed data, was used to assess the significance of the difference in mean overgrowth on control versus extract-treated gels (Zar 1996).

RESULTS

Crude organic sponge extracts diffused gradually from gels (Fig. 2), with a mean of 51.9% of the starting material remaining after 21 d in the field. At the Wrightsville Beach, North Carolina, assay site, gels were exposed to the highest tidal flows that assay plates experienced in this study.

In assays conducted in North Carolina, the tunicate *Diplosoma listerianum* was inhibited from overgrowing gels containing a natural volumetric concentration of a crude extract of the co-occurring sponge *Aplysilla longispina*. In each case, the overgrowth by *D. listerianum* was significantly less on extract-treated gels relative to the overgrowth on control gels (Fig. 3).

In assays conducted in Florida, crude extracts of 6 sponge species inhibited sponge overgrowth (Fig. 4), extracts of 3 species promoted sponge overgrowth (Fig. 5), and extracts of 11 species had no effect (Fig. 6). Although the effects of the crude extracts revealed this level of interspecific variability, each extract gave a consistent result across the 3 overgrowth sponge species (Figs. 4 to 6). The mean percentage coverage of the 3 overgrowth sponges *Tedania ignis*, *Lissodendoryx isodictialis*, and *Haliclona hogarthi* over control gels was 31.8 ± 8.6, 35.9 ± 9.1, and 26.5 ± 8.5%, respectively.

DISCUSSION

Many studies have described putative allelopathic interactions among sessile marine invertebrates (Jackson & Buss 1975, Sheppard 1979, Sammarco et al. 1983, Thompson et al. 1985), yet few have demonstrated the involvement and activity of chemical compounds in field experiments (Porter & Targett 1988, Turon et al. 1996, Thacker et al. 1998). To date, all field assays have used tissue necrosis or regeneration as indicators for the allelopathic activity of applied treatments. This study is the first to use overgrowth by a sessile invertebrate to assess the allelopathic properties of crude organic extracts from invertebrates.

The technique described in this study represents a more ecologically relevant method for assessing the allelopathic activity of extracts. One of the main advantages of this technique is that gels made with Phytagel™ remain hard and stable in the field. We found that both extract-treated and control gels provided a suitable overgrowth substratum for the sponges and tunicate used in this study. Henrikson (1996) also found that the bryozoan *Schizoporella unicornis* overgrew gels of this type. Other gelling agents such as acrylamide, alginic acid, and carageenan did
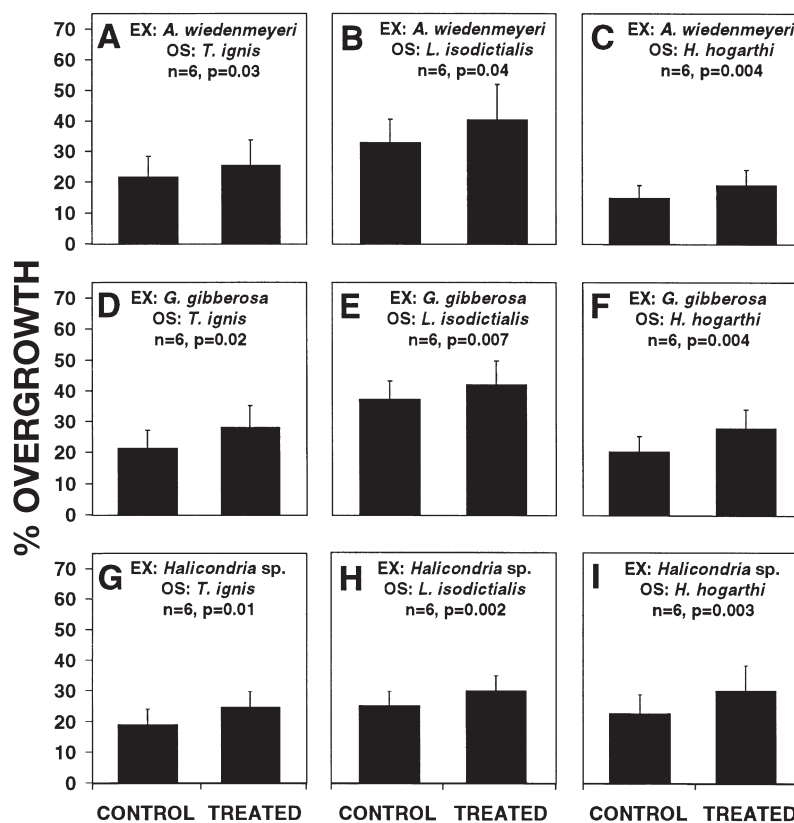

Fig. 5. Sponge extracts that promoted the lateral growth of sponges. Percentage overgrowth on paired control and extract-treated gels. Data presented as in Fig. 4
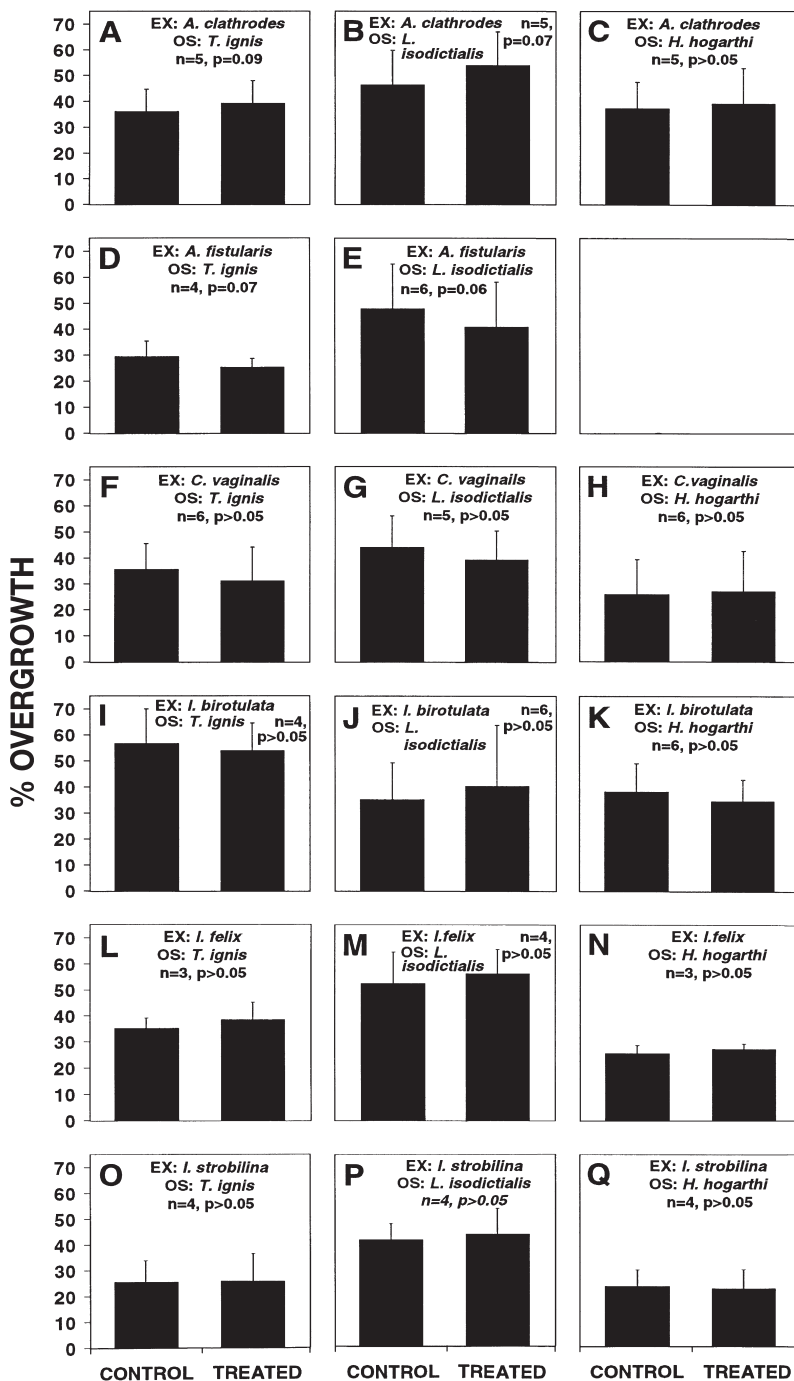
Fig. 6. Sponge extracts that had no effect on the overgrowth of sponges. Data presented as in Fig. 4

dimensional tissue and painted onto a 2-dimensional surface can potentially alter surface chemistry as well as the physical surface characteristics of the substratum (Mihm et al. 1981, Brewer 1984, Henrikson & Pawlik 1995). Extracts incorporated into gels slowly diffuse from the substratum, in a manner that may mimic the slow release of metabolites from the surface of living organisms. Although little is known about the mechanisms of release of secondary metabolites by organisms producing them, there is evidence that some sponges exude secondary metabolites in the field (Thompson 1985, Walker et al. 1985, Nishiyama & Bakus 1999).

Overgrowth organisms responded in 1 of 3 ways to sponge extracts. The majority of extracts (55%) had no effect on overgrowth, while 30% inhibited and 15% promoted overgrowth. Despite the variability in the responses of overgrowth organisms to extracts of different sponge species, their responses were consistent within a treatment. Extracts of the sponges *Amphemidon compressa*, *Aplysilla longispina*, *Aplysina cauliformis*, *Dysidea etheria*, *Ectyoplasia ferox*, and *Phorbas amaranthus* all inhibited overgrowth (Fig. 4). Because none of these species are in the same sponge family, it appears that the production of overgrowth inhibiting metabolites is not taxonomically restricted. Moreover, each of these 6 extracts inhibited all 3 overgrowth sponges, suggesting that the effect is not directed in a species-specific manner. Extracts of the sponge *A. longispina* not only inhibited growth of the 3 sponge species, but also inhibited the growth of the colonial tunicate *Diplosoma listerianum* (Figs. 3 & 4A–C).

This study is part of a broader research program investigating the defensive functions of secondary metabolites from marine organisms. Henrikson & Pawlik (1995, 1998) used gels of the same composition to conduct fouling assays in the field and determined that gels treated with an extract of *Aplysilla longispina* were less fouled than control gels. If secondary metabolites have a cost associated with production (Pawlik
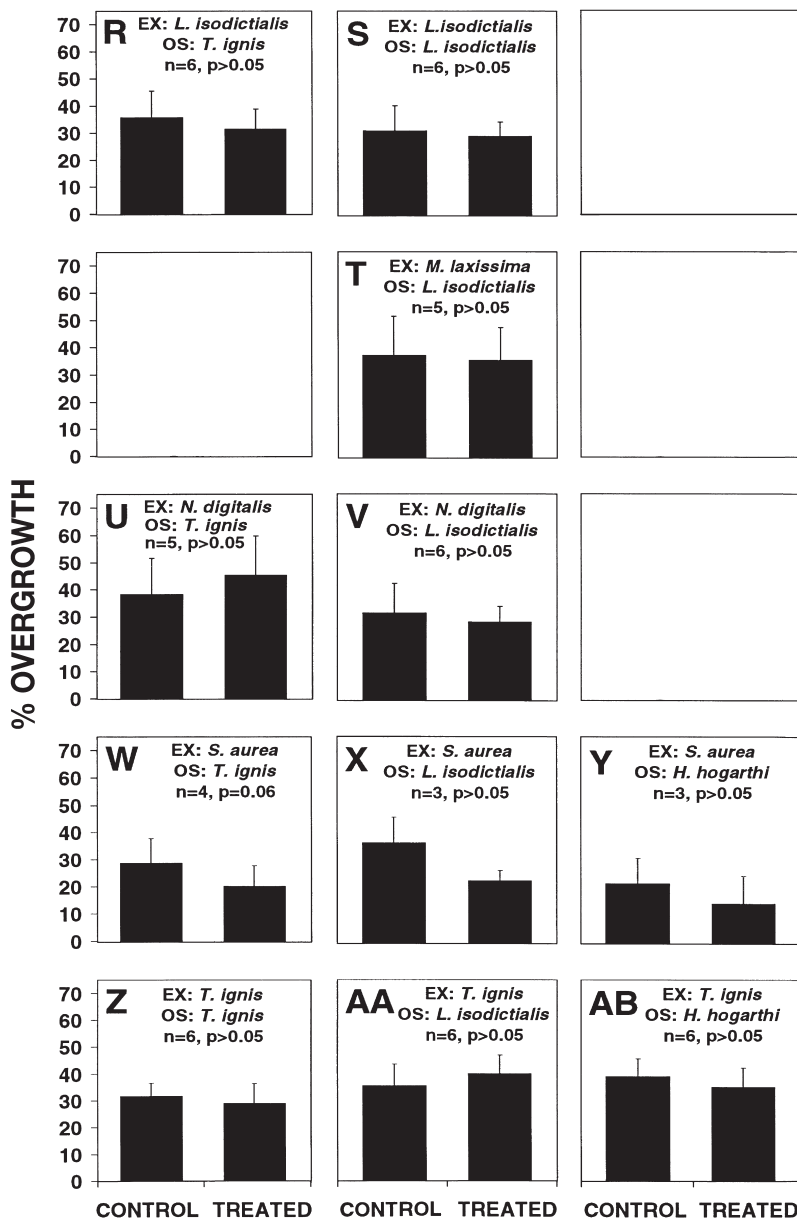
not solidify when extracts were added, or disintegrated under natural field conditions (Henrikson & Pawlik 1995). In addition to gel hardness and stability, the 3-dimensional structure of the overgrowth gel is advantageous over a 2-dimensional overgrowth surface (Bakus et al. 1991). Extracts that are taken from a 3-

Fig. 6 (continued)

al. 1999), and deterred feeding by the reef fish *Thalassoma bifasciatum* (Pawlik et al. 1995). Extracts of the sponge *Ectyoplasia ferox* inhibited all overgrowth sponges (Fig. 4L–N), and deterred predation by fish (Pawlik et al. 1995). Bioassay guided fractionation has yet to be performed to determine the metabolite or group of metabolites responsible for these defensive effects.

Extracts of the sponges *Agelas wiedenmeyeri*, *Geodia gibberosa*, and *Halichondria* sp. promoted overgrowth. Interestingly, *Geodia gibberosa* is frequently found to be thinly encrusted by the sponge *Amphimedon erina*. *G. gibberosa* is chemically undefended from fish predators (Pawlik et al. 1995) and is a preferred food of spongivorous fishes and turtles (Dunlap & Pawlik 1996, 1998). *A. erina*, on the other hand, is strongly chemically defended from fish predation (Pawlik et al. 1995). Allelopathic agents are grouped into allomones, which benefit the organisms producing them, and kairomones, which benefit the organisms receiving them (Whittaker & Feeny 1971). The relationship between *G. gibberosa* and *A. erina* may represent a mutualistic, associational defense. The secondary metabolites produced by *G. gibberosa* may act as both allomones and kairomones, in that promoting the overgrowth of *A. erina* benefits both *G. gibberosa*, by providing a protective, unpalatable covering, and *A. erina*, by providing space for growth.

The majority of sponge extracts had no allelopathic effect on overgrowth organisms, and the effects of these extracts in previous studies on bacterial growth and fish predation were inconsistent. While extracts of the sponges *Aplysina fistularis*, *Ircinia felix*, and *Ircinia strobilina* had no effect on overgrowth, they exhibited antimicrobial (Newbold et al. 1999) and fish antipredatory activity (Pawlik et al. 1995). In addition, extracts of the sponges *Mycale laxissima* and *Smenospongia aurea* also had no allelopathic effect on overgrowth, but were deterrent in fish feeding assays (Pawlik et al. 1995). On the other hand, extracts such as *Callyspongia vaginalis*, *Iotrochota birotulata*, and *Niphates digitalis* (Fig. 6F–K,U,V) neither inhibited overgrowth nor deterred feeding by *Thalassoma bifasciatum* (Pawlik et al. 1995). While

1993), it seems probable that compounds may have evolved to function in multiple defensive roles. Although fouling and overgrowth are different processes, in that the former involves settlement of larval propagules, while the latter involves the growth of one adult organism over another, both play important roles in spatial competition.

Other sponge extracts may also have multi-functional defensive properties. Extracts of *Amphimedon compressa* not only inhibited the lateral growth of sponges in our assay (Fig. 4D–F), but also inhibited bacterial growth in antimicrobial assays (Newbold et

these 3 sponges seem to lack any allelopathic secondary metabolites, it is interesting to note that they are among the most widely distributed and abundant sponges on Caribbean coral reefs (Pawlik et al. 1995). In addition, these sponges are frequently infused with polyps of zoanthids of the genus *Parazoanthus*. While it has been hypothesized that commensal zoanthids provide an associational defense (West 1976, Lewis 1982), Pawlik et al. (1995) did not find any evidence to suggest that zoanthids provided a chemical defense. While the ecological role of commensal zooanthids associated with sponges remains elusive, it appears that this interaction may be possible because these sponges lack allomones that inhibit the growth of such epibionts. Furthermore, sponges that inhibited overgrowth in our assays were never found to be overgrown by any zooanthids.

The absence of an allelopathic effect of an extract using this assay system does not rule out allelopathy by the living sponge. Despite its advantages over previous techniques, this assay system is nevertheless only an approximation of the delivery of sponge metabolites under natural conditions. Sponges that concentrate allelopathic metabolites near their surfaces, those that turn-over metabolites rapidly, or those that produce highly water-soluble metabolites might all have yielded inactive extracts using this assay system. In these respects, the results reported in this study represent a conservative estimate of allelopathy in Caribbean sponges. Moreover, this study provides good candidate species for bioassay-directed isolation of metabolites responsible for allelopathy. Future research directions also include the localization of allelopathic agents and the mechanisms of their release.

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Larval settlement: chemical markers for tracing production, transport, and distribution of a waterborne cue

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ABSTRACT: Dissolved chemical signals mediate many ecological interactions in the marine environment, but little is known about how rates of production and distribution of waterborne cues affect life-history processes in the field. Larvae of the specialist marine herbivore *Alderia modesta* settle and metamorphose in response to complex carbohydrates produced exclusively by the adult host alga *Vaucheria longicaulis*, but the natural cue cannot be detected in sea water by current methods of analytical chemistry. The simple carbohydrates mannitol and glucose, which are highly concentrated in *V. longicaulis* tissue, were tested as possible markers for the settlement cue in laboratory and field experiments. In production experiments, both mannitol and glucose were released by patches of the algae and accumulated in the surrounding water over time, as did bioactivity due to the settlement cue. Pore water trapped within patches of *V. longicaulis* during low tides contained high concentrations of mannitol and glucose, and induced a high level of larval metamorphosis even at a 1:5 dilution. The bioactive pore water was released from algal patches into overlying water following immersion by a flood tide; water collected above the surface of *V. longicaulis* induced significant metamorphosis and changes in larval swimming behavior. Glucose content was significantly correlated with bioactivity in water collected above algal mats throughout the first 30 min of a flood tide, and also 2 h later, during the peak of a high tide. Mannitol and glucose concentrations were high in sea water above the center of an algal patch, but diminished rapidly at the edges and outside of the patch. Pore water collected from mats of the co-occurring alga *Enteromorpha clathrata* did not induce metamorphosis or changes in larval swimming behavior, and contained only background levels of the markers. The combined results show that mannitol and glucose are indeed released and transported along with complex carbohydrates from *V. longicaulis*, and can be used to define patterns of distribution of the dissolved settlement cue on both spatial and temporal scales. Ecologically, the data suggest that settlement rates of larvae of *A. modesta* may vary widely during a tidal cycle, as a function of the release and subsequent hydrodynamic transport of waterborne cues from the host alga.

KEY WORDS: Larval settlement · Chemical cue · Metamorphosis · Recruitment · *Alderia modesta* · *Vaucheria longicaulis*

INTRODUCTION

Marine chemical ecology has traditionally focused on the ecological roles of non-polar secondary metabolites, which are generally contained on or within the surface of living tissue (Hay 1996). However, polar molecules can function as waterborne chemical signals, and dissolved cues mediate many interactions in

the ocean environment, including predation (Zimmer-Faust 1989, Leonard et al. 1999), mating (Gleeson et al. 1984), and habitat selection (Hadfield & Scheuer 1985, Lambert & Todd 1994, Krug & Manzi 1999). The distribution of water-soluble molecules in the field is determined by rates of production and release of the chemicals, followed by transport through advection and molecular or turbulent diffusion (Zimmer & Butman 2000). To understand the importance of soluble chemical cues to ecological processes, it is thus necessary to

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define the spatial and temporal patterns of waterborne molecules in the field.

Larval recruitment plays a critical role in benthic ecosystems, structuring communities and regulating population dynamics (Grosberg 1982, Roughgarden et al. 1988, Underwood & Fairweather 1989, Strathmann 1990). Many marine invertebrates produce dispersing larvae that eventually settle to the bottom and metamorphose, often in response to chemical cues of habitat suitability (Grahame & Branch 1985, Pawlik 1992, Levin & Bridges 1995). Habitat selection by the larvae of benthic marine invertebrates is often determined by the interaction of signal molecules, hydrodynamics of the natural environment, and larval behavior (Butman 1987, Pawlik et al. 1991, Turner et al. 1994). Recent studies have demonstrated that waterborne signal molecules can trigger changes in larval swimming that can increase rates of settlement under natural flow conditions. For example, larvae of the oyster *Crassostrea virginica* responded to a dissolved chemical cue by swimming more slowly and turning more frequently in the water column, behavior that increased settlement rates in flowing water (Turner et al. 1994, Tamburri et al. 1996). Larval swimming trajectories were quantified through computer-assisted video motion analysis, demonstrating that larvae stimulated by the soluble cue swam downwards towards the bottom, whereas larvae in control sea water swam upwards towards the surface (Tamburri et al. 1996). To extend the results from laboratory flume studies to the field, larval collectors were engineered to release soluble molecules from acrylamide gel, at a controlled rate which was both theoretically calculated and empirically verified by sampling water above the apparatus; when deployed in the field, collectors releasing an inductive peptide caused significantly higher settlement rates of barnacle cyprids than those releasing non-inductive peptide controls (Browne & Zimmer unpubl. results). Quantifying the distribution of natural waterborne chemicals is thus the next step for delineating the role of dissolved cues in mediating settlement under field conditions (Eckman et al. 1994).

The specialist marine herbivore *Alderia modesta* (Lovén, 1844) has several features that make it a useful organism for investigating the distribution and ecological effects of dissolved settlement cues in the field. This opisthobranch mollusc occurs in temperate estuaries, feeding exclusively upon yellow-green algae of the genus *Vaucheria* (Xanthophyta: Xanthophyceae) (den Hartog & Swennen 1952, Bleakney & Bailey 1967, Trowbridge 1993). Lecithotrophic (non-feeding) larvae of *A. modesta* metamorphose in response to complex carbohydrates produced specifically by the adult host alga *V. longicaulis* (Krug 1998b, Krug & Manzi 1999, Krug in press). Water trapped within absorbent mats of

the algae during low tides (hereafter termed 'pore water') becomes saturated with the soluble form of the carbohydrate settlement cue; pore water induced immediate changes in larval swimming behavior and triggered metamorphosis, even when diluted 5-fold or more (Krug & Zimmer 2000). However, previous studies did not examine the flux of dissolved compounds out of algal mats into the surrounding water, where the cue would be substantially diluted by turbulent mixing and advection.

The bioactive oligosaccharides produced by the alga *Vaucheria longicaulis* exist in a complex mixture varying in composition and molecular weight (Krug 1998a, Krug & Manzi 1999). The most sensitive method for detection and isolation of carbohydrates is high pH anion-exchange chromatography with pulsed-amperometric detection (HPAEC-PAD, Dionex Systems). This method has a sub-nanomole detection limit for complex carbohydrates and is relatively robust to salt levels in samples from sea water (Kerhervé et al. 1995). However, most likely due to their polyanionic nature, the bioactive compounds from *V. longicaulis* were found to bind irreversibly to the ion-exchange columns used in HPAEC-PAD; the active carbohydrates could not be detected in sea water samples with existing analytical methods (Krug 1998a). We therefore sought to identify other compounds naturally produced by the algae that could be easily measured in sea water samples, and that could serve as markers for the more complex, bioactive molecules.

Two potential markers were the free sugar alcohol mannitol and the monosaccharide glucose. Mannitol, a primary photosynthetic product of many algae, comprised 1.5% of *Vaucheria longicaulis* by dry weight (Krug 1998a). Glucose represented 60% of the low molecular weight carbohydrate pool of *V. longicaulis*, with no other sugar representing more than 12% of the carbohydrate mass of the algae (Krug 1998a). Mannitol and glucose were expected to be released into pore water by the algae, along with the bioactive oligosaccharides, in detectable amounts. Our hypothesis was that mannitol and/or glucose would correlate with the presence of bioactivity in the water column, functioning as natural markers for the carbohydrate settlement cues produced by *V. longicaulis*. By tracing the distribution of these markers, we could thus estimate the efflux and distribution of chemical cues from mats of *V. longicaulis* into the overlying water, where the cues could mediate larval settlement in the field.

MATERIALS AND METHODS

Study site. Field work was conducted in the back of Mission Bay, a shallow estuary located in San Diego,

California, 21 km north of the border between the USA and Mexico. For a full description and photographs of the study site, see Levin (1982, 1984). The mudflat at this site is exposed to air for at least 5 h during daily low tides. The exposed surfaces of the mud are colonized year-round by mats of the yellow-green alga *Vaucheria longicaulis* (Abbott & Hollenberg 1976). Mats of *V. longicaulis* have a moss-like appearance, forming thick, spongy mats that grow as discrete patches or continuous sheets covering the mud.

Carbohydrate analysis. Carbohydrate markers were quantified by analytical HPAEC-PAD and UV detection at 254 nm, at the Glycobiology Core Facility at the University of California, San Diego. Samples were injected onto a CarboPac MA1 column (4 × 250 mm), eluting with 60 mM NaOH for 5 min, followed by a gradient from 60 to 660 mM NaOH over 30 min, and ending with a 660 mM NaOH wash for 5 min. Standard runs using 1 nmol each of mannitol and glucose were done at the beginning and end of each analysis, to calibrate response factors for integration, and to control for changes in retention times over the course of each run. The limit of sensitivity was approximately 100 picomoles of a given sugar per injection; analyses could thus be run for 1 µMolar solutions without concentration, or 0.1 µMolar solutions with desalting and concentration prior to analysis.

Pore water trapped within *Vaucheria longicaulis* mats was analyzed by direct injection of 5 to 50 µl of filtered sample onto the HPAEC-PAD column. Sea water collected above algal patches was desalted by pouring 0.5 ml of each sample over a short column of Dowex AG-50+ cation exchange resin (1 ml); neutral material, including carbohydrates, was eluted with 10 ml Milli-Q water and lyophilized. Concentrated samples were then dissolved in 50 to 100 µl degassed water and analyzed for mannitol and glucose by HPAEC-PAD. Milli-Q water was processed in parallel as a control for the desalting procedure.

Carbohydrate markers in pore water from algal mats. Patches were sampled in the field to determine the variability in carbohydrate marker content between mats of *Vaucheria longicaulis*. The algae forms absorbent mats that trap water, which becomes highly bioactive over the course of a low tide (Krug & Zimmer 2000). Initially, pore water was collected from 4 different mats during the final hour of a low tide (October 1999). Gentle pressure was applied to the algal mat, and exuded water was aspirated from the surface of the algae, filtered to 0.22 µm, and frozen prior to chemical analysis and bioassays. Each sample was analyzed for mannitol and glucose by directly injecting 10 µl onto a CarboPac HPAEC-PAD column. Field-collected pore water was diluted to 20% with filtered sea water (FSW) for use in larval bioassays, as prior results had

indicated pore water bioactivity was highly concentrated (Krug & Zimmer 2000). A further 8 patches were sampled and tested for mannitol and glucose during the final hour of a low tide (January 2000). All pore water collections were made within a 20 min period, and each patch was at least 5 m distant from all other surveyed algal mats. The density of algal mats was appreciably lower in January 2000; the sampled mats contained much less pore water than in October 1999, and insufficient water was collected to permit testing in metamorphosis bioassays. The 8 samples from January 2000 were therefore tested for marker content only, by directly injecting 50 µl onto a CarboPac HPAEC-PAD column.

Marker content was determined over time from a single patch to determine whether there was a detectable increase in concentration of carbohydrates during the course of a low tide. Water from within a patch of algae was sampled 1 h after the algae was exposed to air by receding waters of an ebb tide, and again immediately before the algae was immersed by the flood tide, after approximately 5 h exposure to air. Aliquots were tested for marker content by direct injection of 10 µl onto a CarboPac HPAEC-PAD column. Water samples were diluted to 20% with FSW prior to use in larval metamorphosis bioassays.

Larval metamorphosis assay. Water samples were tested for bioactivity using a modified form of a previously described larval metamorphosis assay (Krug & Manzi 1999). Adult specimens of *Alderia modesta* were collected from the field and held in petri dishes under 1 cm of sea water in the laboratory overnight. Lecithotrophic egg masses were harvested the following morning and were transferred to plastic dishes containing 0.45 µm FSW; water was changed every other day until hatching. Upon hatching, larvae were maintained in FSW for 2 d and were then subsampled for use in the bioassay. For each experimental treatment, 15 larvae were added to replicate assay dishes containing 4 ml of a given sea water sample. Larvae were scored for metamorphosis after 3 d. Each experiment included a FSW-only treatment as a negative control and live *Vaucheria longicaulis* as a positive control. The percentage of metamorphosis for each replicate was arcsine transformed to normalize the data. Bartlett's test for homogeneity of variances was used to test for heteroscedasticity in each data set; t' t-tests with a Bonferroni adjustment and an overall significance level of 5% were used for multiple comparisons when variances between treatments were significantly different, or for increased sensitivity over the more conservative post-hoc Scheffé test (Snedecor & Cochran 1980). Treatments with homogenous variances were compared using a 1-way ANOVA (Sokal & Rohlf 1981). Unplanned comparisons of means were

done using the Scheff procedure when the *F*-ratio was significant (Day & Quinn 1989).

Rates of production and release of markers and bioactivity in the laboratory. The release of (1) bioactive compounds and (2) the carbohydrate markers mannitol and glucose was measured from small patches of *Vaucheria longicaulis* in laboratory experiments. Mats of algae attached to the natural sediment base (5 cm^2) were collected from the field and maintained in an incubator held at 18°C on a 16:8 h light:dark cycle. Algae was moistened daily with sea water. In a preliminary experiment, 1 mat was submerged in sea water and stirred for 30 min. Small patches of *V. longicaulis* measuring 1×1 cm were then cut from the mat with a razor blade, slicing immediately below the point of attachment of basal filaments of the algae to the underlying sediment. Patches were placed in 4 ml FSW in plastic assay dishes for the following time intervals: 10 s, 1, 5, 10, and 20 min. At the end of a given time, patches were removed by sterile forceps and pressed gently against the side of the dish to remove trapped water. Three replicates were done for each time interval. The *V. longicaulis*-conditioned water in each dish was filtered to 0.22 µm and tested in the larval metamorphosis bioassay. The relationship between the time algae was bathed in sea water and the percentage of metamorphosis induced in the larval bioassay was estimated using a Model 1 regression; percentages of metamorphosis were arcsine transformed prior to analysis.

A subsequent experiment was run to quantify the release of both bioactivity and carbohydrate markers over shorter time periods in the laboratory. Small patches of algae were cut from an algal mat, as described above, and incubated for varying time periods in 3 separate trials, yielding the same pattern in each case. Results are presented for the trial in which patches were placed in 15 ml FSW in glass dishes for the following time intervals: 1, 2.5, 5, 7.5, and 10 min. Patches were removed after the allotted time as before, and were then blotted dry. Three replicate patches were used for each time interval. Patches of sediment without *Vaucheria longicaulis* were also cut from the mud and were incubated in sea water for 10 min, as controls for any carbohydrate content of the sediment base. One aliquot of conditioned sea water (1 ml) was removed from each dish for chemical analysis to quantify mannitol and glucose content. A second aliquot (4 ml) was removed from each dish, filtered to 0.22 µm, and tested for bioactivity using the larval metamorphosis assay. Patches were lyophilized and weighed after the experiment to quantify release of carbohydrates as a function of both mass of algae and time. The release rate of markers was ultimately calculated as micromoles of mannitol and nanomoles of glucose produced per gram of algae. The relationship between (1) the timed duration over which algae was incubated in sea water and (2) µmol or nmol of carbohydrate marker released per gram of algae was estimated using a Model 1 regression for both mannitol and glucose. A Model 1 regression was also used to compare the bioactivity of water samples from the different timed exposures, using arsine-transformed percentages of metamorphosis. Water exposed to mud for 10 min was used as a negative control for both bioactivity and release of carbohydrate markers.

Time course of release of markers and bioactivity during a flood tide. Based on the preceding results, the release of bioactivity and markers from algal pore water into the water column was measured during the initial immersion of algal patches during a flood tide (October 1999). A sample of pore water was collected from within one representative patch of *Vaucheria longicaulis* immediately prior to inundation by the rising water. The initial sample from the water column was drawn as soon as the rising tide covered the algae to a depth of 2 mm (termed 'time 0'). Samples (60 ml) were subsequently collected at 5 min intervals, by slowly drawing sea water through a 20-gauge needle into a syringe from a height of 2 mm above the algal patch. The syringe was mounted on a ruler, such that the tip of the needle was fixed at a height of 2 mm above the bottom edge of the ruler, which was rested on the surface of the algal mat for sampling. A control sample of Mission Bay water was collected from the low water mark, over an area of mudflat 50 m distant from the nearest *V. longicaulis*, to represent water that larvae in the bay would encounter prior to tidal inundation of the high mudflat and *V. longicaulis* mats. Samples were filtered to 0.22 µm and frozen at –80°C prior to use in bioassays. Carbohydrate marker content was determined as described above, by desalting and concentrating samples 5-fold. Water samples were tested in the larval metamorphosis assay and in the behavioral assay, to measure (1) induction of metamorphosis and (2) rate of change in direction (RCD).

For statistical analysis, percentages of metamorphosis were arcsine transformed to normalize the data. Multiple comparisons were performed for time 0 and 5 min water samples against all subsequent time points, using a *t'* *t*-test with a Bonferroni adjustment and an overall significance level of 5% (Snedecor & Cochran 1980). This method was sensitive to differences between the 5 min sample and subsequent time points, compared with the more conservative post-hoc Scheffé test. RCD data were normally distributed and were compared directly using a 1-way ANOVA. For water taken at each time point, Spearman rank correlation analyses were used to test for a relationship between glucose content and (1) percent metamorpho-

sis or (2) RCD of larval swimming paths. A non-parametric correlation test was used to avoid biasing the analysis by extreme values from the initial time point.

Larval swimming behavior assay. The swimming behavior of larvae was recorded using a Cohu infrared-sensitive video camera with a Pentax 100 mm macro lens. All video recording was carried out at 22°C, in a darkroom using only infrared light. Larvae were filmed while swimming ≤1 mm above the bottom of a round chamber made of a Plexiglas cylinder with a 32 mm internal diameter. The chamber was rested on a clear Plexiglas sheet with the camera set up beneath. Video recordings were processed at 10 frames s^{-1} through a computer-assisted video motion analyzer (Motion Analysis Corp. model VP 320 and ExpertVision software) interfaced with a Sun Microsystems SPARC 2 computer workstation. Lecithotrophic larvae (15 replicate^{-1}) were placed in 5 ml of FSW (negative control) or a sample of sea water collected from the field, and after a 5 s delay, larval behavior was filmed for 1 min. For each treatment and control, 10 to 12 replicates were run, and all larval paths were recorded and analyzed. Angular velocity (measured as RCD) and swimming speed were quantified for all larval paths during each filmed treatment. Differences in mean values for all paths from treatments and controls were compared using a 1-way ANOVA with a post-hoc Scheffé test for unplanned comparisons of means. Data are given in the text as means ± SE.

Spatial distribution of markers above a patch of *Vaucheria longicaulis*. Water was sampled 2 mm above the surface of *Vaucheria longicaulis* at various distances from the center of a roughly circular patch, 16 mm in diameter, to determine spatial distribution of the markers. Nine segments of Teflon tubing (0.5 m length) were used to draw water into sterile syringes. One end of each length of tubing was mounted on a wooden plank, with the tip of the tubing protruding 5 mm over the edge of the plank. The end of one length of tubing was mounted in the center of the plank, and the ends of the remaining 8 pieces of tubing were positioned 2, 4, 8, and 16 mm to the left and right of the center. The other end of each piece of tubing was attached to a needle secured onto a separate luer-lock syringe. The plank was placed over the top of a patch of *V. longicaulis* at low tide, such that the tips of the tubing were positioned 2 mm above the dry surface of the algal mat. Both ends of the plank were rested on elevated areas of the mud surface, such that the plank itself was above the surface of the patch and out of the water at all times. When the rising water of the next flood tide had immersed the algae to a depth of 2 mm, 5 ml of water was slowly drawn into each syringe.

Samples were transferred from syringes to sterile centrifuge tubes, and were then filtered to 0.22 µm and frozen at −80°C prior to analysis. An aliquot of each sample (0.5 ml) was desalted and analyzed for mannitol and glucose content as described above.

Correlation of markers and bioactivity in water collected during a high tide. To determine whether the cue was present above algal patches during subsequent hours of a high tide, sea water was collected above each of 8 different patches of *Vaucheria longicaulis* during the peak of a high tide. The 8 mats of algae were randomly selected, and each patch was at least 2 m distant from the nearest sampled patch. Samples were collected by syringe through a 20-gauge needle from a height of 2 mm above the surface of the algae, 2 h after tidal immersion when water depth was ~50 cm above patches of *V. longicaulis*. A control sample of sea water was collected from Mission Bay during a low tide, from an area of mudflat 50 m distant from any patches of *V. longicaulis*. Bioactivity was measured by adding larvae directly to replicate assay dishes containing the 8 water samples filtered to 0.22 µm. Glucose content was measured for each sample of field-collected water, and a Spearman rank correlation analysis was used to test for a relationship between glucose concentration and the percent metamorphosis induced by each water sample.

Specificity of markers and bioactivity in *Vaucheria longicaulis* pore water. The only macroalgal species that commonly co-occurs with *Vaucheria longicaulis* at the Mission Bay field site is the filamentous green alga *Enteromorpha clathrata* (Krug in press). To determine whether the carbohydrate markers and bioactivity were specific to water conditioned by *V. longicaulis*, water samples were collected from a small pool containing a dense, filamentous mat of *E. clathrata*. This *E. clathrata* pore water was filtered to 0.22 µm and frozen at −80°C prior to analysis. Mannitol and glucose content were measured by desalting and concentrating 0.5 ml of *E. clathrata* pore water followed by HPAEC-PAD analysis. Bioactivity was measured (1) in the larval metamorphosis assay, by adding larvae directly to replicate dishes containing filtered pore water, and (2) in the behavioral assay, by recording larval swimming in pore water. Results were compared with the sample of sea water collected 2 mm above a patch of *V. longicaulis* immediately upon tidal immersion ('time 0' sample) and with filtered sea water controls, using a 1-way ANOVA with a post-hoc Scheffé test. A second collection of *E. clathrata*-conditioned sea water was made by compressing mats of *E. clathrata* in the field, and collecting the algal exudate. This water sample was only analyzed for carbohydrate markers, as the high organic content led to microbial contamination in metamorphosis assays.

RESULTS

Carbohydrate markers in pore water from algal mats

The free sugar alcohol mannitol and the monosaccharide glucose are the primary low molecular weight carbohydrates in the tissue of *Vaucheria longicaulis*. To determine whether these 2 compounds could be used as detectable markers for complex carbohydrates produced and released by *V. longicaulis* into sea water, we collected samples of sea water from within the spongy mats of *V. longicaulis* during low tide in the field. These samples of 'pore water' were then analyzed for mannitol and glucose content by analytical chromatography using HPAEC-PAD. In preliminary assays, both mannitol and glucose were detected in pore water samples at µM concentrations (Table 1). It was not necessary to concentrate or desalt pore water prior to analysis for detection of these compounds. The background concentrations of both molecules in Mission Bay water were below the limit of detection (<100 pmol of carbohydrate per injection) when a comparable volume of water was analyzed directly by HPAEC-PAD.

Variation in concentration of the markers between patches of *Vaucheria longicaulis* was measured by sampling 4 mats in October 1999 and an additional 8 mats in January 2000. High levels of both mannitol and glucose were measured in all 4 pore water samples taken in October 1999 (Table 1). A 2-fold difference in

Table 1. Carbohydrate analysis of pore water collected from within patches of *Vaucheria longicaulis*. Samples of sea water were initially collected from 4 mats in October 1999; an additional 8 mats were sampled in January 2000. All samples were filtered to 0.22 µm and frozen prior to analysis. An aliquot (10 to 50 µl) of each sample was injected directly onto an HPAEC-PAD column with no prior desalting; a second injection at a different volume was used to confirm each result. Because pore water was not concentrated before analysis, markers were below the sensitivity limit for some samples (nd = not detected)

Algal patch	Mannitol (µM)	Glucose (µM)
October 1999		
1	392	82
2	372	80
3	782	186
4	732	200
January 2000		
5	nd	nd
6	nd	nd
7	9.8	5.2
8	31.0	8.4
9	17.2	2.4
10	19.2	5.8
11	nd	nd
12	14.4	2.6

concentration was found between mats with the highest and lowest marker content. In contrast, the mean abundance of both markers was approximately 50-fold lower in pore water collected in January 2000 (Table 1). The thickness of *V. longicaulis* mats varies seasonally at the Mission Bay study site, and the density of algal patches was much lower in January 2000. Markers were only detected in 5 of 8 samples by direct injection of pore water onto the HPAEC-PAD column; however, pore water was not desalted and concentrated, which would have permitted the detection of lower levels of each marker. The concentration of mannitol decreased significantly from a mean of 570 ± 109 µM SE in October 1999 to a mean of 11 ± 4 µM SE in January 2000 (unpaired 2-tailed t-test: df = 10, t = 7.6, p < 0.0001). The mean concentration of glucose also decreased significantly between October (137 ± 32 µM SE) and January (3 ± 1 µM SE) (unpaired 2-tailed t-test: df = 10, t = 6.1, p < 0.0001). Although the concentration of each marker decreased significantly between the October and January samples, the mean ratio of mannitol to glucose in pore water remained consistent. There was no significant difference in the mean ratio of mannitol to glucose in pore water from October 1999 (mean ratio = 4.32, n = 4 mats) and January 2000 (mean ratio = 4.35, n = 5 mats).

A single collection of pore water was previously shown to induce high levels of metamorphosis when tested at field strength. To test for between-patch variation in bioactivity, pore water was collected from 4 different mats of *Vaucheria longicaulis*. Each sample was assayed for induction of larval metamorphosis at a 1:5 dilution of the field concentration, and compared with background water from Mission Bay. All samples of 1:5-diluted pore water induced significantly higher levels of metamorphosis than did Mission Bay water (Fig. 1, and results of a 1-way ANOVA: $F_{4,14}$ = 9.43, p = 0.0006). There was no significant difference in bioactivity between pore water from any of the 4 patches (Fig. 1).

To determine whether the concentration of markers increased in pore water over the course of a low tide, samples were collected from one algal mat after the algae had been exposed to air for 1 h, and again from the same mat 4 h later, immediately before tidal inundation of the algal mats. A detectable increase was found in the pore water concentration of both mannitol and glucose over the 4 h sampling interval (Table 2). The concentration of glucose was found to double during the course of the low tide, while mannitol showed a clear but less dramatic increase. The percentage of metamorphosis induced by 1:5-diluted water did not change significantly over the course of the low tide, due to the high activity of pore water after the tide had initially receded.

Production and release of markers and bioactivity in the laboratory

The production and release of both markers and bioactivity were quantified in the laboratory for small patches of *Vaucheria longicaulis*. When mats of the algae were immersed in sea water, both mannitol and glucose were released into the surrounding water and
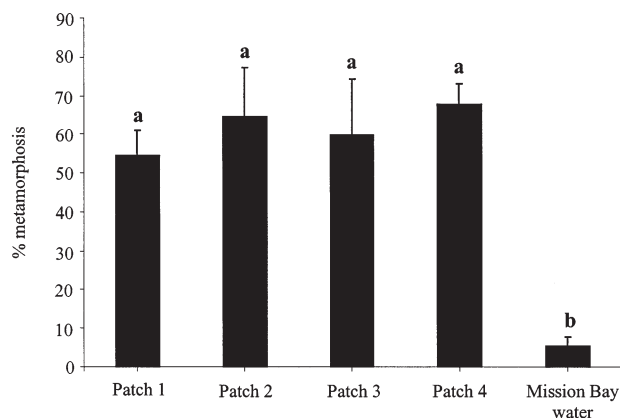

Fig. 1. Induction of larval metamorphosis of *Alderia modesta* by diluted pore water from patches of the alga *Vaucheria longicaulis*. Pore water trapped within spongy mats of *V. longicaulis* was collected from 4 different patches of the alga during a low tide, when the algae was exposed to air. Pore water was diluted 1:5 with filtered sea water and bioassayed for induction of metamorphosis using lecithotrophic larvae of *A. modesta*. Water collected from an area of Mission Bay devoid of *V. longicaulis* was used as a negative control. Data are mean (+SE) percentages of metamorphosis. Means marked with the same letter did not differ significantly (1-way ANOVA on arcsine-transformed percentages, followed by a post-hoc Scheffé test for unplanned multiple comparisons)

Table 2. Accumulation of carbohydrate markers and bioactivity in pore water over the course of a low tide. Samples of pore water were collected from within one patch of *Vaucheria longicaulis* 1 h after exposure to air on a receding tide, and 4 h later, immediately prior to immersion on the flood tide. An aliquot (10 to 50 µl) of each sample was injected directly onto an HPAEC-PAD column with no prior desalting to quantify mannitol and glucose concentration. Aliquots were diluted to 20% field strength with filtered sea water and used in the larval metamorphosis bioassay to test for changes in bioactivity, expressed as percent metamorphosis ± SE (n = 3), over the low tide

Time sampled	Mannitol (µM)	Glucose (µM)	Bioactivity (%)
Initial exposure to air (slack tide)	312	38	59.2 ± 2.6
4 h later (before flood tide)	372	80	67.7 ± 5.5

accumulated over time (Fig. 2). The relationship between immersion time and release of mannitol and glucose was estimated to determine whether markers increased in concentration with time. Time of immer-
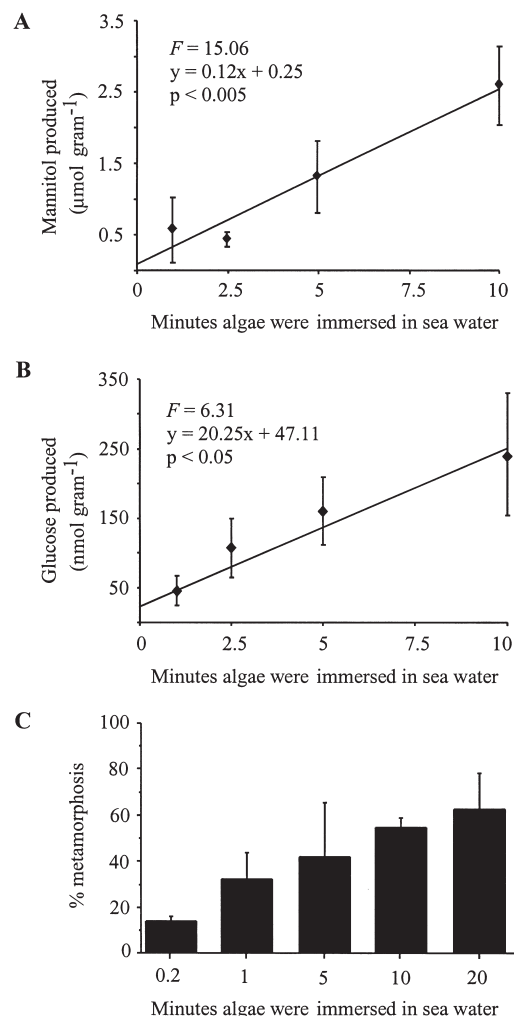

Fig. 2. Laboratory production and release of carbohydrate markers and bioactivity from small patches of *Vaucheria longicaulis*. Replicate patches of algae (n = 3) were immersed in filtered sea water for timed intervals. The concentration of both markers and the bioactivity of the resulting solutions were then determined, and algal patches were dried and weighed. (A) Production of mannitol by *V. longicaulis*. Data are given as mean (±SE) µmol of mannitol produced per gram of algal patch (dry weight) in the indicated amount of time, plotted with the results of a linear regression. (B) Production of glucose by *V. longicaulis*. Data are given as mean (±SE) nmol of glucose produced per gram of algal patch (dry weight) in the indicated amount of time, plotted with the results of a linear regression. (C) Release of bioactivity by *V. longicaulis*. Sea water was conditioned by patches of the alga for the indicated time interval, and was then filtered to 0.22 µm. Larvae of *Alderia modesta* (15 per replicate dish) were added to the *V. longicaulis*-conditioned water and were scored for metamorphosis after 3 d. Data are mean (+SE) percentages of metamorphosis

sion accounted for a significant percentage of the variance in mannitol content of conditioned sea water (Fig. 2A, and results of a Model 1 regression of effects of time, x, on mannitol release, y: $y = 0.12x + 0.25$; $F_{1,11} = 15.06$, p < 0.005, $r^2 = 0.60$) and a significant but lower percentage for glucose (Fig. 2B, and results of a Model 1 regression: $y = 20.25x + 47.11$; $F_{1,11} = 6.31$, p < 0.05, $r^2 = 0.41$). After 5 min of immersion, a mean 1.3 μg of
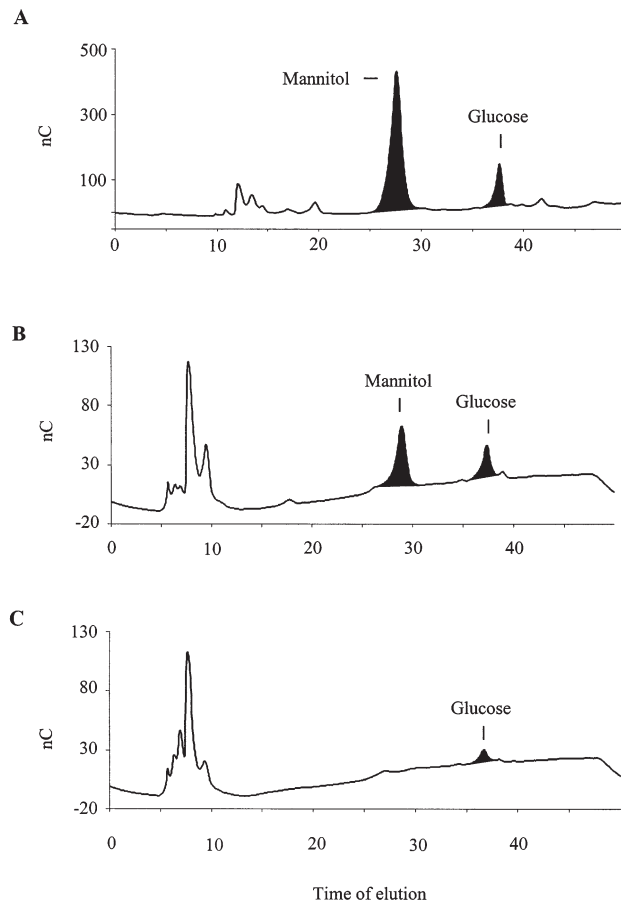

A

B

C

Time of elution

Fig. 3. Carbohydrate analysis of sea water samples using high-pH anion exchange chromatography with pulsed-amperometric detection (HPAEC-PAD). Carbohydrates were eluted from an HPAEC-PAD column using a gradient of NaOH over a 40 min run with electrochemical post-column detection. Units on the *y*-axis are arbitrary absorption units given as nano-coulombs. (A) Carbohydrate marker content of pore water collected within a patch of *Vaucheria longicaulis* during a low tide. Presence of mannitol and glucose was detected by direct injection of pore water without concentrating the sample. (B) Marker content of water sampled 2 mm above the same patch of *V. longicaulis* immediately after the rising tide covered the patch to a depth of 2 mm. The water sample was desalted and concentrated 5-fold prior to analysis. Note the change in scale of the *y*-axis compared with (A). (C) Marker content of water sampled 2 mm above the same patch 10 min after initial tidal immersion. This sample was also concentrated 5-fold prior to analysis

mannitol was released per gram of *V. longicaulis*; after 10 min, the amount had doubled to a mean 2.6 μg of mannitol per gram of *V. longicaulis*. Time of immersion also accounted for a significant percentage of the variance in bioactivity of *V. longicaulis*-conditioned sea water produced in 2 separate trials (Fig. 2C, and results of a Model 1 regression: $y = 0.31x + 0.06$; $F_{1,13} = 24.08$, p < 0.0005, $r^2 = 0.65$). Neither mannitol nor glucose was released by control patches of mud with the overlying mat of *V. longicaulis* removed, and water conditioned by mud alone had no significant bioactivity. The same general pattern was observed with different patches of *V. longicaulis* collected from the field at different times, indicating that different mats consistently release both markers and bioactivity into the surrounding water over time.

Time course of release of markers and bioactivity during a flood tide

The preceding results indicated that mannitol, glucose, and the more complex bioactive carbohydrates were produced and released by absorbent mats of *Vaucheria longicaulis* in the field, and that markers and bioactivity accumulated over time in pore water in both field and laboratory experiments. We therefore hypothesized that during a low tide markers and bioactivity would accumulate in pore water, and would subsequently be released in a concentrated pulse into overlying waters following immersion on the next flood tide. To test this prediction, we sampled water 2 mm above the surface of a representative mat of *V. longicaulis* immediately upon immersion by a flood tide, and at 5 min intervals for the next 30 min. Water samples were bioassayed for (1) induction of larval metamorphosis, (2) induction of changes in larval swimming behavior, and (3) concentration of mannitol and glucose. Both mannitol and glucose were present in high concentrations in the pore water prior to immersion by the flood tide (Fig. 3A). When the rising water had covered the *V. longicaulis* mat to a depth of 2 mm (time 0), both markers were detectable in the water column 2 mm above the surface of the algae (Fig. 3B). After 10 min, only glucose was detectable in the water column, at a significantly reduced concentration (Fig. 3C); glucose was detectable in all water samples taken over the first 30 min following tidal inundation. In subsequent analyses, we therefore focused on glucose as a natural marker, and tested for correlations between glucose concentration and bioactivity of water samples taken above *V. longicaulis* patches.

Water samples collected above the algal mat at sequential 5 min intervals differed significantly in the percentage of metamorphosis induced in larval bioas-

says (Fig. 4A). Water collected at time 0 (when water first reached a depth of 2 mm) induced a significantly higher mean level of metamorphosis (58.2 ± 9.4 % SE) than all water samples collected after the 5 min time point (t' t-test with a Bonferroni correction, $p < 0.05$). The 5 min water sample also stimulated significantly higher metamorphosis than the 30 min water sample and control Mission Bay water (t' t-test with a Bonferroni correction, $p < 0.05$). Most bioactivity was therefore released in a concentrated burst during the initial immersion, and was rapidly diluted by rising water after the first 5 min of the flood tide.

Vaucheria longicaulis pore water induced changes in larval swimming behavior, causing larvae to turn with increased frequency in response to the dissolved settlement cue (Krug & Zimmer 2000). However, larval response to water collected above algal patches was not previously tested. We therefore analyzed larval swimming behavior in response to the water samples collected at 5 min intervals above the *V. longicaulis* patch. Motion analysis revealed significant variation in larval behavior, quantified as RCD (Fig. 4B, and results of a 1-way ANOVA: $F_{4, 271} = 6.36$, $p < 0.0001$). The initial water sample induced the highest RCD, which was significantly greater than the turning rate induced by the 30 min sample and background bay water (Scheffé test, $p < 0.05$). Water sampled at 10 min after submersion still induced a significantly higher RCD than background bay water (Scheffé test, $p < 0.05$). Over the

first 30 min of the flood tide, water sampled at each successive time point induced progressively lower RCD values in larvae. The behavioral and metamorphosis bioassay data therefore show the same trend, with a spike in bioactivity at time 0 that progressively diminished to background levels over time. The percent metamorphosis and RCD induced by field water samples were significantly correlated (Spearman's rank correlation: $\rho = 0.89$, $Z = 1.98$, $p < 0.05$). There was no change between treatments in larval swimming speed ($F = 1.33$, $p > 0.25$), which is less sensitive to the presence of the *V. longicaulis*-associated chemical cue than RCD (Krug & Zimmer 2000).

The glucose content of the bioassayed water samples was determined, and showed a dramatic pulse of the marker out of the patch at time 0, followed by an exponential decay of the marker until background levels of bay water (0.1 μM) were reached at the 30 min time point (Fig. 4C). The concentration of glucose in water samples was significantly correlated with the percent-

Fig. 4. Bioactivity and carbohydrate marker content of sea water sampled 2 mm above a patch of *Vaucheria longicaulis* in the field. Water was collected beginning at the time of initial immersion of the patch (time 0) by a flood tide, and at subsequent 5 min intervals for the next 30 min. (A) Bioactivity of water collected in the field above a patch of *V. longicaulis*. Lecithotrophic larvae of *Alderia modesta* (15 dish⁻¹) were added to water samples in replicate dishes (n = 6 replicate dishes per time point). Data are mean (+SE) percentages of larval metamorphosis after 3 d exposure to the sea water sample. Percentages were arcsine transformed and compared using a t' t-test with a Bonferroni correction for multiple comparisons and an overall significance level of 5 % (*$p < 0.05$ compared with sea water controls). (B) Behavioral response of larvae of *A. modesta* to sea water sampled above a patch of *V. longicaulis*. Rate of change in direction (RCD) was quantified using computer-assisted video motion analysis for lecithotrophic larvae in response to field-collected water samples and background Mission Bay water. Data are mean (+SE) RCD for all larval paths recorded for a given treatment. Means marked with the same letter did not differ significantly (1-way ANOVA on arcsine-transformed percentages, followed by a post-hoc Scheffé test for unplanned multiple comparisons). (C) Glucose content of water samples collected above a patch of *V. longicaulis*. Water samples were concentrated 5-fold and analyzed for carbohydrate content by analytical HPAEC-PAD. Data are plotted with the results of a logarithmic function showing the exponential decay of glucose concentration over time
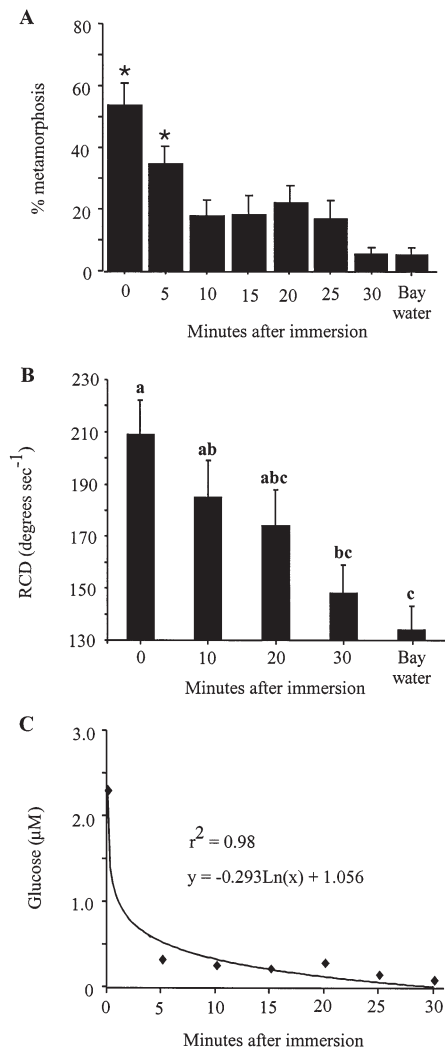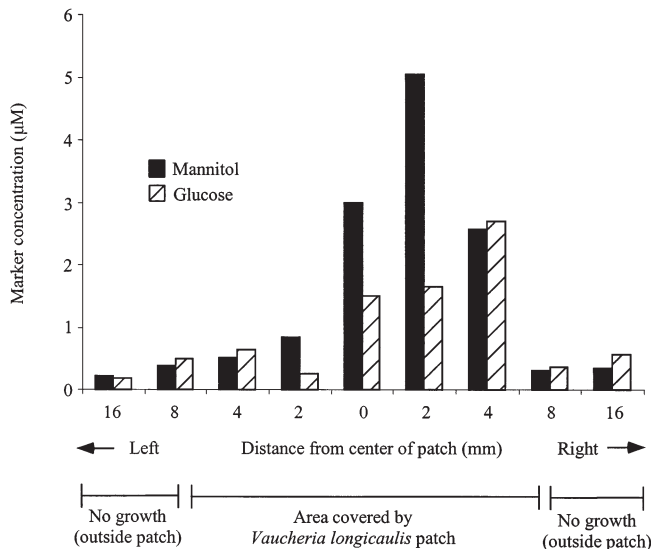
Fig. 5. Spatial distribution of carbohydrate markers above and around a patch of *Vaucheria longicaulis*. Marker content of water was measured 2 mm above the bottom immediately following immersion by a flood tide, at various distances from the center of a patch of *V. longicaulis*. The patch extended 8 mm to the left and right of the center point; water sampled 16 mm from the center was above uncolonized mud. Data are concentrations of mannitol and glucose measured by analytical HPAEC-PAD

age of metamorphosis induced in larval bioassays (Spearman's rank correlation: $\rho = 0.98$, $Z = 2.58$, $p < 0.01$). The correlation was significant even if the data for the water sampled at time 0 were omitted from the analysis. Glucose was also significantly correlated with changes in larval swimming behavior, quantified as increased RCD (Spearman's rank correlation: $\rho = 0.89$, $Z = 1.98$, $p < 0.05$).

Spatial distribution of markers above a patch of *Vaucheria longicaulis*

When water was sampled 2 mm above the surface of a patch of *Vaucheria longicaulis* in March 2000, both markers were again present at high concentrations over the center of the patch immediately following tidal immersion (Fig. 5). Marker concentrations were highest in water collected within 2 to 4 mm of the center of the patch, but declined rapidly near the edge. The concentrations of mannitol and glucose above both edges of the algal patch (4–8 mm to the left, 8 mm to the right of center) were in the same range as water sampled above the mud surface well outside of the patch (16 mm left and right of center) (Fig. 5). Lower marker levels were measured above the left side of the study patch at distances of 2 and 4 mm, compared with

the right side of the patch; variable thickness of the algal mat or other differences that were not observed may explain the asymmetry in the release patterns for soluble compounds from this patch. Glucose and mannitol are thus spatially distinct markers of algal exudate, present in high concentrations immediately above the center of a given patch of *V. longicaulis*, but absent at the edges and over adjacent areas of the mudflat surface.

Correlation of markers and bioactivity in water collected during a high tide

The marker glucose was positively correlated with the bioactivity of water collected above algal patches during the first 30 min of a flood tide. To determine whether glucose was a useful marker during the subsequent hours of a high tide, water samples were collected 2 h after algal patches were submerged by a flood tide, when water depth was approximately 50 cm. Samples were assayed for induction of larval metamorphosis and for glucose content as before. There was significant variation in the bioactivity of the water samples (Fig. 6, and results of a 1-way ANOVA: $F_{8,18} = 4.71$, $p < 0.005$). Water collected above one patch induced significantly more metamorphosis than sea water controls and the least-active sample of field water (Scheffé test, $p < 0.05$). A significant concentration of the dissolved chemical settlement cue was thus present in water above certain *Vaucheria longicaulis* mats even during the peak of a high tide. The source of the variation between patches was likely a combination of varying thickness of different algal mats, and local hydrodynamics affecting dilution and distribution
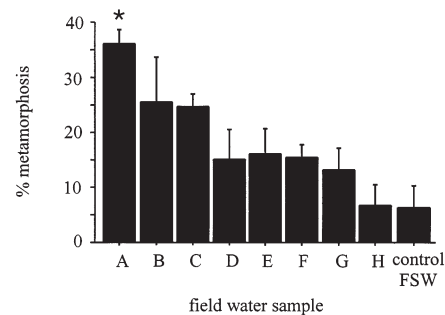

Fig. 6. Bioactivity of water collected above 8 distinct patches (labeled A to H; FSW: filtered sea water) of *Vaucheria longicaulis* during the peak of a high tide, 2 h after tidal immersion. Data are mean percentages of larval metamorphosis (+SE) in response to a 3 d exposure to the indicated water sample. Arcsine-transformed percentages were compared using a 1-way ANOVA with a post-hoc Scheffé test (*$p < 0.05$ compared with sea water controls)

of the dissolved cue. The significantly active sample also contained the highest concentration of glucose (0.6 µM), and the concentration of glucose was significantly correlated with bioactivity for the overall data set (Spearman's rank correlation: $\rho = 0.73$, $Z = 2.07$, p < 0.05).

Specificity of markers and bioactivity in *Vaucheria longicaulis* pore water

The macroalga *Enteromorpha clathrata* sometimes co-occurs with *Vaucheria longicaulis*, and may cover the majority of the mudflat surface at certain times of the year at the Mission Bay field site. To determine whether the carbohydrate markers and bioactivity were specific to water conditioned by mats of *V. longicaulis*, water samples were collected from a small pool containing filamentous mats of *E. clathrata*; this *E. clathrata* pore water was tested for bioactivity in metamorphosis and behavioral assays, as well as for mannitol and glucose content. Results were compared with the sample of sea water collected 2 mm above a patch of *V. longicaulis* immediately upon tidal immersion (time 0; see Fig. 4A). There was significant variation in the percentage of metamorphosis induced by water collected above *V. longicaulis*, water trapped in mats of *E. clathrata*, and control sea water (Fig. 7A, and results of a 1-way ANOVA: $F_{2,12} = 25.30$, p < 0.0001). Water collected above *V. longicaulis* induced a significantly higher percentage of metamorphosis than did pore water from *E. clathrata* or a filtered sea water control (Scheffé test, p < 0.001). Significant variation was also seen in the behavioral assay (Fig. 7B, and results of a 1-way ANOVA: $F_{2,198} = 12.36$, p < 0.0001). Water collected above *V. longicaulis* induced larvae to turn significantly more frequently than did *E. clathrata* pore water or control sea water (Scheffé test, p < 0.005).

The carbohydrate marker content was determined for 2 separate collections of pore water from *Enteromorpha clathrata*. Markers were identified in trace amounts, near the limits of detection of HPAEC-PAD, for both samples of *E. clathrata* pore water. In water collected among filaments of *E. clathrata* growing in a shallow pool, mannitol was present at a concentration of 0.44 µM and glucose at 0.22 µM; water trapped within a damp, air-exposed mat of *E. clathrata* contained 0.13 µM mannitol and 0.12 µM glucose. These concentrations, close to background levels for Mission Bay water, were 1 order of magnitude below the levels of both markers detected in water collected 2 mm above a patch of *Vaucheria longicaulis* on the same day, and were 1 to 3 orders of magnitude lower than concentrations found in pore water collected within mats of *V. longicaulis* (Table 1). High levels of markers
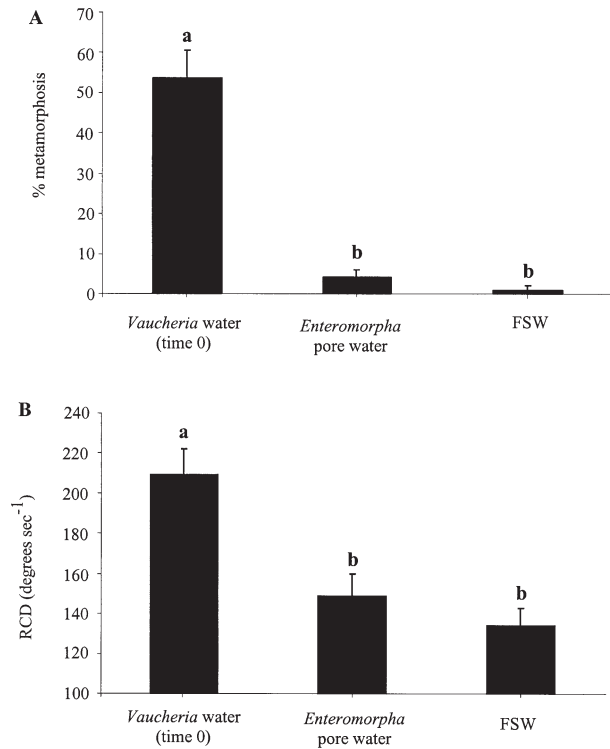

Fig. 7. Specificity of larval response to exudate from *Vaucheria longicaulis*. (A) Larval metamorphosis in response to water collected above a patch of *V. longicaulis*, water sampled from a pool densely packed with filaments of the macroalga *Enteromorpha clathrata*, and control filtered sea water (FSW). *Vaucheria*-conditioned water was collected above a mat of the algae immediately following immersion by a flood tide (time 0 sample). Data are mean (+SE) percentages of metamorphosis. Means marked with the same letter did not differ significantly (1-way ANOVA on arcsine-transformed percentages, followed by a post-hoc Scheffé test). (B) Behavioral response of larvae to water conditioned by *V. longicaulis*, *E. clathrata*, or control FSW. Rate of change in direction (RCD) was quantified using motion analysis for larvae added to each of the water treatments in (A). Data are mean (+SE) RCD values. Means marked with the same letter did not differ significantly (1-way ANOVA followed by a post-hoc Scheffé test)

and bioactivity are therefore specific to pore water from patches of *V. longicaulis*, and are not present above background levels in pore water collected from mats of *E. clathrata*.

DISCUSSION

Natural markers for tracing complex settlement cues in the field

Despite decades of research, no structure has been published for any natural, water-soluble inducer of

larval metamorphosis (Pawlik 1992). Limitations of analytical chemistry make it difficult, if not impossible, to identify trace amounts of polar metabolites in dilute sea water samples, confounding the isolation of dissolved cues (Zimmer-Faust & Tamburri 1994). This is particularly true for carbohydrates, where separation and detection both present technical challenges (Krug 1998a). Thus, there have been no reports to date quantifying the release of any natural chemical cue for larval settlement, or tracing the distribution of soluble cues in the water column. While continuing to work towards identifying the structures of the complex carbohydrates responsible for the bioactivity of *Vaucheria longicaulis* pore water, we aimed to identify detectable markers with chemical and physical properties similar to the bioactive compounds. By quantifying production and release of such markers in the field, we sought to determine the distribution of the dissolved cue under natural conditions without direct detection of the cue itself. This study focused on the production of mannitol and glucose by *V. longicaulis*, 2 simple carbohydrates that are abundant in algal tissue but contain no intrinsic bioactivity in larval assays (Krug 1998a).

Glucose and its isomer fructose are the major free monosaccharides in sea water (Mopper et al. 1980, Ittekkot et al. 1981). Reported concentrations of glucose from different oceans ranged from 0.03 μM to 0.67 μM, with a mean of approximately 0.3 μM (Mopper et al. 1980); a similar mean value can be approximated from the data of Ittekkot et al. (1981). The background concentration of free glucose in Mission Bay water was measured at 0.1 μM or less in the present study; concentrations of glucose in *Vaucheria longicaulis* pore water were thus 1 to 3 orders of magnitude above background levels in Mission Bay. The free sugar alcohol mannitol, a reduced form of mannose, has not been reported as a major free carbohydrate component of sea water samples, and was not detectable in Mission Bay water. The high concentration of mannitol in *V. longicaulis* pore water (nearly mM in some patches) is thus a clear indicator of water that has been trapped within an algal mat. Mannitol is the predominant low molecular weight carbohydrate contained in tissue of *V. longicaulis*, representing 1.5% of the dry mass of the algae (Krug 1998a). The consistent mean 4.3 ratio of mannitol to glucose in pore water averaged across several patches, regardless of the absolute concentrations of the 2 markers, appears to be a distinctive chemical signature of the algae, and may be a diagnostic feature of *V. longicaulis*-conditioned sea water in the field.

In samples taken above one *Vaucheria longicaulis* mat, the mannitol signature of diffusing pore water was evident at time 0, but rapidly diminished over the first 5 min of the flood tide to near-background levels. In contrast, elevated levels of glucose were apparent in water above the algal patch for the first 20 to 30 min of the flood tide. This discrepancy may be due to different sources for the 2 markers. Mannitol is a fully reduced sugar alcohol with no reducing end, and cannot be polymerized into oligosaccharides; all mannitol is therefore located intracellularly within *V. longicaulis* tissue. The high levels of mannitol measured in laboratory production experiments and in field samples of pore water are most likely due to cell lysis or leakage of cellular contents. *V. longicaulis* is a siphonaceous alga, with a continuous cytoplasm inside each filament (Abbott & Hollenberg 1976); damage to the algae could lead to considerable leakage of intracellular products into the surrounding medium. In the field, feeding by adult specimens of *Alderia modesta* may damage algal blades and contribute to the subsequent leakage of mannitol into pore water. Adult slugs feed by puncturing the algal cell wall and then sucking out the cytoplasm; damaged thalli may continue to leak mannitol until wound healing occurs (Tornbom & Oliveira 1993a,b). In contrast, glucose exists both as the most abundant free monosaccharide inside the cytoplasm and also as the major component of both soluble oligosaccharides and cell wall polysaccharides of the alga (Krug 1998a). The continuous sloughing off of cell-surface polysaccharides and subsequent microbial degradation will contribute to the glucose concentration of *V. longicaulis* pore water. This may explain why the glucose signature of pore water persists in the overlying water column for much longer periods than mannitol, even during later hours of a high tide: glucose can be continually released from the surface of the algal tissue through degradation of glucose-containing polymers, whereas mannitol can only accumulate during low tides as a product of cell leakage.

The results demonstrate that even when the chemical structure of a settlement cue is unknown, more easily detectable compounds may be useful as proxy molecules for field research. To our knowledge, this is the first use of natural markers to track the release and transport of active compounds from a benthic source into the water column. Additional research is needed to examine the release of dissolved compounds from replicate patches of algae in different seasons, to fully characterize how these processes function in the natural habitat. This study serves as a proof of the concept that natural markers can be a useful tool for understanding how polar molecules are distributed in the field. Furthermore, these compounds provide insight into the natural rates of production by the algae and subsequent dilution of waterborne cues by local hydrodynamic forces.

Role of dissolved cues: settlement in the field

The results demonstrate that water-soluble chemical cues from the alga *Vaucheria longicaulis* accumulate in pore water during low tides, and are subsequently released in a concentrated burst when patches of the algae are initially immersed by the next flood tide. Both marker concentration and bioassay data indicated that the soluble cue was largely diluted by rising tidal waters within 10 min of immersion, and reached background levels within 30 min for the patch analyzed in Fig. 4. However, 1 out of 8 patches (Fig. 6) still released a significant amount of glucose and the dissolved cue during the second hour of a high tide, suggesting that some algal mats may continue to release carbohydrates well into a given tidal cycle. These results may have important implications for the settlement of larvae of *Alderia modesta* in the field. If dissolved cues are important in mediating settlement in this species, larvae carried over the algal patches during the first few minutes of a high tide should have a greater chance of settling in response to waterborne cues than larvae that are transported over patches later in a high tide.

Future work will involve determining whether larvae 'ride' the front of the rising tide, which would result in the maximum exposure to dissolved settlement cues. Both long- and short-lived larvae of *Alderia modesta* showed immediate and dramatic changes in swimming behavior in response to the soluble cue (Krug & Zimmer 2000). Such swimming behaviors would have the best chance of increasing settlement under conditions of slow water flow. We have begun to characterize the hydrodynamic environment of settling larvae, using acoustic Doppler velocimetry to measure flow in 3 dimensions over patches of *Vaucheria longicaulis* in the field. The mean velocity 1 cm over a patch of algae during the first 100 min of a high tide was 1.13 cm s^{-1}, and the mean u_* value during this time interval was 0.19 cm s^{-1} (authors' unpubl. data). These results indicate that the back bay habitat in which larvae of *A. modesta* settle is characterized by very slow flow and low turbulence, conditions which should enable larvae to respond to dissolved cues and increase their chance of successful settlement onto an algal patch. Further field work will indicate how biology, chemistry, and physics interact to mediate settlement during the initial period of each flood tide, as predicted from the present data.

To fully characterize the distribution of waterborne cues in the field, we are expanding this study by sampling above replicate patches of *Vaucheria longicaulis*, at various heights in the water column and at multiple distances from the centers of different algal mats. By combining marker concentration profiles with bioassay data, it will be possible to estimate production and flux out of algal mats; measurement of hydrodynamic forces such as turbulent mixing over algal mats will contribute to our understanding of how transport of the dissolved settlement cue occurs over both space and time. The ultimate goal is to determine where in the mudflat habitat, and during what times in a tidal cycle, soluble cues may influence patterns of larval settlement. Larval collectors can then be used to release known amounts of the dissolved algal compounds, to demonstrate larval availability and response to soluble cues in the field. In a previous study, collectors were engineered to release inductive and non-inductive peptide cues from acrylamide gels, over time at a controlled and monitored rate; release of the inductive cue at trace concentrations significantly enhanced settlement of barnacle cyprids in the field (Browne & Zimmer unpubl. results). Using a similar approach, our aim is to release the dissolved cue from *V. longicaulis* at different places and times during a tidal cycle, to quantify the settlement response of larvae under natural conditions.

To our knowledge, this is the first report to suggest that patterns of settlement in an intertidal habitat may vary widely over a short time frame, due to dilution of waterborne cues during a rising tide. Similar situations may exist for larvae of other species which settle on mudflats and in estuarine environments, as well as in rocky intertidal habitats where cues could accumulate in isolated tide pools and disperse during flood tides. Even in rocky subtidal or coral reef environments, chemical cues could accumulate in areas of low flow speed and low turbulent mixing, such as within crevices, dense kelp beds, or coral heads. The current study thus demonstrates the value of the marker approach, highlighting a potentially important ecological phenomenon that might be overlooked if the chemistry of dissolved molecules and the physics of the environment were not taken into account. By combining studies of the behavior of organisms, the distribution of signal molecules, and the hydrodynamics of field habitats, it may ultimately be possible to define the role of dissolved compounds in mediating complex processes such as larval settlement in the marine environment.

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Velocity and concentration distributions in turbulent odor plumes in the presence of vegetation mimics: a flume study

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ABSTRACT: Many marine animals use olfaction to search for vital resources (e.g. food, mates), and their success depends on the complex structure of turbulent odor plumes. To better understand chemically mediated interactions it is necessary to empirically measure the relationship between turbulent water flow and odor distribution in a variety of settings. Marsh edge habitats are important nursery and foraging areas for resident and transient estuarine species. The presence of emergent vegetation in these habitats can have a strong influence on the ambient turbulence regime, as well as the dispersal of water-borne odors. To quantify the effects of vegetation-like structure on odor dispersal, I measured velocity and concentration fluctuations in artificial 'odor' plumes created in a laboratory flume. Time-series measurements of velocity and concentration were made simultaneously at the same spatial location using a thermistor flowmeter combined with a conductivity microprobe. Flow speed (advection) controlled odor dilution and odor filament dispersal throughout the plume; plumes generated in 5 cm s^{-1} flows had higher mean concentrations, and were characterized by fewer odor filaments that were more concentrated and of longer duration than plumes generated in 12 cm s^{-1} flows. In contrast, adding *Spartina alterniflora* mimics upstream of the plume source primarily altered odor filament properties; the presence of upstream structure created more abundant odor filaments that were more dilute and of shorter duration relative to smooth-bottomed treatments. Importantly, such changes to the odor plume structure can alter the success of odor mediated search, although further studies are needed to show if this effect will be positive or negative.

KEY WORDS: Odor plume · Hydrodynamics · Turbulence · Microscale

INTRODUCTION

Chemical information permeates marine environments and the importance of this information for the ecology of marine organisms is undisputed. Dissolved and adsorbed compounds mediate processes ranging from larval settlement (positive and negative cues, metamorphosis induction) to foraging (attraction and deterrence) and mating (mate attraction, mass spawning). Classically, the study of chemical ecology has focused on the identification and purification of the myriad compounds used in these interactions (e.g.

Valentinčič 1991, Pawlik 1992, Nevitt et al. 1995, Decho et al. 1998). While this research is critical to our understanding of compound synthesis and detection, the factors which contribute to the dispersal and transport of chemical signals are equally important to understanding the ecological and behavioral components of chemoreception. This is especially true for dissolved compounds (e.g. odors), whose distributions in space and time are intimately tied to the ambient flow regime.

Research focusing on the link between hydrodynamics, odor signal structure, and animal performance has led to a fundamental shift in our understanding of chemical plumes and how they are perceived by navigating organisms. It is now commonly accepted that the

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distribution of odorant within plumes is patchy, rather than the stable gradient predicted by time-averaged (Gaussian) models (e.g. Murlis & Jones 1981, Atema 1985, Moore & Atema 1988, 1991, Zimmer-Faust et al. 1988, 1995, Moore et al. 1991, Finelli et al. 1999). Furthermore, properties associated with individual odor patches (Moore & Atema 1988, Zimmer-Faust et al. 1988, Moore et al. 1994) and mechanical information provided by flowing water (Matthewson & Hodgson 1972, Weissburg & Zimmer-Faust 1993, Zimmer-Faust et al. 1995, Atema 1996, Finelli et al. 2000) may be used during orientation to plumes. These findings, among others, necessitate a more general understanding of hydrodynamics and odor dispersal processes in a variety of habitats. Unfortunately, our knowledge of odor plume structure, especially with reference to prevailing hydrodynamic conditions, is restricted to a handful of studies conducted in the laboratory (Moore & Atema 1988, 1991, Weissburg & Zimmer-Faust 1993, Moore et al. 1994) or simplified field settings (Zimmer-Faust et al. 1995, Finelli et al. 1999). As such, manipulative studies are critically needed to determine how odor dispersal patterns respond to the fluid dynamics that characterize different habitat types.

Salt marshes serve as critical habitat for adult and juvenile crustaceans and fishes (e.g. Kneib 1997, Minello & Webb 1997). The marsh edge, in particular, is an area of focused activity for estuarine predators and prey (Baltz et al. 1993, Minello et al. 1994, Peterson & Turner 1994). For example, Micheli (1996, 1997) showed that blue crabs *Callinectes sapidus* use marsh edge habitats more frequently and have higher predation rates in these habitats than in adjacent sandy bottom areas. She hypothesized that blue crabs were benefitting from increased prey density and protection from predatory birds among the shoots of emergent vegetation. Micheli's findings are particularly intriguing because blue crabs seem to be exploiting a habitat traditionally regarded as an area of prey refuge (Baltz et al. 1993, Micheli 1996, Kneib 1997). In this study I begin to address whether the physical-chemical environment of the marsh edge facilitates the odor-mediated foraging of blue crabs and other organisms.

Partially submerged beds of vegetation (e.g. *Spartina alterniflora*) are characteristic of tidal marshes and play an important role in the hydrography of these habitats (e.g. Eckman 1983, Nowell & Jumars 1984, Leonard & Luther 1995, Sand-Jensen & Mebus 1996). For example, Leonard & Luther (1995) showed that both mean velocity and turbulence intensity decrease among stands of *S. alterniflora* and other marsh vegetation. They argued that stands of emergent vegetation act as a baffle that retards flow through the marsh canopy and redistributes turbulent energy from larger to smaller eddies.

Such habitat-specific changes in mean flow and turbulence properties can have pronounced effects on both odor dispersal and orientation behavior. For example, Finelli et al. (1999) demonstrated how gross plume morphology was related to the variable eddy sizes within tidal flows. Moreover, Weissburg & Zimmer-Faust (1993) showed that foraging by blue crabs is sensitive to both mean flow and turbulence. Specifically, blue crabs were less efficient and less successful in orienting to odor plumes in flows with increased turbulence. They concluded that increased turbulent mixing caused more rapid dilution of the plume, thereby decreasing the ability of blue crabs to detect the odor signal. Thus, depending on the nature and magnitude of changes in turbulence induced by stands of emergent vegetation, the success of odor-mediated search may be enhanced or diminished.

To determine how emergent vegetation affects odor dispersal, I performed a flume study during which odor concentration and velocity were measured simultaneously throughout tracer plumes. I conducted these studies at 2 flow speeds and in the presence or absence of structures mimicking emergent vegetation. To simplify this initial flume study, I consider the effects of structure on odor dispersal in downstream clearings rather than inside the canopy where searching may be further constrained. As did Leonard & Luther (1995), I found that vegetation-like structure decreased turbulence intensity and redistributed turbulent kinetic energy from larger to smaller eddies. Turbulence properties in the presence of upstream structure resulted in more diffuse plumes and more numerous individual odor filaments. Such changes in plume structure are likely to alter the ability of organisms to successfully search in vegetated habitats compared to adjacent smooth-bottomed habitats, although further studies are needed to tease apart these effects.

MATERIALS AND METHODS

The experiment. I tested the effects of flow speed and emergent vegetation on odor dispersal in a laboratory flume using 2 flow speeds (~5 and ~12 cm s^{-1}) and 2 vegetation mimic treatments (present and absent). Tracer plumes of an ethanol-salt solution were introduced into flowing freshwater while concentration and velocity were measured at 40 locations in each plume. The ethanol-salt mixture was used as an easily measurable odor analog with dispersal properties similar to other water soluble compounds (for similar examples of this technique see Moore & Atema 1988, Bara et al. 1992).

I performed these studies in a racetrack flume housed at the Louisiana Universities Marine Consortium (LUMCON) Defelice Marine Center in Cocodrie,
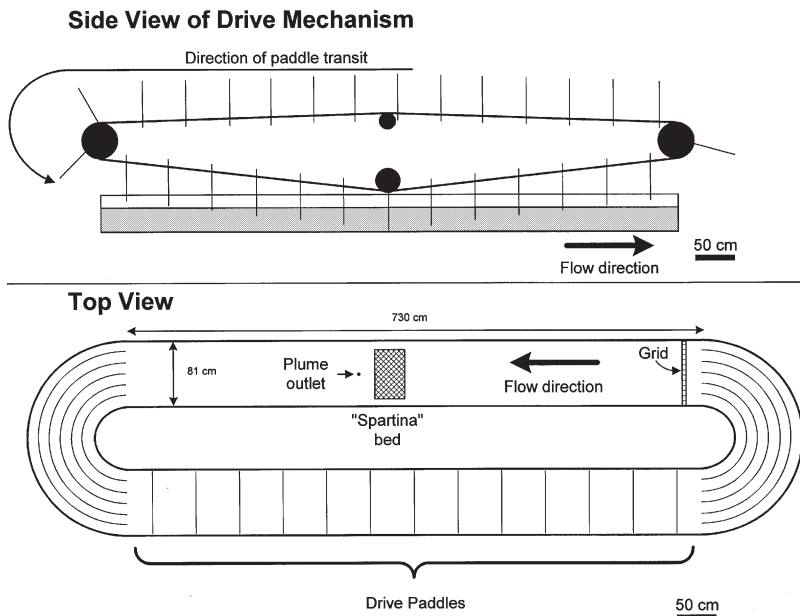
Side View of Drive Mechanism

Fig. 1. Design and layout of the LUMCON flume. Side view of drive mechanism shows the vertical entry and exit of paddles. Top view of the flume shows relative positions of experimental apparatus

thermistor flow meter. Two vegetation mimic treatments, present and absent, were also conducted. For these preliminary studies, I chose to control 'vegetation' geometry and distribution by using 0.8 cm diameter wood dowels to simulate *S. alterniflora* stems. I arranged 57 dowels in a 61.2×34.5 cm staggered grid with 7 cm between dowels (Fig. 2), resulting in a final stem density of 270 m^{-2}. This density falls well within natural densities studied elsewhere (Eckman 1983, Leonard & Luther 1995).

I created 'odor' plumes by continuously releasing a dyed salt-ethanol solution into the flowing freshwater. The tracer solution consisted of a 77:23 mixture, by volume, of 50‰ brine (Crystal Sea Marine Mix, Marine Enterprises International, Baltimore, MD, USA, dissolved in distilled water) and 100% ethanol (Louisiana State University Medical School Supply). Fluorescein dye was added at 0.1 g l^{-1} and the final solution was filtered to remove undissolved salt. This mixture was developed by trial and error to produce a solution equal in density to flume water but with an increased conductivity (Table 1). The nominal conductivity of the tracer solution was measured with an Orion Model 125 conductivity meter, and its density matched that of the flume water to within 0.15% on each trial day (Table 1).
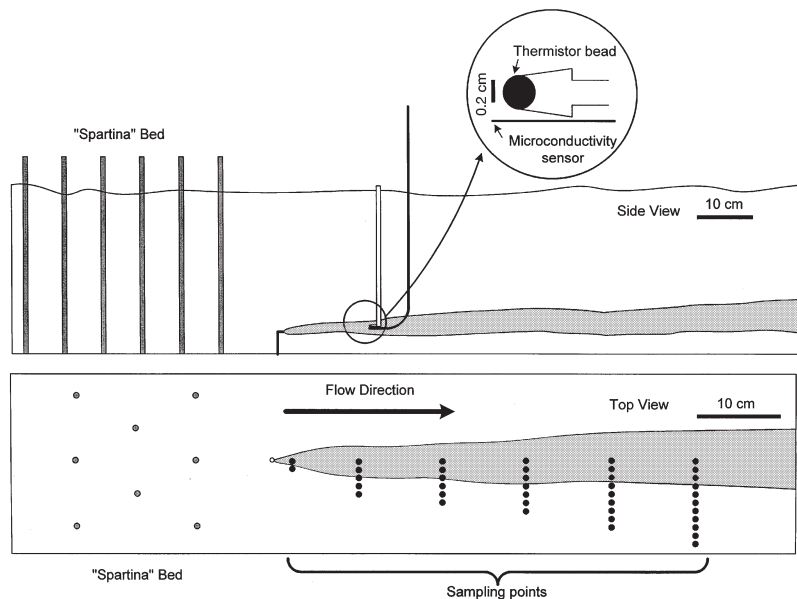
LA, USA (Fig. 1). The LUMCON flume is oval, with the straight portions of the flume measuring 730 cm in length (Fig. 1). The channel width is 81 cm at all points and depth was held at 30 cm. At this depth, the water volume in the flume is ~4900 l. Water flow is driven by a series of paddles attached to a caterpillar-type drive mechanism. The paddles enter and exit the water vertically to minimize the creation of secondary flows (Fig. 1). A 5.5 cm thick plastic baffle (1×1 cm grid) is located at the extreme upstream position on the 'working' side of the flume to reduce large-scale flow disturbances introduced by paddle motion or flume turns. The flume was filled with fresh tap water at least 48 h prior to each trial. Three replicate trials were completed on separate days with all 4 treatments being performed in a single day.

Flow speed treatments were chosen to represent high (12 cm s^{-1}) and low (5 cm s^{-1}) flows present in *Spartina alterniflora* dominated high marsh areas (Leonard & Luther 1995), and to present conditions under which blue crabs can successfully forage in the field (Zimmer-Faust et al. 1995, Finelli et al. 2000). Flow speed was set each morning by measuring the transit time of dye released in a 50 cm section of the flume, and subsequently confirmed using a

Fig. 2. Top and side views of the experimental array showing the relative size and position of the plume outlet, vegetation mimics ('*Spartina* bed'), sensors, and sampling points

Table 1. Temperature, density, and conductivity of flume water and conductivity solution for each trial day. Temperature, density, and conductivity of the tracer solution were measured at the start of each day. Mean densities (±1 SD) are taken from 10 replicate measurements, while mean conductivities for flume water are taken from measurements made at the beginning and end of each treatment run (n = 8)

Trial	Flume water			Conductivity solution		
	Temp. (°C)	Density (g l^{-1})	Conductivity (mS)	Temp. (°C)	Density (g l^{-1})	Conductivity (mS)
1	19.5	0.9950 (±0.0012)	0.559 (±0.008)	19.9	0.9963 (±0.0010)	32.7
2	17.1	0.9974 (±0.0011)	0.563 (±0.007)	17.6	0.9988 (±0.0023)	32.1
3	18.0	1.0003 (±0.0010)	0.502 (±0.008)	20.8	1.0018 (±0.0025)	33.9

The tracer solution was gravity fed from a medical enteral feeding bag (Ross Industries Toptainer, Columbus, OH, USA) at a constant rate of 5.5 ml min^{-1} through an orifice located 4.0 cm from the flume bottom and 410 cm from the upstream plastic baffle (Figs. 1 & 2). Given a plume outlet diameter of 0.2 cm, the inlet velocity was ~2.9 cm s^{-1} and the jet Reynolds number (Re_{jet}) was ~58. Once the tracer plume was established, conductivity (linearly proportional to salt concentration) was measured at 40 locations within the plume (Fig. 2) using a microscale conductivity instrument (MSCI, Precision Measurement Engineering, Encinitas, CA, USA). The MSCI sensor is a 4 conductor-type conductivity electrode measuring 500 µm in width. The MSCI was calibrated each day using 3 NIST (National Institute of Standards and Technology) traceable conductivity standards (0.1, 1.413, and 10 mS) and the working 'odor' solution. The resulting linear calibrations had an average r^2 = 0.9993 (±0.0006 SD, n = 3).

At each measurement location, I used a thermistor bead velocimeter (LaBarbara & Vogel 1976) to measure flow velocity and turbulence intensity. For these studies turbulence intensity is defined as the root-mean square (RMS) turbulence intensity, which is the standard deviation of the velocity time series. The sensor was temperature compensated and calibrated each day. Velocity calibrations were performed by repeatedly moving the probe through a still water tank, while recording the output of the thermistor along with the output of a linear velocity transducer (LVT). Subsequently, the output of the thermistor was regressed against that of the LVT using King's Law (Hart et al. 1996). The resulting linear calibrations had a mean r^2 = 0.97 (±0.02 SD, n = 3).

Conductivity and velocity measurements were collected at a rate of 8 Hz for 32 s with a Campbell CR10X datalogger. By choosing sensors with miniature components, velocity and conductivity were measured at essentially the same spatial location (Fig. 2). Paired measurements of velocity and conductivity were made at a single location during each sample period (32 s), after which the probes were moved to the next sample location. Sampling was begun at the plume center, 2 cm downstream from the plume source, and progressed cross-stream before moving to the next downstream location. To avoid confounding conductivity measurements with heat from the thermistor probe, the MSCI sensor was placed slightly upstream and below the thermistor bead (Fig. 2).

Data analysis. Mean velocities and RMS turbulence intensities from each measurement location were used to evaluate spatial variation in flow conditions within the plume. In addition, treatment mean velocities and turbulence intensities for each trial were calculated and are presented in Table 2. I used a 2-factor analysis of variance (ANOVA) to evaluate the influence of flow treatment and vegetation mimic treatment on the mean velocities and turbulence intensities.

Spectral analysis of each velocity time series was performed to determine the frequency characteristics of velocity for each treatment combination (e.g. Box et al. 1994). Each velocity time series was normalized with respect to its mean by subtracting the mean velocity from each individual data point. This allows spectral densities to be compared between treatments without regard for differences in mean velocity (SAS Institute 1993, Finelli et al. 1999). Each normalized time series was then windowed using a 256-point triangular (Bartlett) window (Press et al. 1992) before spectral analyses were completed using Fast-Fourier Transform methods (SAS Institute 1993). Composite spectral density plots were constructed for each day and treatment combination from the 40 individual plots generated from the raw data.

Concentration mean and standard deviation for each spatial location in each plume were calculated from individual time series. In addition, relative dilution rates between treatments were examined by normalizing each mean to the source conductivity, thus placing all concentration data on a relative scale from 0 to 1. Using this scheme, cross-stream and downstream profiles can be compared regardless of the source conductivity. A 2-factor analysis of covariance (ANCOVA),

Table 2. Flow speed and conductivity conditions for each replicate treatment on each trial. Composite mean (±1 SD) flow speeds and root-mean square turbulence intensities (RMS TI) are taken from the 40 individual flow records collected during each trial. Conductivity data are background levels measured from the flume channel

Trial	Flow	Vegetation mimic	Flow speed (cm s^{-1})	RMS TI (cm s^{-1})	Beginning cond. (mS)	End cond. (mS)
1	Slow	Smooth	4.43 (±0.22)	0.55 (±0.16)	0.547	0.553
	Slow	'Vegetated'	3.59 (±0.25)	0.37 (±0.17)	0.554	0.558
	Fast	Smooth	11.28 (±0.34)	0.60 (±0.14)	0.564	0.571
	Fast	'Vegetated'	10.88 (±0.47)	0.47 (±0.21)	0.560	0.565
2	Slow	Smooth	6.24 (±0.39)	0.84 (±0.17)	0.551	0.556
	Slow	'Vegetated'	6.37 (±1.00)	0.58 (±0.14)	0.558	0.563
	Fast	Smooth	12.26 (±0.43)	0.62 (±0.20)	0.568	0.572
	Fast	'Vegetated'	12.26 (±0.26)	0.47 (±0.15)	0.563	0.569
3	Slow	Smooth	5.56 (±0.34)	0.89 (±0.15)	0.490	0.496
	Slow	'Vegetated'	5.51 (±0.77)	0.73 (±0.21)	0.496	0.500
	Fast	Smooth	13.60 (±0.80)	0.85 (±0.28)	0.508	0.514
	Fast	'Vegetated'	12.09 (±0.45)	0.78 (±0.24)	0.502	0.508

with the x- or y-position included as a covariate, was used to test for flow and vegetation mimic effects on normalized concentrations in the profiles. The data were arcsine transformed for statistical testing (Zar 1996).

Properties of individual odor filaments may be important to navigating organisms; therefore, I investigated the effects of flow speed and vegetation mimics on some of these properties. Individual odor filaments (peaks) were identified in each conductivity time series. The number of peaks, mean peak height, mean peak width, and intermittency were calculated for each conductivity time series. Intermittency is defined as the proportion of time conductivity remains above baseline values (Finelli et al. 1999). Centerline and cross-stream profiles are presented for all treatments. A series of 2-factor ANCOVAs, using the x- or y-position as a covariate, was used to test the effects of flow and vegetation mimics on odor filament properties in these profiles. For statistical testing, intermittency was arcsine transformed, while peak height and peak width were log transformed (Zar 1996).

Because of the low sample size (n = 3), I conducted a post-hoc power test (Zar 1996) to determine the probability of wrongly accepting the null hypotheses. Power for the tests described above was generally very low (<0.30); consequently the chance of falsely accepting a null hypothesis was >70%. Given this low statistical power, I considered tests with a p < 0.05 as statistically significant, and p < 0.15 as marginally significant results. In addition, for each 2-factor ANOVA or ANCOVA a vegetation mimic × flow speed interaction term was tested and shown to be non-significant; therefore this interaction was not included in any further analyses.

Spectral analyses of each conductivity time series were also completed as described above for velocity time series. Spectral analyses were conducted on raw conductivity data, not the normalized values used to compare dilution rates. Because of spatial gradients in the spectral properties of time series of conductivity, composite plots of spectral density from the 3 trials were produced for each treatment and spatial location in the plume.

RESULTS

Flow properties

Mean velocity and turbulence intensity showed nearly constant distributions throughout the simulated odor plumes (Figs. 3 & 4). Flow velocity was slightly decreased and RMS turbulence intensity slightly increased at the center of the plume just downstream (x = 2 cm) from the plume source, reflecting the influence of the plume outlet on flow conditions (Figs. 3 & 4). This effect was consistent for all treatments and days, and flow properties return to background levels within the first 10 cm of the plume, indicating that for distances >10 cm plume transport was dominated by general flume flow conditions.

The 2 flow treatments had significantly different mean flow speeds (ANOVA; $F_{1,9}$ = 128.13, p < 0.001), while vegetation mimics had no effect on mean flow speeds (ANOVA; $F_{1,9}$ = 0.56, p = 0.474). RMS turbulence intensities did not differ between flow treatments (ANOVA; $F_{1,9}$ = 0.11, p = 0.752); however, the presence of vegetation mimics marginally reduced turbulence intensity (ANOVA; $F_{1,9}$ = 2.86, p = 0.125).

Spectral analyses of velocity time series further illustrate the effects of vegetation mimics on turbulence in the flume. Patterns in the spectral density were consistent throughout each plume; therefore, I combined
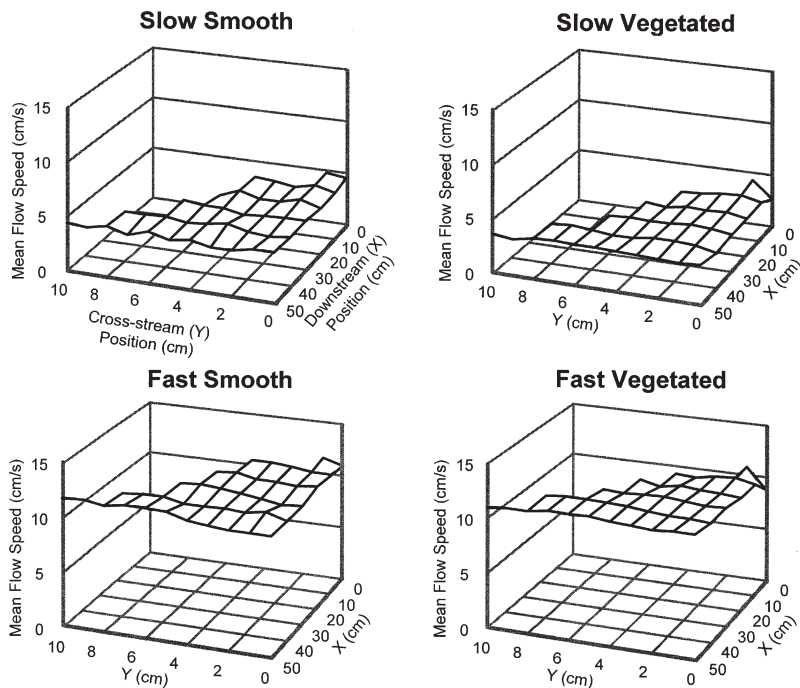
Fig. 3. Spatial distributions of mean flow velocity for each treatment combination during Trial 1. Note the nearly uniform distributions throughout the plume. A slight decrease in mean velocity is detected at the source ($x = 0$, $y = 0$), reflecting the influence of the slower moving odor solution at the plume outlet

results in peaks in the time-series data (Fig. 6). Such time series vary within each plume and between treatments. However, they can be summarized by calculating mean conductivity (= concentration), variance, number of peaks, peak heights, peak widths, and the intermittency (Fig. 6).

Regardless of flow or vegetation treatment, concentration mean and variance is highest at the source and gradually decreases with distance from the source and from the plume center (Figs. 7 & 8). In the fast flow treatment, mean concentration near the plume source is about one-half that in the slow flow treatment (Fig. 7). This increased rate of dilution is more clearly seen in centerline profiles of relative conductivity (Fig. 9). In all treatments, mean conductivity was ~1% of source values within 10 cm from the outlet (Fig. 9). At $x = 2$ cm from the plume source, mean conductivities in the slow treatments were more concentrated (8 to 16% of source concentration) than in the high

data from all 40 locations into a single plot for each treatment combination and each day (Fig. 5). In each case, the plots show the majority of spectral energy to be concentrated at the lower frequencies, corresponding to larger eddies in the flume channel. In all treatments there is a sharp break in the spectral density at ~1 Hz; however, this break is more pronounced in the high flow treatment (Fig. 5). The action of vegetation mimics on turbulence is further evidenced in the slow flow treatment by a proportional increase in spectral energy at the highest frequencies during Trial 2 and Trial 3 (Fig. 5). The net effect of the vegetation mimics is to break up large energetic eddies to produce a more uniform flow environment (Fig. 4) dominated by smaller, less variable eddies (Fig. 5).

Plume properties

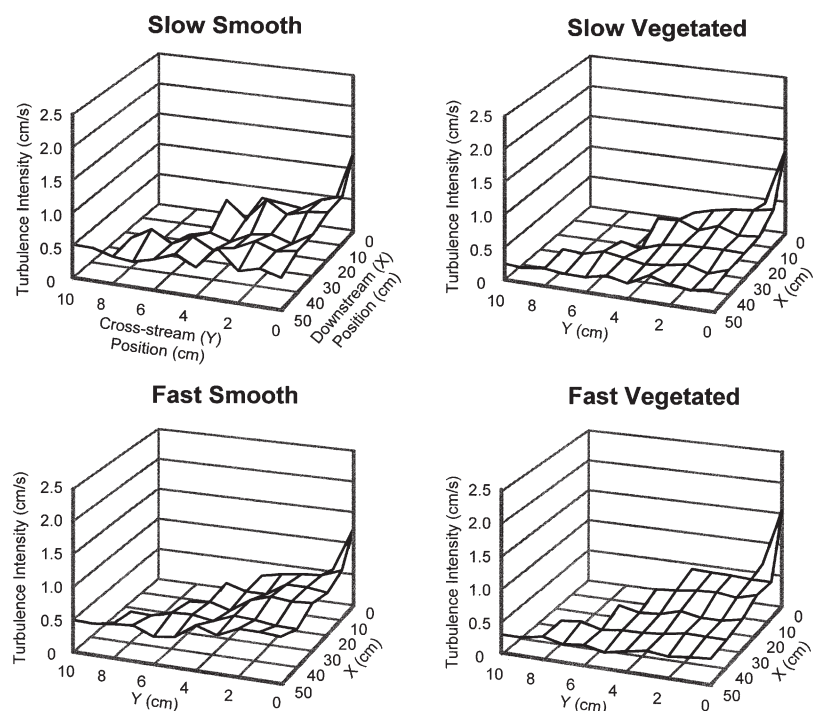The movement of individual plume filaments past the conductivity sensor

Fig. 4. Spatial distributions of the root-mean square turbulence intensity for each treatment combination during Trial 1. Turbulence intensity is highest near the plume source, reflecting the influence of the slower moving odor solution at the plume source
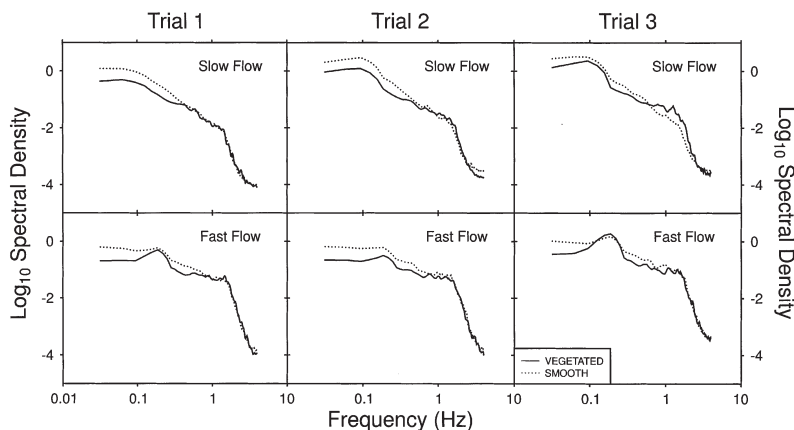
Fig. 5. Effects of flow speed and vegetation mimics on turbulent eddy structure during each trial. Each line reflects the mean spectral density computed from the 40 analyses performed for each plume

flow treatments (2 to 3% of source concentration). Close to the plume source (e.g. $x = 2$ cm) vegetation mimics appear to be important as well, especially in the slow flow treatment. However, a 2-factor ANCOVA reveals a significant effect of flow treatment only ($F_{1,67} = 4.38$, p = 0.040), not vegetation treatment ($F_{1,67} = 0.57$, p = 0.453). The downstream position (along x-axis) is a significant covariate in this analysis. When cross-stream profiles are considered, neither flow treatment nor the presence of vegetation mimics affected normalized conductivity (ANCOVA; flow treatment: $F_{1,67} = 1.40$, p = 0.242; vegetation treatment: $F_{1,67} = 0.34$, p = 0.559); however, the cross-stream position (along the y-axis) is a significant covariate in the model. In both analyses, the presence of a significant spatial covariate indicates that the relative conductivity is decreasing with distance from the plume source or the plume center.

Centerline profiles of odor filament properties show sharp decreases in the number of peaks, mean peak height, mean peak width, and intermittency from 2 to 10 cm from the plume outlet (Fig. 10). These parameters then remain fairly constant further downstream (Fig. 10). Both vegetation mimics and flow treatments significantly increase peak number (ANCOVA; flow treatment: $F_{1,67} = 150.17$, p < 0.001; vegetation mimic treatment: $F_{1,67} = 34.05$, p < 0.001) and intermittency (ANCOVA; data arcsine transformed; flow treatment: $F_{1,67} = 15.86$, p < 0.001; vegetation mimic treatment: $F_{1,67} = 9.50$, p = 0.003). In contrast, only flow treatment had a significant effect on peak height (ANCOVA; data log transformed; flow treatment: $F_{1,67} = 13.62$, p < 0.001; vegetation mimic treatment: $F_{1,67} = 1.04$, p = 0.311) and peak width (ANCOVA; data log transformed; flow treatment: $F_{1,67} = 19.99$, p < 0.001; vegetation mimic treatment: $F_{1,67} = 0.01$, p = 0.941), causing

a decrease in both cases. In general, plumes in the slow flow treatments are characterized by fewer peaks that are taller and of longer duration than in high flow treatments. The presence of vegetation mimics serves to further break up the plume, creating more odor filaments in both flow treatments. These odor filament properties combine to create patterns in the intermittency, which is higher in fast flows and in the presence of vegetation than in slow flows or smooth bottomed treatments. In all cases the downstream distance from the plume source (x-position) was a significant negative covariate, indicating that plume properties decreased with distance from the plume source.

Both vegetation mimics and flow speed were important factors in cross-stream dispersal, showing significant or marginally significant effects in all analyses (Fig. 11). Both flow speed and vegetation cause significant increases in peak number (ANCOVA; flow treatment: $F_{1,67} = 15.72$, p < 0.001; vegetation mimic treatment: $F_{1,67} = 6.73$, p = 0.012) and intermittency (ANCOVA; data arcsine transformed; flow treatment: $F_{1,54} = 3.25$, p < 0.077; vegetation mimic treatment: $F_{1,54} = 5.63$, p = 0.021). In contrast, increased flow speed and the presence of vegetation mimics cause a decrease in peak height (ANCOVA; data log transformed; flow treatment: $F_{1,54} = 26.03$, p < 0.001; vegetation mimic treatment: $F_{1,54} = 7.71$, p = 0.008) and peak width (ANCOVA; data log transformed; flow treatment: $F_{1,54} = 33.84$, p < 0.001; vegetation mimic
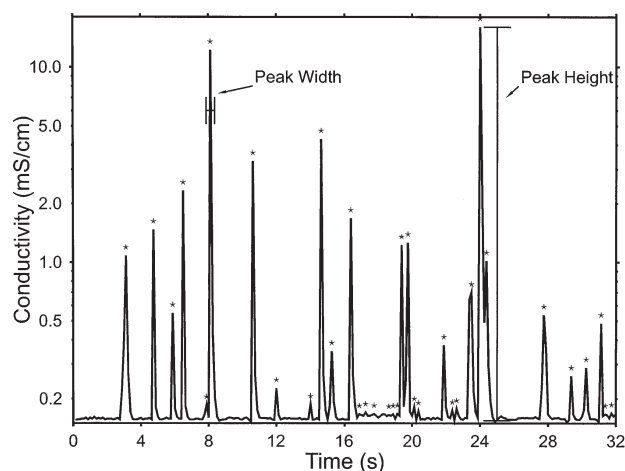

Fig. 6. Sample of conductivity data showing the filamentous nature of turbulent plumes. Stars represent the location of individual peaks. Peak height and peak width are also noted
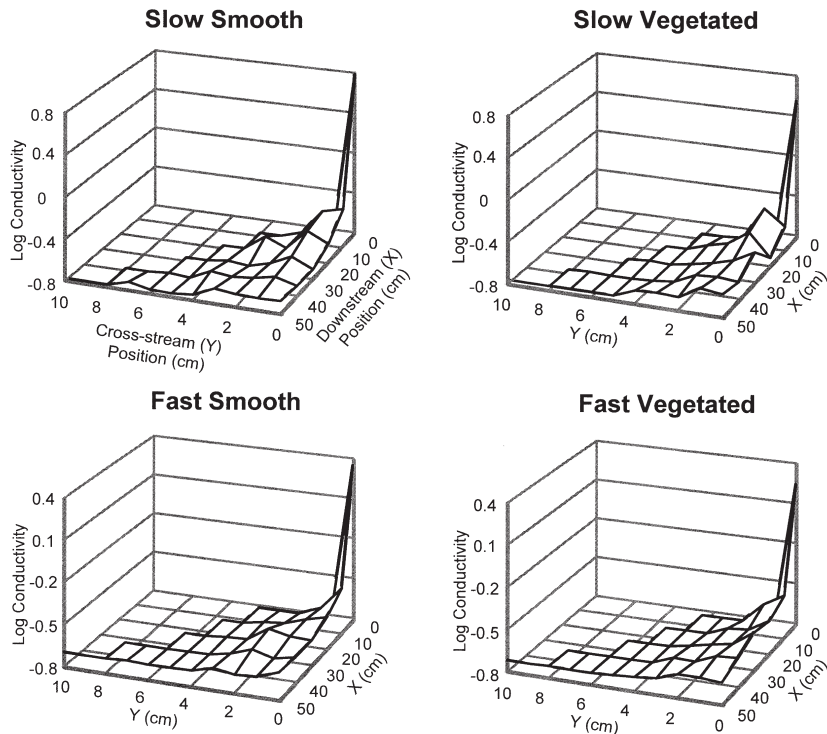
Fig. 7. Spatial distributions of mean conductivity (= concentration) for all 4 treatments during Trial 1

treatment: $F_{1,67}$ = 3.83, p = 0.056). In all analyses except that of peak height, the cross-stream position (*y*-position) was negatively correlated with with plume filament properties. Peak heights do not change substantially with distance from the plume center.

Spectral analyses of conductivity time series from the plume mid-line show diminishing plume heterogeneity with increasing distance from the plume source. Spectral densities are higher at all frequencies close to the odor source, indicating greater variation in the time series of conductivity (Fig. 12). As the plume is mixed and concentrations become more homogeneous, spectral density decreases with distance from the source. In addition, the proportion of spectral energy found at high frequencies decreases with increasing distance from the plume source, reflecting a gradual decrease in the number and magnitude of odor peaks. The more homogeneous nature of plumes at high flow and in vegetated treatments is characterized by the generally lower spectral densities (slow vs fast) and overall com-

pression of the data (smooth vs structured) (Fig. 12).

Diminishing temporal heterogeneity in conductivity is also noted in the cross-stream dimension. Spectral density plots are relatively flat close to the plume center, with a steady decrease in high frequency energy towards the plume edges (Fig. 13). This pattern reflects the gradual decline in the number of peaks with increasing distance from the plume center. The cross-stream decay of the odor plume is more gradual in faster flows than in slow flows, as indicated by the more graded decline in spectral density. The presence of vegetation mimics seems to enhance this gradient in both flow treatments.

DISCUSSION

Spatial patterns of odor plume dispersal result from 2 factors associated with flowing water: advection and turbulent mixing. Advection is directly
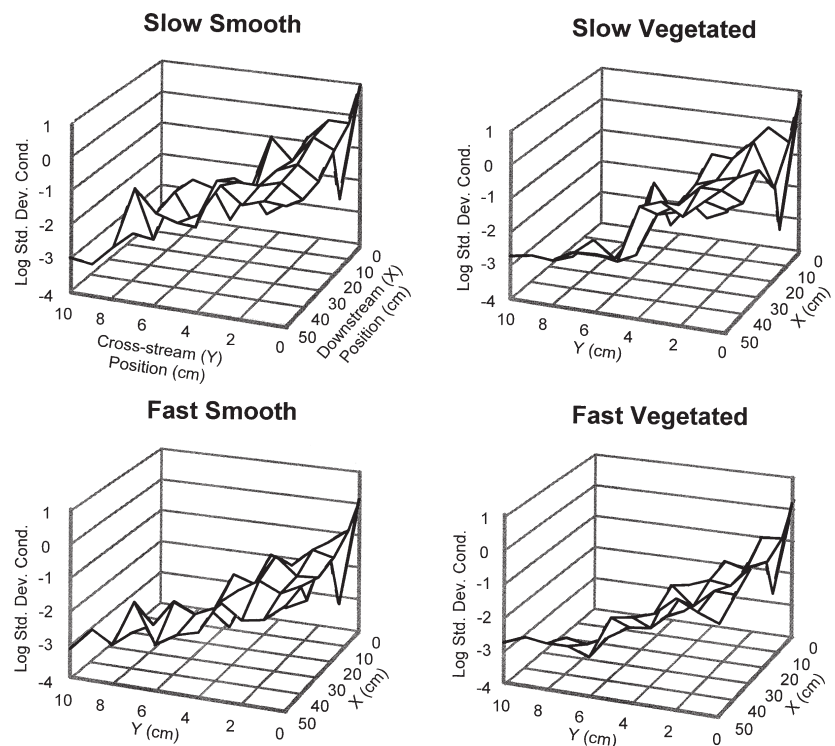

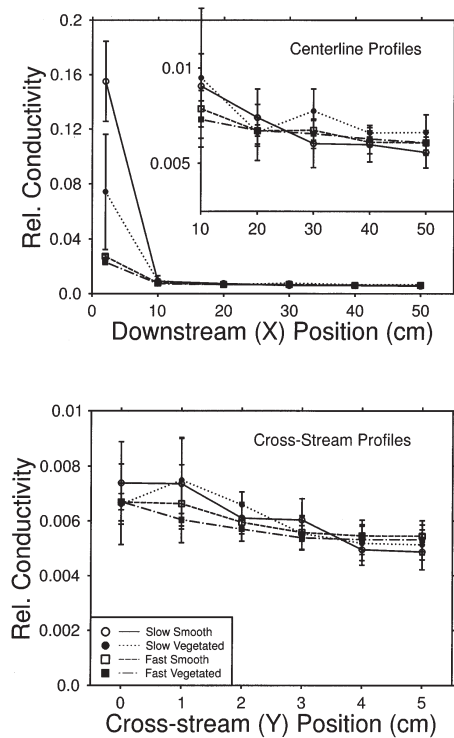Fig. 8. Spatial distributions of the standard deviation of conductivity from each treatment during Trial 1

Fig. 9. Centerline and cross-stream profiles of relative conductivity (mean conductivity/source conductivity) showing the greater rates of dilution caused by increased flow speed. Treatment means computed from all 3 replicate days are plotted with standard deviations

Turbulent mixing, the principle mechanism of vertical and lateral plume spread, can compound advective plume dilution and is primarily responsible for the genesis of odor filaments. The Principle of Local Exchange provides a mechanistic basis for understanding turbulence effects on plume dispersal (McNair et al. 1997). This theoretical construct specifies that the water column is divided into discreet water packets that move relative to each other due to the action of turbulent eddies. To preserve continuity, for each water parcel that moves an adjacent water parcel must fill its place. Hence, turbulent mixing occurs as the by-product of the reciprocal exchange of adjacent water packets, some of which contain dissolved material and some of which do not (McNair et al. 1997).

The mechanism suggested by the Principle of Local Exchange can be applied with success to the present study. For example, at the plume source initial exchange will be between odor-laden water in the plume axis and adjacent clean water, leading directly to the formation of odor filaments (e.g. List 1982, Mylne 1992). Because these filaments are cleaved from the main plume, they will initially retain characteristics of the main plume. So, in the high flow treatment where advection created plumes with lower overall concentration, the concentration within odor filaments (peak heights) was reduced as well (Figs. 10 & 11). The process of local exchange is repeated and the plume gradually grows laterally and vertically as turbulent eddies exchange water parcels from the plume axis with those farther on the plumes edges. Not all exchange occurs at the plume edges, and some mixing within the plume can be expected. For example, the number of odor

related to the downstream component of water velocity and dictates the rate at which odor molecules are transported downstream from the plume source. Given equal release rates of dissolved material, plume concentrations in fast flows will be decreased relative to those in slower flows because the finite amount of odorant released per unit time is dispersed within a greater volume of water. This mechanism is consistent with my finding that flow speed, rather than the presence of vegetation mimics, was the primary control on mean concentration and peak height (Figs. 7, 9, 10 & 11). For example, in the present study, odorant was released at ~5.5 ml min^{-1}. In the slow flow treatment (~5 cm s^{-1}), after 1 s, 0.09 ml of odorant will be contained in a plume measuring 5 cm in length. In the high flow treatment, the same 0.09 ml will be spread over a linear distance of 12 cm, effectively decreasing the concentration by more than one-half (without considering the effects of lateral and vertical plume spread).
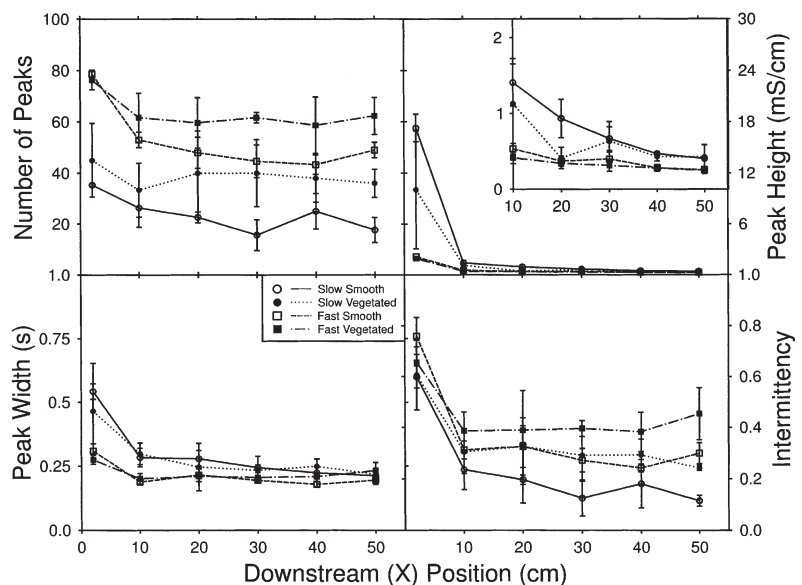

Fig. 10. Centerline profiles of odor filament properties. Treatment means computed from all 3 replicate days are plotted with standard deviations
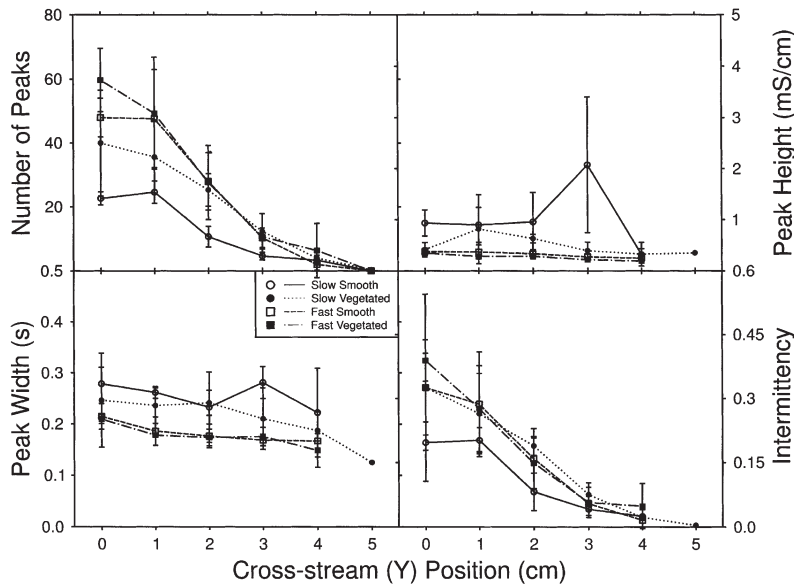
Fig. 11. Cross-stream profiles of odor filament properties. Profiles are taken from 20 cm from the plume source (e.g. $x = 20$). Treatment means computed from all 3 replicate days are plotted with standard deviations

the intermittency (Figs. 10 & 11). These results confirm the importance of eddy structure and local exchange on the creation and dispersal of odor filaments.

Whether changes in odor plume structure induced by the presence of emergent vegetation, such as those documented here, will be reflected in the navigation abilities of organisms has yet to be seen. Examples from the literature point to different conclusions. For example, Willis et al. (1994) show that male moths have more direct flght paths to a pheromone source when an upstream obstruction (e.g. plastic cylinder mimicking a tree trunk) is directly upwind. They show that moths are better able to orient to the wider, more evenly distributed ('diffuse') plumes created by eddies shedding from the cylinder. In contrast, Weissburg & Zimmer-Faust (1993) demonstrated that blue crabs have difficulty foraging in the presence of increased turbulent mixing. In their experiment, increased turbulence resulted in odors being rapidly diluted to below detectable levels. In this flume study the presence of *Spartina alterniflora* mimics created a more evenly distributed flow field and a more

filaments in the centerline of a given plume remained fairly constant at distances >10 cm from the plume source (Fig. 10). This pattern stems directly from the reciprocal exchange of like water parcels (e.g. each containing odorant) within the main axis of the plume. In contrast, the number of peaks declined in the cross-stream direction as local exchange progressively transports odor filaments laterally (Fig. 11).

The size of water parcels exchanged and the rate at which the exchange occurs is determined by the spatial and temporal scales of turbulent eddies. The model *Spartina alterniflora* stand acted as a baffle that absorbed turbulent kinetic energy at low frequencies (large eddies) through friction, and released energy at higher frequencies (small eddies) via eddy shedding (Fig. 5, see also Laws & Livesey 1978, Leonard & Luther 1995). Moreover, because I chose a uniform spacing and stem size, velocity fluctuations in the vegetated treatments were more uniform than in the smooth bottomed treatments. These alterations in the eddy structure of the carrier flow increased the rate of local exchange and resulted in a significant increase in the number of odor filaments and in
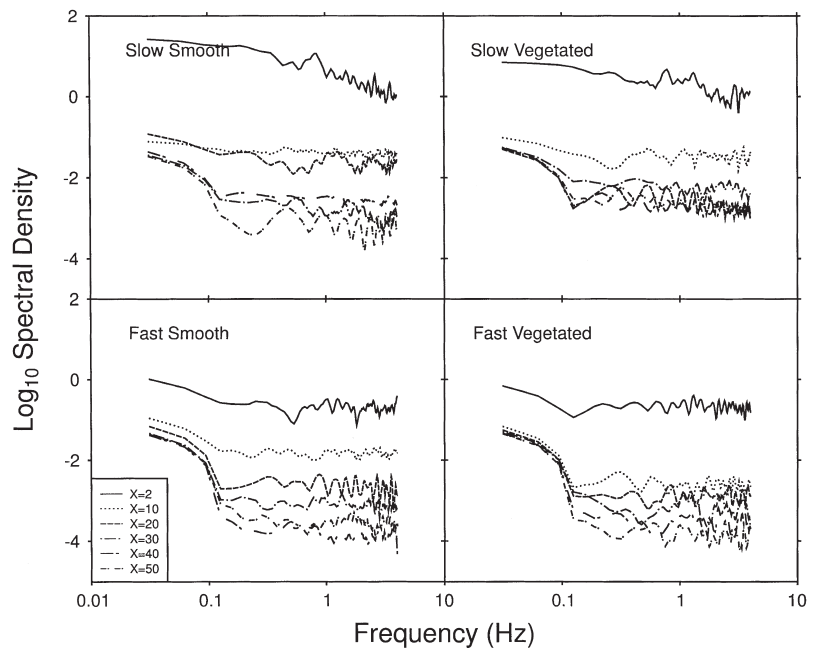

Fig. 12. Spectral density plots from the plume centerline in each treatment. Each plot is the composite mean for each treatment and downstream distance. The decrease in peak number and/or height with distance from the odor source is noted as a steady decline in spectral density at high frequencies. x = downstream distance from plume source
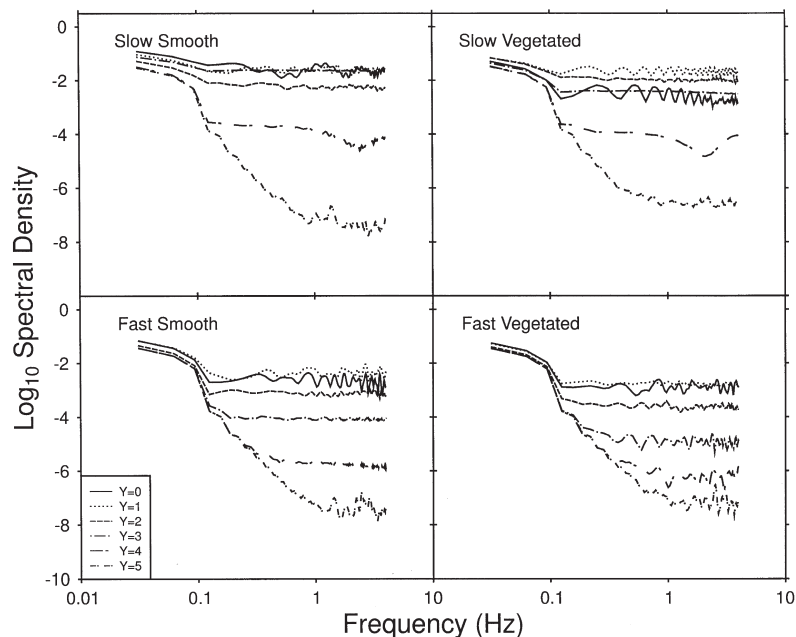
Fig. 13. Spectral density plots as a function of cross-stream distance. The decrease in peak number with distance from the plume center is noted as a steady decline in spectral density at high frequencies. *y* = cross-stream distance from plume centerline

evenly distributed, although more dilute, plume. Therefore, marsh edge organisms are confronted by a more dilute, but potentially more stable chemical signal. Further experiments are needed to determine the conditions under which the benefits of a more evenly distributed plume are undermined by increased dilution in the presence of natural vegetation.

Navigating organisms are faced with 2 fundamental challenges when orienting to odor plumes. First, they must be able to detect when they are in a plume. This problem is one of 'edge detection', and is a function of both the timing of odor filaments and the concentration within each odor filament. Of course, odor concentration must be above a minimum threshold for it to be detected at all. The threshold for behavioral response to an odor is highly variable depending on the species involved and the immediate history of the particular individual (e.g. Zimmer-Faust et al. 1996). Previously, I (along with my colleagues) have argued that the signal intermittency might be a potent cue for organisms navigating turbulent plumes (Finelli et al. 1999). Edge detection via the intermittency would require that an organism only detect concentration greater than background levels. In the present study, the signal intermittency clearly defines the plume edge without regard for the absolute concentrations in the odor filament.

The second challenge faced by searching organisms is the determination of the direction to the plume source. Given the patchy distribution of odorant, mechanisms

that rely on stable gradients in odor, such as classical chemotaxis, are not likely to be effective. Atema, Moore, and their colleagues have argued that the American lobster *Homarus americanus* can use information coded in individual odor filaments, especially the peak slope, to successfully follow an odor plume (Moore & Atema 1988, 1991, Moore et al. 1991, 1994, Atema 1996). Atema (1996) refers to this mechanism as 'eddy chemotaxis' to distinguish it from classical chemotaxis. Because the lobster's chemoreceptors are held on long antenules away from the flow perturbations caused by the presence of its body and because some odor filament properties vary consistently within plumes, eddy chemotaxis may be an effective strategy (Atema 1996). However, there is some question as to whether lobsters and other marine organisms can sample quickly enough to detect variation in peak slopes (Finelli et al. 1999, but see Gomez et al. 1994, Gomez & Atema 1996).

For organisms such as blue crabs, in which chemosensors (on legs and antenules) are held within the boundary layer surrounding the body, individual odor filaments may be smeared and some characteristics lost due to diffusional boundary layer effects (Cheer & Koehl 1988, Moore et al. 1991, Berg et al. 1992, Gleeson et al. 1993). Under these circumstances reliance on odor filament properties may be ineffective, and the use of alternative strategies such as odor-conditioned rheotaxis may be more suitable (Weissburg & Zimmer-Faust 1993, Zimmer-Faust et al. 1995, Finelli et al. 2000). Using this strategy, an organism needs to detect only the edge of the plume to trigger upstream locomotion. This, too, could be accomplished by detection of intermittency.

The efficacy of both of the mechanisms described above (edge detection via intermittency and odor-conditioned rheotaxis) may be enhanced in the presence of emergent vegetation. For example, the intermittency is increased in the presence of vegetation mimics regardless of flow speed, indicating that concentration is above background levels for a greater proportion of time. Moreover, the presence of vegetation mimics homogenized the flow field, perhaps making it easier to determine more precisely the direction of the odor source. If such enhancements to odor-mediated search can be demonstrated, then we must re-evaluate the utility of the marsh edge as a prey refuge from olfactory predators.

This study demonstrates the potential for emergent vegetation to alter flow conditions and odor dispersal

marsh edge habitats. Both flow speed and vegetation-like structure, through their influence over local exchange and eddy sizes, alter the size, frequency, and concentration of odor filaments. Further studies are needed to test whether these changes will be reflected in the behavior of navigating organisms, and if so, whether searching will be enhanced or degraded. Such studies may help to understand the mechanisms that drive habitat use and ecological interactions among the many animals that use olfaction to find food, mates, and shelter.

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