

Seaweed sex pheromones and their degradation products frequently suppress amphipod feeding but rarely suppress sea urchin feeding

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Summary. A diverse group of brown seaweeds produce bouquets of C₁₁ metabolites, some of which act as pheromones that cue gamete release or attract sperm to eggs following release. We demonstrate that these C₁₁ metabolites and their degradation products also frequently and strongly deter feeding by the herbivorous amphipod *Ampithoe longimana*, but rarely by the herbivorous sea urchin *Arbacia punctulata*. Across the range of concentrations tested, seven of twelve C₁₁ metabolites or mixtures that we tested deterred feeding by the amphipod, but only two of eleven deterred the sea urchin. For those compounds where we could rigorously contrast the magnitude of deterrence against the amphipod with the magnitude of deterrence against the urchin, the amphipod was deterred significantly more than the urchin by five of six metabolites. Thus, C₁₁ compounds were more frequently and more strongly deterrent to the amphipod than to the sea urchin. These findings for C₁₁ metabolites conflict with previous investigations, where other classes of seaweed chemical defenses have been shown to deter feeding by large mobile herbivores like urchins and fishes but to be relatively ineffective against mesograzers, especially the species of amphipod that we used here. Our results suggest that C₁₁ metabolites are unusual among the known seaweed chemical defenses in that they are especially effective against mesograzers, which often consume seaweed spores, zygotes, and juveniles. The high concentrations of C₁₁ metabolites in brown algal eggs could allow these defenses to be especially important in defending gametes, zygotes, or young sporelings from herbivorous mesograzers.

Key words. algal pheromones – amphipods – *Ampithoe longimana* – *Arbacia punctulata* – brown algae – C₁₁ compounds – *Dictyopteris* – marine ecology – plant-herbivore interactions – sea urchins

Introduction

Seaweeds can experience extremely high rates of grazing due to generalist herbivores such as fishes, sea urchins,

and gastropods (Carpenter 1986; Hay 1991; John *et al.* 1992). In habitats impacted by these consumers, many seaweeds have structural, morphological, or chemical traits that significantly deter herbivores (Duffy & Hay 1990; Hay 1996, 1997). Chemical defenses against marine herbivores have been especially well studied (see reviews by Hay & Fenical 1988, 1996; Hay & Steinberg 1992; Paul 1992; Hay 1996), with functionalized terpenoids, acetogenins, and cyclic peptides containing uncommon amino acid residues being especially widespread and common (Faulkner 1997; and his earlier reviews cited therein). However, structurally simple C₁₁ hydrocarbons and C₁₁ sulfur metabolites from brown algae in the genus *Dictyopteris* can also deter grazing by some herbivores (Hay *et al.* 1988a; Schnitzler *et al.* 1998), but the potential defensive properties of these hydrocarbons have not been widely investigated.

Eleven C₁₁ pheromones and more than 50 stereoisomers have been isolated from diverse groups of brown algae; these C₁₁ metabolites have also been detected in diatom cultures, blooms of freshwater microalgae, and in higher plants (Boland 1995). Although they occur in this wide range of organisms, they appear to be most frequent and most abundant in the brown algal genus *Dictyopteris* (Moore 1976, 1977). The function and biosynthesis of these hydrocarbons as mating pheromones for gametes of brown algae have been studied extensively (Boland 1995; Maier 1995; Pohnert & Boland 1996), but little else is known about the origin or ecological significance of the C₁₁ metabolites. In a recent investigation focused on C₁₁ sulfur compounds produced by species of *Dictyopteris*, Schnitzler *et al.* (1988) found that each of the four metabolites they studied strongly deterred feeding by the amphipod *Ampithoe longimana* but had no effect on feeding by the sea urchin *Arbacia punctulata*, even when tested at concentrations that were 2–8 times greater than those that deterred amphipods. These results were a dramatic contrast to several previous investigations where a variety of seaweed chemical defenses had been shown to deter feeding by large mobile herbivores like urchins and fishes, but to be relatively ineffective against mesograzers, especially the amphipod *Ampithoe longimana* (see Hay *et al.* 1987a, 1988a,b; Duffy & Hay 1994; Hay & Fenical 1996).

Because C_{11} compounds are known to occur in the eggs of brown algae (Boland 1995) and because some brown algae spend seasonal periods in juvenile resting stages (Richardson 1979) when defense of these small stages might be especially important, it is possible that C_{11} compounds could function as defenses against mesograzers like amphipods that can affect community composition by consuming algal zygotes, spores, or germlings as well as larval invertebrates (Zimmerman *et al.* 1979; Oliver *et al.* 1982; Brawley & Adey 1981; Parker & Chapman 1994). In addition to the four C_{11} sulfur compounds studied by Schnitzler *et al.* (1988), there are numerous non-sulfur C_{11} metabolites in brown algae or their eggs. If these metabolites, or their break-down products, functioned first to attract sperm to eggs and then to defend the developing zygotes from consumption by marine mesograzers, this could represent a system where particular metabolites were serving multiple ecological functions, or where metabolites used for one, time-sensitive function (gamete attraction) were then converted to metabolites that defend fertilized zygotes from consumers. Chemical defense of juvenile or larval stages is known to occur among benthic marine invertebrates (Lindquist & Hay 1996), but chemical defense of algal spores, sporelings, or juveniles has not been investigated (Hay 1996).

We tested the possibility that C_{11} metabolites, their degradation products, or related compounds might be especially deterrent to mesograzers like the amphipod *Ampithoe longimana* that could limit juvenile seaweeds by foraging within cracks and crevices that serve as refuges from larger herbivores. We reasoned that chemically defending juveniles from mesograzers might be possible because mesograzers may be able to feed at spatial scales that would allow chemical discrimination among very small individuals. This level of discrimination (and thus the ability to defend juveniles chemically) seems unlikely for large grazers like urchins, because they take bites that may include many separate juveniles per bite (Hay & Fenical 1992).

Our assays used the same amphipod and sea urchin that were used by Schnitzler *et al.* (1988) in their study of C_{11} sulfur compounds. Because only one of our metabolites contained sulfur, this allowed us to determine whether the general patterns found by Schnitzler *et al.* were restricted to sulfur containing C_{11} metabolites, or also occurred for the non-sulfur C_{11} pheromones and related metabolites.

Materials and methods

Species and metabolites

C_{11} metabolites have been isolated from more than 100 species and 28 genera of brown algae, including several that co-occur with the herbivores we used for our bioassays (*e.g.*, *Dictyopteris*, *Dictyota*, *Zonaria*, *Sargassum*, *Sporochnus*, *Scytosiphon*, *Colpomenia*, *Ectocarpus*, *Ascophyllum*). The C_{11} compounds appear to be biogenetically related to simple, olefinic C_{11} hydrocarbons previously identified in the essential oils of *Dictyopteris*, and other brown algae (Moore 1976, 1977).

The metabolites we tested were obtained via purification from algal extracts (rarely) or via synthesis (commonly). The general methodological approach for purification or synthesis paralleled that described in Schnitzler *et al.* (1988). Several of the C_{11} metabolites we assayed are known to be naturally produced by species of *Dictyopteris* (compounds **1**, **5**, **6**, **7**, & **9**; see Figs. 1–5 for structures), *Dictyota* (**1**, **9**), or *Sargassum* (**1**), or to be natural degradation products (**2**, **3**, **4**, **7**, **9**, **11** & **11'**) of the initial pheromone (Piel 1994; Boland 1995). All the known C_{11} pheromones are biotically or abiotically degraded by ubiquitous oxidative pathways involving singlet oxygen or hydroxyl radicals, which can be produced via heavy metals, humic acids, or light (Boland 1995). Natural oxidative degradation of the pheromone dictyotene **1** has been studied in volatiles of egg extracts from *Dictyota dimensis* (Phillips *et al.* 1990) and in volatiles produced by the thalli of the Mediterranean phaeophyte *Dictyopteris membranacea*. This degradation produces compounds **2**, **3**, **4**, **7**, **9**, **11** & **11'**. Oxidative degradation also has been studied extensively using purified dictyotene **1** as the substrate and iodosylbenzene and manganese tetraphenylporphyrin (TPPMn) as the oxidant (McMurry & Groves 1986). Alternatively, dictyotene was readily oxidized as a thin film by oxygen (air) leading to the hydroperoxides **11** and **11'** (Piel 1994) which, especially in the presence of Fe(III), decomposed to all of the above compounds. The chemical model systems appeared to mimic the natural degradation process, but provided larger amounts of the degradation products that could be more carefully analyzed and tested. Pure samples of **7**, **10**, **11** and **11'** were obtained by air oxidation of dictyotene (**1**), and the ketones were prepared from (**1**) by Sarret-oxidation followed by separation of the complex reaction mixture. The alcohol (**7**) was obtained from the hydroperoxide (**11'**) by reduction with PPh_3 or $NaBH_4$ according to standard procedures. For spectroscopic data see Moore (1976, and references cited therein).

Air oxidation of dictyotene: Dictyotene (0.50 g, 3.3 mmol) was distributed as a film on the inner surface of a 1L flask by slow rotation (rotovap) at 60°C in the dark while a stream of air (500 ml min^{-1}) passed through the system. After 2 h the crude mixture was separated by flash chromatography on silica gel using pentane/ether (95:5, v:v) for elution. The regioisomeric hydroperoxides **11** and **11'** (mixture of *syn,anti* isomers each) were obtained in 11% (38.0 mg) and 24% yield (84.0 mg), respectively. For spectroscopic data see Piel (1994).

Sarret-oxidation of dictyotene: A suspension of the CrO_3 ·pyridine complex (15.0 g) in dry CH_2Cl_2 (50 ml) was treated with stirring under argon with a solution of dictyotene (1.0 g, 6.0 mmol) in the same solvent (10 ml). An efficient stirrer was necessary to cope with the rapidly ensuing tar-like complex. After 20 h the solids were removed by filtration and carefully extracted with several portions of CH_2Cl_2 . The organic extracts were diluted with the same volume of ether and washed with a solution of sat. aq. $NaHCO_3$ (6 × 50 ml). The aqueous layer was re-extracted with ether and, then, the combined organic extracts were successively washed with dil. HCl (5%, 3 × 10 ml), sat. aq. $NaHCO_3$ (10 ml) and brine (10 ml). After drying ($MgSO_4$) and removal of solvent in vacuo, the residue was separated by chromatography on silica gel using pentane/ether (90:10, v:v) for elution. Yield of isomeric ketones was: (**2**) 119 mg (12%), (**3**) 358 mg (36%), and (**4**) 126 mg (13%). For spectroscopic data see Moore (1976, and references cited therein) and Piel (1994).

In addition to testing the degradation products as pure metabolites, we also tested the complete mixture resulting from air oxidation of dictyotene (**1**). The mixture contained many compounds including alcohols, ketones, and hydroperoxides, many of which are still unknown. Testing this mixture allowed us to assess the possibility that some very minor, and thus overlooked, degradation products might be active at very low concentrations, or that the mixed products of degradation might have unexpected synergistic effects. We also tested a heptadeca-1,8,11,14-tetraene **8** that may be considered a lower homologue to the widely distributed, highly unsaturated C_{19} and C_{21} hydrocarbons from thalli of many marine brown, as well as several red and green, algae (Hallsall & Hills 1971; Youngblood *et al.* 1971). A C_{11} sulfur compound **6** found in *Dictyopteris* (I. Schnitzler & W. Boland, unpublished) and a stable analogue of a thermolabile cis-disubstituted pheromone precursor **12** were also assayed. This last compound is not a natural metabolite; it is an analogue that is stable at room temperature, thus allowing testing.

The herbivores we used in our assays were collected in coastal North Carolina, USA, where they co-occur with a diverse assemblage of abundant brown algae (Schneider & Searles 1991). Many local seaweeds are genera that produce C_{11} metabolites (compare genera listed in Boland [1995] with those described in Schneider & Searles [1991]), but local populations of the Dictyotalean seaweed *Dictyopteris membranacea* are the only ones in which C_{11} metabolites have been explicitly studied (Schnitzler *et al.* 1998). This species produces dictyotene **1**, as well as several other C_{11} metabolites. The concentrations of these compounds have rarely been carefully quantified, but some have been isolated from this alga at yields of about 0.1% of algal dry mass (Schnitzler *et al.* 1998). To evaluate the potential of C_{11} compounds to function as defenses against herbivores, we tested their effects on feeding by the generalist sea urchin *Arbacia punctulata* and the generalist amphipod *Ampithoe longimana*. *Arbacia* is common and wide-spread on both sides of the Atlantic and is the most abundant sea urchin in coastal North Carolina where these assays were conducted. Feeding by *Arbacia punctulata* has rarely been studied, but it is known to readily consume several species of red and green seaweeds and to eat only limited quantities of the brown seaweeds (*Sargassum*, *Dictyota*, and *Padina*) that have been tested (Hay *et al.* 1986). Dictyotalean brown algae in the genus *Dictyota* are avoided by this urchin, and various diterpene alcohols produced by *Dictyota* significantly deter feeding by *Arbacia* (Hay *et al.* 1987a; Cronin & Hay 1996a,b). Feeding by omnivorous and herbivorous fishes is also deterred by these metabolites (Hay 1991), suggesting that *Arbacia* may serve as a model for how algal secondary metabolites affect feeding by several larger generalist herbivores.

The herbivorous amphipod *Ampithoe longimana* is common in marine and estuarine habitats along the east coast of North America (Bousfield 1973) and occurs on a wide variety of algae and seagrasses (Nelson 1979; Duffy 1990; Duffy & Hay 1991, 1994). Like several marine mesograzers that live on the seaweeds that they consume (see reviews by Hay 1992, 1996), *A. longimana* selectively consumes and lives on chemically defended brown algae that are avoided by fishes; by living on seaweeds that are chemically repugnant to omnivorous fishes, *A. longimana* reduces its susceptibility to fish predation (Hay *et al.* 1987a,b, 1988b; Duffy & Hay 1991, 1994). Thus, the feeding behavior of *Ampithoe longimana* contrasts with the feeding behavior of generalist urchins and fishes in the Western Atlantic in that *Ampithoe* tends to selectively consume seaweeds that are avoided by the generalist urchins and fishes. Additionally, the brown algal chemical defenses that have been tested have had minimal effects on feeding by *Ampithoe longimana* but often strongly suppressed feeding by *Arbacia*, herbivorous fishes, or even other species of amphipods (Hay *et al.* 1987a,b, 1988b; Duffy & Hay 1991a, 1994; Cronin & Hay 1996a,b). Thus, *Ampithoe longimana* appears to be representative of small mesograzers that have evolved a high tolerance to seaweed chemical defenses, selectively consume and associate with chemically-defended algae, and by doing so, minimize their exposure to consumers (Hay 1992, 1996).

Bioassays

Because hunger stress can alter herbivore feeding responses (Cronin & Hay 1996a), both amphipods and sea urchins were fed with a mixed algal-diet immediately before being used in feeding bioassays. The amphipods were kept in 20-L flow-through containers at the University of North Carolina's Institute of Marine Sciences and fed a mixture of freshly collected, local algae (*Sargassum filipendula*, *Padina gymnospora*, *Dictyota ciliolata*, *Dictyota menstrualis*, *Ulva* sp. *Enteromorpha* sp. and *Hypnea musciformis*). Sea urchins were held in flow-through seawater tanks and fed with fresh algae that they find palatable (*Gracilaria tikvahiae*, *Agardhiella ramosissima*, or *Codium fragile*).

The effects of the assay compounds on feeding by the urchin and amphipod were assessed by comparing feeding on an artificial food that contained (*i.e.*, treatment food) or did not contain (*i.e.*, control food) the secondary metabolite, or mixture of metabolites, being assayed. Artificial foods were made by mixing freeze-dried and finely powdered algae into an agar base and forming this onto fiberglass window screening material. This provided support and an internal uniform grid that allowed us to quantify the amount eaten by

counting the squares of the screen that had been cleared of algae (see Hay *et al.* 1994 or 1998 for detailed methods). Before mixing the freeze dried algal powder into the agar, the algal powder to be used for the treatment food was covered with diethyl ether containing the desired amount of the test metabolite, and the algal powder to be used for the control food was covered with diethyl ether alone. The solvent was then removed from each of these powders using rotary evaporation, and the dried powder was incorporated into the experimental foods. Each separately housed urchin ($N = 20-40$) or separately housed group of 4-6 amphipods ($N = 22-50$) was then offered equal amounts of both a control and treated food. Feeding was monitored periodically, and food was removed from individual replicates whenever half or more of either food had been consumed, or at the end of the experimental period. Duration of assays varied as a function of feeding rate, but assays with sea urchins generally ran for 1-24h and those with amphipods for 12-29h. Feeding was quantified by counting the number of window-screen squares that had been cleared of food. If there was no feeding in a replicate or if both foods were consumed completely between our monitoring intervals, then these replicates provided no information on relative palatability and were thus excluded from the analyses. Such exclusions resulted in actual sample sizes of 22-46 for the amphipod assays and 16-35 for the urchin assays.

To make food for urchins, we used freeze dried *Ulva fasciata*, which urchins consumed readily. In preliminary assays, amphipods consumed this food only reluctantly until we mixed it with equivalent portions of *Enteromorpha linza* and *Hypnea musciformis*. Because the effects of chemical defenses on feeding can vary as a function of the palatability of the assay food (Duffy & Paul 1992), we used these differing diets in an effort to equilibrate palatability rather than using identical diets, but ones that these two herbivores perceived and valued differently. Additionally, our procedure of stopping individual replicates within an experiment whenever we found that 50% or more of either food had been consumed in that replicate often produced a group of replicates that ran for a short period (*e.g.*, 1-2h; for those individuals that fed rapidly) and another group of replicates in that same treatment that ran for a longer period (*e.g.*, 18-29h). We contrasted these short- versus long-duration replicates as one way of assessing whether compounds might be degrading significantly in longer duration assays (even though being bound in the agar should have reduced exposure to oxidation) and producing altered feeding patterns early versus late in the assay. None of our data suggested altered feeding patterns as a function of assays duration.

The natural concentrations of most of the metabolites we used are unknown. However, the yield of specific C_{11} metabolites from *Dictyopteris membranacea* (a species that co-occurs with the amphipod and sea urchin we used in feeding assays) is known to be about 0.1% of plant dry mass (Schnitzler *et al.* 1998). Given the losses and inefficiencies involved in extraction, isolation, and purification of small quantities of metabolites, and that the few studies that have addressed intraspecific or intra-plant variation in seaweed secondary metabolites have often found considerable variation (reviewed in Hay 1996), we reasoned that natural concentrations could equal 2-4x this yield. This might be especially true of concentrations in eggs, zygotes, or juvenile stages because C_{11} metabolites are concentrated in eggs, as would be expected given their role as pheromones. We therefore initially tested compounds at a concentration of 0.2% of food dry mass. If quantities of compounds were sufficient for additional tests, we retested at lower concentrations for deterrent compounds and at higher concentrations for compounds that were inactive at a concentration of 0.2%. By taking this approach, we tested some compounds over concentrations ranging from 0.025 to 0.4% of plant dry mass and hoped to bracket the variations in concentration that might occur naturally between different populations, plant parts, or ontogenetic stages of a plant. This was the basic procedure used by Schnitzler *et al.* (1998) in previous assays with four C_{11} sulfur metabolites, so this also facilitated direct comparisons of our findings with that previous investigation.

A comparative assay of each metabolite against both the sea urchin and the amphipod was not always possible. Some metabolites were available in only limited quantities and some degraded with time - both factors limiting the number of assays that could be conducted. These same limitations constrained the range of concentrations over which we could assay each metabolite. We were able to assay 9

compounds against both urchins and amphipods, 3 against only amphipods, and 2 against only urchins.

Initial statistical analyses of differences in feeding were evaluated using 2-tailed, paired sample t-tests (or Wilcoxon's paired tests if differences were not normally distributed). In 7 contrasts where both the amphipod and the urchin were assayed at the same metabolite concentration and where one herbivore appeared to be deterred more than the other, we performed additional analyses to determine if these apparent herbivore-specific differences in effects of the compounds could be statistically verified. Because all of our assays were paired, each replicate in each assay produced a control minus treatment difference in the percentage of food eaten. Thus, differences in preference from an urchin assay could be contrasted with differences from an amphipod assay at the same metabolite concentration and evaluated with an unpaired, 1-tailed t-test. We used 1-tailed tests for these analyses because both previous work on C_{11} sulfur compounds (Schnitzler *et al.* 1998) and the initial assays in this experiment led us to hypothesize that the C_{11} metabolites would deter amphipod feeding more than sea urchin feeding.

Results

Of the nine metabolites or mixtures tested at equal concentrations against both the amphipod and sea urchin, five significantly decreased amphipod, but not urchin, feeding, one decreased urchin, but not amphipod feeding, and three had similar, nonsignificant effects on feedings (Figs. 1–4). Of the compounds or groups of compounds that were tested against either amphipods or sea urchins at 0.2% of food dry mass, four of twelve compounds significantly deterred am-

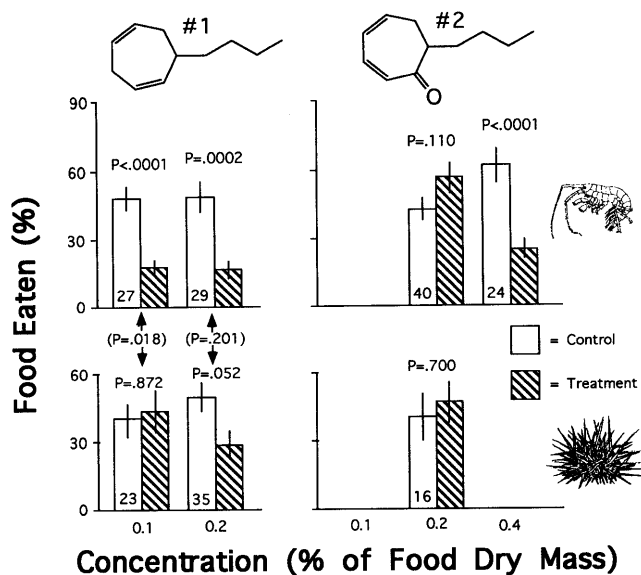


Fig. 1 Effects of various concentrations of C_{11} metabolites on feeding by the amphipod *Ampithoe longimana* (top histograms) and the sea urchin *Arbacia punctulata*. Bars show means \pm SE. Numbers in the base of each histogram pair give the sample size for that assay. P-values above histograms evaluate the metabolite effect on feeding by that herbivore at that concentration. P-values with arrows pointing to two sets of histograms evaluate the relative deterrence of that compound for the amphipod versus the sea urchin (see text for a discussion of statistical procedures). Compound 1 is the pheromone dictyotene, which is found in the genera *Dictyopterus*, *Dictyota*, *Dilophus*, *Culteria*, and *Sargassum*. Compound 2 is a natural degradation product of 1.

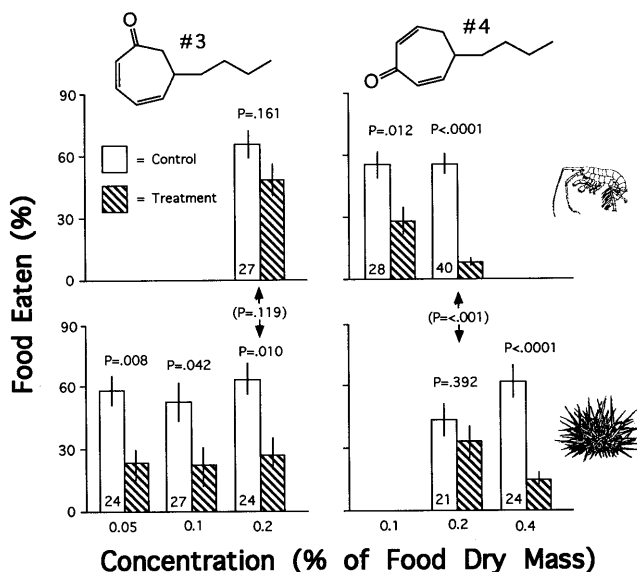


Fig. 2 Symbols and analyses are as in Fig. 1. Compound 3 and 4 are natural degradation products of 1.

phipod feeding, while only one of eleven deterred urchin feeding (Figs. 1–5). At a concentration of 0.4% or below, seven of twelve compounds deterred amphipods, while two of eleven deterred the sea urchin. For those compounds where we could rigorously contrast the magnitude of deterrence against the amphipod versus the urchin (marked by contrasting arrows in Figs. 1–4), the amphipod was deterred significantly more than the urchin for five of six metabolites. Thus, as a group, the metabolites tested here had a greater frequency and magnitude of deterrence against the amphipod *Ampithoe longimana* than against the sea urchin *Arbacia punctulata*.

Although no compounds could rigorously be demonstrated to deter the urchin more than the amphipod, compound 3 appeared to be more deterrent to the urchin. At 0.2% of food dry mass, compound 3 significantly deterred the urchin, but not the amphipod; but, the magnitude of deterrence between these assays did not differ significantly (Fig. 2; $P = 0.119$, t-test, $df = 49$). However, at a concentration of only 0.05% of food dry mass, this compound was still strongly depressing urchin feeding ($P = 0.008$). The compound was not tested against the amphipod at these lower concentrations because it did not have a significant effect on amphipod feeding at 0.2% of food dry mass (Fig. 2). Given its non-significant effect on amphipod feeding at 4x the concentration that strongly deterred the urchin, it is unlikely that it would have deterred feeding at lower concentrations.

The natural pheromone 1 strongly suppressed amphipod feeding, with no obvious decline in potency when assay concentrations were decreased from 0.2% (66% decrease in feeding) to 0.1% (64% decrease in feeding) of food dry mass (Fig. 1). This compound appeared to deter urchin feeding at a test concentration of 0.2% (feeding declined by 42%; but this was not

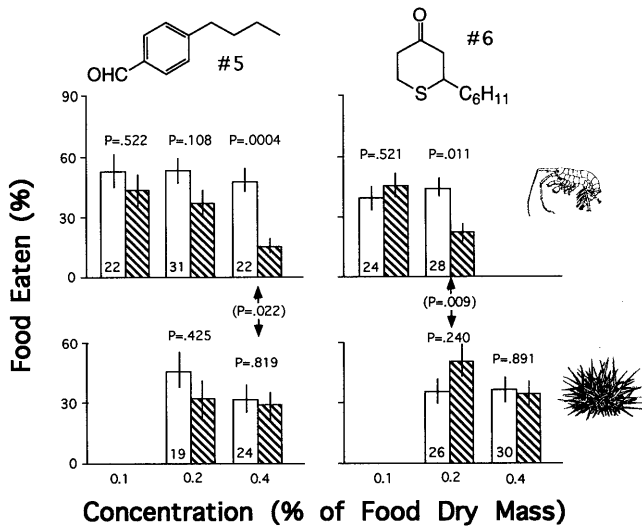


Fig. 3 Symbols and analyses are as in Fig. 1. Compound 6 was recently found in extract from *D. membranacea* (Schnitzler & Boland, unpublished). Compound 5 is a minor constituent of the TPPM catalysed oxidation of dictyotene 1.

quite significant, $P = 0.052$), but it had no effect at 0.1% ($P = 0.872$). When compound 1 degrades, degradation products 4 and 11 + 11' still deterred amphipod feeding as strongly as the parent compound (Figs. 2, 5). Degradation products 2, 5, and 7 were less potent feeding deterrents than the parent compound, but still deterred feeding at a concentration 0.4% of food dry mass (Figs. 1, 3, 4). Degradation products 3, 9, 10, and their air oxidation mixture of degradation products lost all effects against the amphipod (Figs. 2, 5). Oxidation to compound 3 strongly increased activity against the

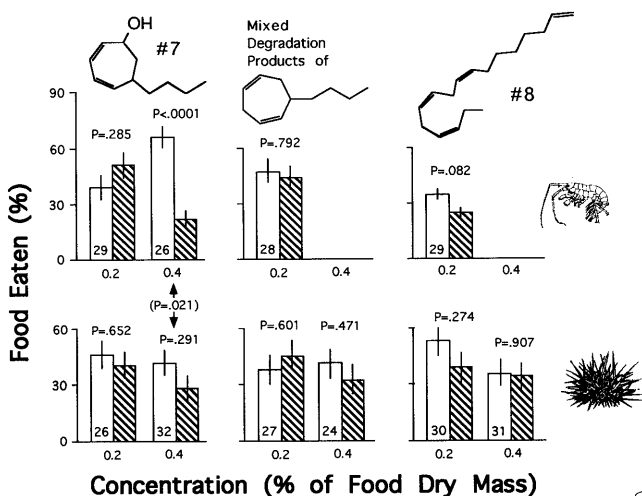


Fig. 4 Symbols and analyses are as in Fig. 1. Compound 7 is found in species of *Dictyopteris* and is a natural degradation product of 1. The middle figures test the effects of the complete mixture of compounds resulting from air oxidation of 1. This mixture contained many undefined metabolites including alcohols, ketones, hydroperoxides. Compound 8 may be considered as a lower homologue of the widespread C_{19} and C_{21} penta- and hexaenes from thalli of many seaweeds.

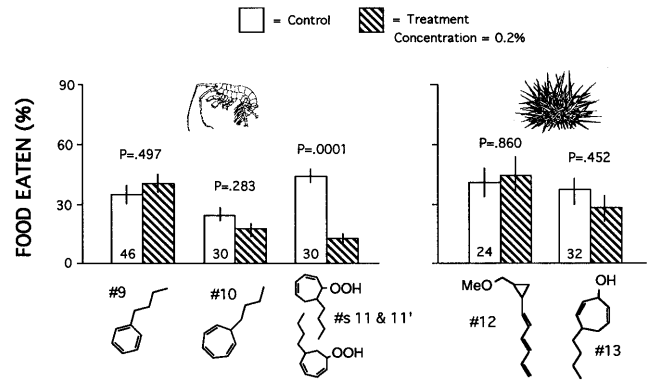


Fig. 5 Symbols and analyses are as in Fig. 1. Each of these metabolites was tested at only one concentration (0.2% of food dry mass). Compounds 9, 10, 11, 11', and 13 are degradation products of 1. 9 has also been isolated from species of *Dictyopteris* and *Dictyota*. The hydroperoxides 11 and 11' appear to be major compounds during the earliest stages of the degradation of 1. 12 is a thermolabile analogue of a thermolabile cis-disubstituted pheromone precursor (Hertweck & Boland, unpublished).

urchin while decreasing activity against the amphipod (Fig. 2). Compound 3 deterred urchin feeding by a significant 59% at a concentration of only 0.05% of food dry mass. The precursor of compound 3 (*i.e.*, compound 1) had no effect on urchin feeding at 2X this concentration (Fig. 1). Compound 3 lost activity against the urchin when the test concentration was decreased to 0.025% (control = 57.6% eaten, treatment = 43.3% eaten, $N = 26$, $P = 0.243$, paired t-test). No other degradation product deterred urchin feeding at a concentration of 0.2% but 4 did deter the urchin at a concentration of 0.4% (Fig. 2).

The single sulfur containing C_{11} metabolite from *D. membranacea* (Schnitzler & Boland, unpublished) that we tested was significantly more deterrent to the amphipod than to the urchin; it had no effect on the urchin (Fig. 3). The C_{17} metabolite showed a non-significant trend toward deterring the amphipod ($P = 0.082$) but had no effect on urchin feeding (Fig. 4). The thermally stable analogue 12 of the natural thermolabile pheromone precursor had no effect on urchin feeding.

Discussion

Our findings that several of the C_{11} compounds from *Dictyopteris* strongly deter amphipod feeding but have little effect on sea urchin feeding provide a stark contrast to numerous previous investigations suggesting that sedentary mesograzers are less susceptible to seaweed chemical defenses than are larger and more mobile herbivores (Hay 1992; Hay & Fenical 1996). Most previous investigations that focused on how seaweed chemical defenses affected feeding by large generalist urchins and fishes versus smaller, more sedentary mesograzers (like tube-building or domicile-building amphipods and polychaetes) found that the more sedentary mesograzers were resistant to seaweed sec-

ondary metabolites that deterred feeding by larger herbivores (reviewed in Hay & Fenical 1996; Hay 1997). Previous investigations, some using the same species of amphipod and sea urchin that we studied here (Hay *et al.* 1987a,b, 1998b, 1990a; Duffy & Hay 1991, 1994; Cronin & Hay 1996a,b), found that diterpene alcohols produced by brown seaweeds in the genus *Dictyota* significantly deterred feeding by both temperate and tropical species of fishes and the sea urchins, but had no effect on, or at some concentrations stimulated, feeding by the amphipod *Ampithoe longimana*, as well as by the tube building polychaete *Platynereis dumerilii* and the domicile building amphipod *Pseudamphithoides incurvaria*. In similar contrasts, both the halogenated monoterpene octadecene from the red alga *Ochtodes secundiramea* (Paul *et al.* 1987) and a mixture of two C₁₁ hydrocarbons from the brown alga *Dictyopteris delicatula* deterred feeding by herbivorous reef fishes but failed to deter feeding by plant-dwelling amphipods (Hay *et al.* 1988a). Several other mesograzers such as plant-dwelling crabs and ascoglossans shown similar patterns of being immune to seaweed chemical defenses that strongly deter larger herbivores (Hay *et al.* 1989, 1990b, Stachowicz & Hay 1996). Patterns from these previous investigations are consistent with the hypothesis that seaweeds have evolved chemical defenses against large generalist herbivores that are known to have a tremendous impact on marine plant communities, and that plant-associated mesograzers have, in turn, evolved a resistance to seaweed chemical defenses because living on chemically-defended plants provides a safe site where they are less susceptible to predation (see Hay 1992, 1997; Hay & Steinberg 1992).

A recent study by Schnitzler *et al.* (1998) used the same herbivores we tested here, but focused on the effects of sulfur containing C₁₁ metabolites from *Dictyopteris*. Each of the four metabolites they studied strongly deterred amphipod feeding at concentrations of $\leq 0.2\%$ of food dry mass, with three of the four metabolites being strongly deterrent at concentrations of only 0.05–0.1%. These metabolites had no effects on urchin feeding, even at concentrations of 0.4%. The single C₁₁ sulfur compound that we tested (**6**) produced a somewhat similar pattern, but so did several of the C₁₁ compounds that did not contain sulfur (Figs. 1–5). For the non-sulfur containing C₁₁ compounds, or mixes, that we tested, three of nine deterred amphipods at $\leq 0.2\%$ concentration and six of nine deterred amphipods at a concentration $\leq 0.4\%$. Similar contrasts for the urchin show only one and two of nine compounds were deterrent at concentrations of $\leq 0.2\%$ and $\leq 0.4\%$, respectively. Thus, C₁₁ metabolites, with or without sulfur, commonly deter the amphipod more than the urchin; however, those containing sulfur appear to be more predictable, and possibly more potent, deterrents.

Although feeding by mesograzers is often considered to have little effect on populations of marine macrophytes (Bell 1991), there are several examples of plant-dwelling mesograzers having significant negative effects on marine macrophytes (Tegner & Dayton 1987;

Duffy 1990; Brawley 1992; Parker *et al.* 1993), and one recent study suggests that herbivorous amphipods can function as keystone species in some marine systems because of their strong negative effects on dominant brown seaweeds that are chemically resistant to fish and urchin grazing (J.E. Duffy & M.E. Hay unpublished). The strength and specificity with which the C₁₁ compounds affected amphipod feeding suggest that selection for these metabolites could have been driven, at least in part, by damage due to mesograzers feeding and that C₁₁ compounds may function as defenses that are especially effective against these types of mesograzers. To our knowledge, the C₁₁ compounds studied here and by Schnitzler *et al.* (1998) represent the first group of seaweed secondary metabolites that appear to be selectively effective against *Ampithoe*-like mesograzers that are commonly resistant to the effects of other brown algal chemical defenses.

The ecological or evolutionary costs of producing chemical defenses against marine herbivores are poorly understood (Hay & Fenical 1988; Hay & Stineberg 1992), but costs could be minimized by using compounds that serve multiple functions (Schmitt *et al.* 1995) or by slightly modifying existing biosynthetic pathways to produce compounds that serve new needs. Some of the C₁₁ compounds that deter amphipod feeding also serve as pheromones that attract sperm to brown algal eggs (Boland 1995; Pohnert & Boland 1996). These particular metabolites serve multiple ecological roles and thus appear to represent examples of molecular economy. We also found that some degradation products or slight variants of pheromones deterred amphipods. This suggests that pheromones could degrade to defensive metabolites and that slight modifications of pathways producing gamete attractants could provide herbivore deterrent metabolites as well. With one exception (the effect of compound **3** on the urchin), degradation of the pheromone dictyotene (**1**) tended to produce less deterrent or equally deterrent products rather than ones with increased deterrence. Thus, if degradation products are to play a defensive role, then seaweeds may need to control the degradation endpoint so that a deterrent molecule is produced. The complex mixture of metabolites produced by the abiotic process of simple air oxidation of dictyotene did not affect feeding by either the amphipod or the urchin.

Although investigations of secondary metabolites in both marine and terrestrial plants have tended to focus on their roles as herbivore deterrents (Rosenthal & Berenbaum 1992), these metabolites may commonly serve multiple functions. Marine examples of this include (1) diterpene alcohols from brown seaweeds serving as both antiherbivore and antifouling metabolites (Schmitt *et al.* 1995) and (2) dimethylsulphonio propionate (DMSP) in a unicellular planktonic alga converting to the deterrent compound dimethylsulfide (DMS) when cells are lysed by protozoan herbivores (Wolfe *et al.* 1997). In addition to being a non-toxic precursor for DMS, DMSP also functions as an osmolyte (Dickson & Krist 1987), a cryoprotectant (Krist 1991), and a methyl donor (Ishida & Kadota 1968). The potential for sec-

ondary metabolites to serve multiple functions (Paul & Fenical 1986) complicates our ability to assess the costs versus benefits of these compounds and the role they play in prey-consumer coevolution (Schmitt *et al.* 1995; Oldham & Boland 1996).

Because some C_{11} compounds that strongly deter amphipod feeding also occur as pheromones in gametes (*e.g.*, **I**), and thus possibly in zygotes and sporelings, they could play critical roles in protecting juvenile stages from mesograzers. It is also possible that C_{11} compounds in eggs that initially serve as gamete attractants could naturally degrade to metabolites that deter consumption of zygotes or germlings. Chemical protection of juvenile stages from mesograzers is feasible and potentially important because mesograzers can feed on scales where they could select among zygotes or germlings based on chemical cues and because they can graze in cracks and crevices that provide juveniles with spatial refuges from larger herbivores. Spatial refuges from large grazers like urchins may be critical for juvenile seaweeds because these herbivores take bites that will include many individuals at a time, making it unlikely that they could select among co-occurring juveniles based on chemical defenses (Hay & Fenical 1992). Additionally, in seasonal habitats, Dictyotalean brown algae may settle and grow to only a few cells tall during the fall and rely on these resting juvenile stages to reestablish the population the following spring (Richardson 1979). Because amphipod abundances often increase dramatically during these cooler portions of the year when fish predators are less active (Nelson 1979; Duffy & Hay 1991, 1994), chemical defense of germlings from mesograzers could be critical.

Our investigation demonstrates that C_{11} compounds could defend brown algal juveniles from mesograzers, but we did not directly investigate chemical defenses of either zygotes or germlings. Studies focused explicitly on the C_{11} compounds of zygotes and germlings, how these compounds affect mesograzers feeding, and how the concentrations of these metabolites change as a function of plant ontogeny would be especially interesting. Recent studies have documented potent chemical defenses among the larvae of many marine invertebrates and have suggested that the presence of larval defenses may affect the evolution of larval size, color, and time of release, the dispersal mode and distance, and the general life-history patterns associated with different marine invertebrates (Lindquist & Hay 1996). Studies of gamete, zygote, and sporeling chemical defenses might provide similar insights into the life-history and reproductive patterns of seaweeds.

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References

- Bell SS (1991) Amphipods as insect equivalents? an alternative view. *Ecology* 72:350–354
- Boland W (1995) The chemistry of gamete attraction; chemical structures, biosynthesis and (a)biotic degradation of algal pheromones. *Proc Natl Acad Sci* 92:31–43
- Bousfield EL (1973) Shallow-Water Gammaridean Amphipoda of New England. NY/Ithaca: Cornell University Press
- Brawley SH (1992) Mesoherbivores. Pp 235–264 in John DM, Hawkins SS, Price JH (eds) *Plant-Animal Interactions in the Marine Benthos*, GB-Oxford: Systematics Association Special Volume, Clarendon Press
- Brawley SH, Adey WH (1981) The effects of micrograzers on algal community structure in a coral reef microcosm. *Mar Biol* 61:167–177
- Carpenter RC (1986) Partitioning herbivory and its effect on coral reef algal communities. *Ecol Monogr* 56:345–365
- Cronin G, Hay ME (1996a) Seaweed susceptibility to herbivores depends on recent history of both the plant and the animal. *Ecology* 77:1531–1543
- Cronin G, Hay ME (1996b) Amphipod grazing and induction of seaweed chemical defenses. *Ecology* 77:2287–2301
- Dickson DM, Kirst GO (1987) Osmotic adjustment in marine eukaryotic algae: the role of inorganic ions, quarternary ammonium, tertiary sulfonium and carbohydrate solutes. *New Phytology* 106:645–655
- Duffy JE (1990) Amphipods on seaweeds: partners or pests? *Oecologia* 83:267–276
- Duffy JE, Hay ME (1990) Seaweed adaptation to herbivory. *Bio-science* 40:368–375
- Duffy JE, Hay ME (1991) Food and shelter as determinants of food choice by an herbivorous marine amphipod. *Ecology* 72:1286–1298
- Duffy JE, Hay ME (1994) Herbivore resistance to seaweed chemical defense: the role of mobility and predator risk. *Ecology* 75: 1304–1319
- Duffy JE, Paul VJ (1992) Prey nutritional quality and the effectiveness of chemical defenses against tropical reef fishes. *Oecologia* 90:333–339
- Faulkner JD (1997) Marine natural products. *Nat Prod Rep* 14:259–302
- Halsall TG, Hills IR (1971) Isolation of Heneicosa-1,6,9,12,15,18-hexaene and -1,6,9,12-pentaene from the alga *Fucus vesiculosus*. *J Chem Soc Chem Comm* (1971) 448–449
- Hay ME (1991) Fish-seaweed interactions on coral reefs: effects herbivorous fishes and adaptations of their prey. Pp 96–119 in Sale PF (ed.) *The Ecology of Fishes on Coral Reefs*. CA/San Diego: Academic Press
- Hay ME (1992) The role of seaweed chemical defenses in the evolution of feeding specialization and in the mediation of complex interactions. Pp 93–118 in Paul VJ (ed.) *Ecological Roles of Marine Natural Products*. NY/Ithaca: Cornell University Press
- Hay ME (1996) Marine chemical ecology: What is known and what is next? *J Exp Mar Biol Ecol* 200:103–134
- Hay ME (1997) The ecology and evolution of seaweed-herbivore interactions on coral reefs. *Coral Reefs* 16 Suppl: S67–S76
- Hay ME, Fenical W (1988) Marine plant-herbivore interactions: The ecology of chemical defense. *Ann Rev Ecol Syst* 19:111–145
- Hay ME, Fenical W (1992) Chemical mediation of seaweed-herbivore interactions. Pp 319–337 in John DM, Hawkins SS, Price JH (eds) *Plant-Animal Interactions in the Marine Benthos*, GB-Oxford: Systematics Association Special Volume, Clarendon Press
- Hay ME, Fenical W (1996) Chemical ecology and marine biodiversity: insights and products from the sea. *Oceanography* 9:10–20
- Hay ME, Steinberg PD (1992) The chemical ecology of plant-herbivore interactions in marine versus terrestrial communities. Pp

- 371–413 in Rosenthal GA, Berenbaum MR (eds) *Herbivores: Their Interactions with Secondary Plant Metabolites*. Second Edition, Vol. II, Evolutionary and Ecological Processes. CA/San Diego: Academic Press
- Hay ME, Lee RR, Guieb RA (1986) Food preference and chemotaxis in the sea urchin *Arbacia punctulata* (Lamarck) Philippi. *J Exp Mar Biol Ecol* 96:147–153
- Hay ME, Duffy JE, Pfister CA, Fenical W (1987a) Chemical defense against different marine herbivores: are amphipods insects equivalents? *Ecology* 68:1567–1580
- Hay ME, Fenical W, Gustafson K (1987b) Chemical defense against diverse coral reef herbivores. *Ecology* 68:1581–1591
- Hay ME, Duffy JE, Fenical W, Gustafson K (1988a) Chemical defense in the seaweed *Dictyopteria delicatula*: differential effects against reef fishes and amphipods. *Mar Ecol Prog Ser* 48:185–192
- Hay ME, Renaud PE, Fenical W (1988b) Large mobile versus small sedentary herbivores and their resistance to seaweed chemical defense. *Oecologia* 75:246–252
- Hay ME, Pawlik JR, Duffy JE, Fenical W (1989) Seaweed-herbivore-predator interactions: host-plant specialization reduces predation on small herbivores. *Oecologia* 81:418–427
- Hay ME, Duffy JE, Fenical W (1990a) Host-plant specialization decreases predation on a marine amphipod: an herbivore in plant's clothing. *Ecology* 71:733–743
- Hay ME, Duffy JE, Paul VJ, Renaud PE, Fenical W (1990b) Specialist herbivores reduce their susceptibility to predation by feeding on the chemically-defended seaweed *Avrainvillea longicaulis*. *Limnol Oceanogr* 35:1734–1743
- Hay ME, Kappel QE, Fenical W (1994) Synergisms in plant defenses against herbivores: interactions of chemistry, calcification and plant quality. *Ecology* 75:1714–1726
- Hay ME, Stachowicz JJ, Cruz-Rivera E, Bullard S, Deal MS, Lindquist N (1998) Bioassays with marine and freshwater macroorganisms. Pp 39–141 in Millar JG, Haynes KF (eds) *Methods in Chemical Ecology*. New York: Chapman and Hall
- Ishida Y, Kadota H (1968) Participation of dimethyl- β -propiothetin in transmethylation reaction in *Gyrodinium cohnii*. *Bull Jpn Soc Sci Fish* 34:699–705
- John DM, Hawkins SS, Price JH (eds) (1992) *Plant-Animal Interactions in the Marine Benthos*, GB-Oxford: Systematics Association Special Volume, Clarendon Press
- Krist GO, *et al.* (1991) DMSP in ice-algae and its possible biological role. *Mar Chem* 35:381–388
- Lindquist N, Hay ME (1996) Palatability and chemical defense of marine invertebrate larvae. *Ecol Monogr* 66:431–450
- Maier I (1995) Brown algal pheromones. *Progress in Phycological Research* 11:51–102
- Moore RE (1976) Chemotaxis and the odor of seaweed. *Lloydia* 39:181–190
- Moore RE (1977) Volatile compounds from marine algae. *Acc Chem Res* 10:40–47
- McMurry TJ, Groves JT (1986) Metalloporphyrin models for cytochrome P-450. Pp 1–28 in *Cytochrome P-450, Structure, Mechanism, and Biochemistry*. Ortiz de Montellano PR (ed.) New York: Plenum
- Nelson WG (1979) Experimental studies of selective predation on amphipods: consequences for amphipod distribution and abundance. *J Exp Mar Biol Ecol* 38:225–245
- Oldham N, Boland W (1996) Chemical ecology; multifunctional compounds and multitrophic interactions. *Naturwissenschaften* 83:248–254
- Oliver JS, Oakden JM, Slattery PN (1982) Phoxocephalid amphipod crustaceans as predators on larvae and juveniles in marine soft-bottom communities. *Mar Ecol Prog Ser* 7:179–184
- Parker T, Chapman ARO (1994) Separating the grazing effects of Periwinkles and amphipods on a seaweed community dominated by *Fucus distichus*. *Ophelia* 39:75–91
- Parker T, Johnson C, Chapman ARO (1993) Gammarid amphipods and littorinid snails have significant but different effects on algal succession in a littoral fringe tidepools. *Ophelia* 38:69–88
- Paul VJ (ed) (1992) *Ecological Roles for Marine Natural Products*. Ithaca: Comstock Press
- Paul VJ, Fenical W (1986) Chemical defense in tropical green algae, order Caulerpales. *Mar Ecol Prog Ser* 34:157–169
- Paul VJ, Hay ME, Duffy JE, Fenical W, Gustafson K (1987) Chemical defense in the seaweed *Ochtodes secundiramea* (Montagne) Howe (Rhodophyta): effects of its monoterpenoid components upon diverse coral-reef herbivores. *J Exp Mar Biol Ecol* 114:249–260
- Piel, J (1994) Abiotic degradation of pheromones of marine brown algae. Diploma thesis, University of Bonn
- Pohnert G, Boland W (1996) Biosynthesis of the algal pheromone hormosirene by the freshwater diatom *Gomphonema parvulum* (Bacillariophyceae). *Tetrahedron* 52:10073–10082
- Richardson JP (1979) Overwintering of *Dictyota dichotoma* (Phaeophyceae) near its northern distributional limit on the east coast of North America. *J Phycol* 15:22–26
- Rosenthal GA, Berenbaum MR (eds) (1992) *Herbivores: Their Interactions with Secondary Plant Metabolites*. Second Edition, Vol. II, Evolutionary and Ecological Processes. CA/San Diego: Academic Press
- Schmitt TM, Hay ME, Lindquist N (1995) Constraints on chemically-mediated coevolution: multiple functions for seaweed secondary metabolites. *Ecology* 76:107–123
- Schneider CW, Searles RB (1991) *Seaweeds of the Southeastern United States: Cape Hatteras to Cape Canaveral*. Durham: Duke University Press
- Schnitzler I, Boland W, Hay ME (1998) Organic sulfur compounds from *Dictyopteria* spp. (Phaeophyceae) deter feeding by an herbivorous amphipod (*Ampithoe longimana*) but not by an herbivorous sea urchin (*Arbacia punctulata*). *J Chem Ecol* (in press)
- Stachowicz JJ, Hay ME (1996) Facultative mutualism between an herbivorous crab and its coralline algal host: advantages of eating noxious seaweeds. *Oecologia* 105:377–387
- Tegner MJ, Dayton PK (1987) El Niño effects on southern California kelp forest communities. *Adv Ecol Res* 17:243–279
- Wolfe GV, Steinke M, Kirst GO (1997) Grazing-activated chemical defense in a unicellular marine alga. *Nature* 387:894–897
- Youngblood WW, Blumer M, Guillard RL, Firoe F (1971) Saturated and unsaturated hydrocarbons in marine benthic algae. *Mar Biol* 8:190–201
- Zimmerman R, Gibson R, Harrington J (1979) Herbivory and detritivory among gammaridean amphipods from a Florida seagrass community. *Mar Biol* 54:41–47

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