

# Activated defense systems in marine macroalgae: evidence for an ecological role for DMSP cleavage

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**ABSTRACT:** Activated defenses against herbivores and predators are defenses whereby a precursor compound is stored in an inactive or mildly active form. Upon damage to the prey, the precursor is enzymatically converted to a more potent toxin or feeding deterrent. In marine systems, activated defenses are only known to exist in a few species of tropical macroalgae. In this study, we examined an activated defense system in temperate marine macroalgae in which the osmolyte dimethylsulfoniopropionate (DMSP) is converted to acrylic acid or acrylate, depending upon the pH, and dimethyl sulfide (DMS) by the enzyme DMSP lyase upon damage to the alga. We surveyed 39 species of red, green, and brown algae from the Washington and Oregon coasts, and found high concentrations of DMSP in the chlorophytes *Acrosiphonia coalita*, *Codium fragile*, *Enteromorpha intestinalis*, *E. linza*, *Ulva californica*, *U. fenestrata*, and *U. taeniata*, and in the rhodophyte *Polysiphonia hendryi*. Concentrations of DMSP ranged from 0.04% of the alga's fresh mass (FM) to 1.8% FM. We found significant DMSP lyase activity in 1 green alga, *U. fenestrata*, and 1 red alga, *P. hendryi*, with DMSP cleavage rates approaching 300 mmol kg<sup>-1</sup> FM min<sup>-1</sup>. Loss of DMSP and the production of DMS when the tissues of *U. californica* and *P. hendryi* were crushed suggested that physical damage results in DMSP cleavage. In laboratory feeding preference experiments, acrylic acid deterred feeding by the sea urchin *Strongylocentrotus droebachiensis* at concentrations of 0.1 to 2% FM and by *S. purpuratus* at 0.25 to 2% FM, while the precursor DMSP functioned as a feeding attractant to both sea urchins. In contrast, feeding by the isopod *Idotea wosnesenskii* was not deterred by acrylic acid even at concentrations as high as 8% FM. Our data suggest that DMSP may function as a precursor in an activated defense system in diverse species of temperate macroalgae and may possibly contribute to the widespread success of the Ulvophyceae. This chemical system is also found in unicellular phytoplankton, and presents an opportunity to compare and contrast the ecological role of chemical defense among micro- and macroorganisms.

**KEY WORDS:** Acrylic acid · Activated defenses · DMSP · Herbivory · Macroalgae

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## INTRODUCTION

Marine macroalgae produce a variety of secondary metabolites, including many that function as herbivore deterrents (Hay & Fenical 1988, Van Alstyne & Paul 1989, Hay & Steinberg 1992, Paul 1992, Targett & Arnold 1998). Secondary metabolite levels in macroal-

gae can be influenced by or correlated with a number of environmental factors including light intensity (Cronin & Hay 1996b,c, Pavia et al. 1997), nutrient levels (Ilvessalo & Tuomi 1989, Yates & Peckol 1993, Arnold et al. 1995, Cronin & Hay 1996a, Peckol et al. 1996), desiccation (Renaud et al. 1990), and salinity (Pedersen 1984). In some species, biological interactions, such as grazing, affect the types and levels of defenses. Several species of marine macroalgae produce induced defenses in which defense production increases after

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algae are damaged by herbivores (Van Alstyne 1988, Yates & Peckol 1993, Cronin & Hay 1996b, Peckol et al. 1996, Hammerstrom et al. 1998). The induction of defenses occurs over a period of days (Hammerstrom et al. 1998) to weeks (Van Alstyne 1988) and may be dependent upon nutrient levels (Yates & Peckol 1993, Peckol et al. 1996).

An alternative mechanism by which grazers influence the potency of defenses in their prey is the production of activated defenses. Activated defenses are compounds that are stored in plants in a non-toxic or mildly toxic form. Upon damage to the tissue, stored compounds are converted to more deterrent compounds. It is suspected that damage activates an enzyme or enzymes that convert the less toxic compound (the substrate or precursor) to the more deterrent compound (the product) and other by-products.

Activated defenses have been reported from a variety of terrestrial plants. For example, many crucifers produce glucosinolates that are converted to mustard oils in a series of reactions involving the enzyme myrosinase (Chew 1988, Louda & Mole 1991). Mustard oils are toxic towards many herbivorous mammals and generalist insect grazers. Native plants producing glucosinolates are typically fast-growing, ephemeral species (Louda & Mole 1991). Similarly, cyanogenic glycosides are non-toxic compounds found in almost all the major groups of vascular plants (Conn 1979, 1981, Seigler 1991). When plant tissues are crushed, cyanogenic glycosides are enzymatically hydrolyzed, usually by  $\beta$ -glycosidases or hydroxynitrile lyases, and hydrogen cyanide (HCN) is released (Seigler 1991). HCN and the other products of this reaction, sugars, aldehydes and ketones, may be toxic to non-adapted herbivores, although many animals are capable of detoxifying limited quantities of HCN (Conn 1979, Seigler 1991). Similar conversions of compounds have also been reported that involve phenolic glycosides in terrestrial vascular plants (Clausen et al. 1989), sesquiterpenes in mushrooms (Sternner 1985, Sternner et al. 1985), and glucosinolates in aquatic vascular plants (Newman et al. 1992). Activated defenses have been described in tropical siphonous green algae (Paul & Van Alstyne 1992). However, there are no published reports of activated defenses in temperate marine macroalgae.

This study examines the role of dimethylsulfonio-propionate (DMSP) in an activated defense system in northern Pacific marine macroalgae. DMSP, a tertiary sulfonium derivative of methionine, is found in many species of green and red macroalgae in habitats ranging from polar (Karsten et al. 1990) to temperate (White 1982, Reed 1983a) to tropical (Bischoff et al. 1994, Dacey et al. 1994). It is also abundant in phytoplankton and some halotolerant vascular plants (Dacey & Wakeham 1986, Keller et al. 1989, Rhodes & Hanson 1993, Otte & Morris 1994). DMSP is a compatible solute (Reed 1983a, Kirst 1989, Kirst et al. 1991, Karsten et al. 1992) and may also play a role in cryoprotection in polar algae (Kirst et al. 1991, Karsten et al. 1992). Although it functions in osmoregulation, DMSP concentrations do not respond to short-term (hourly) salinity changes (Reed 1983a,b, Dickson & Kirst 1986, Edwards et al. 1987, 1988), but only to longer-term adaptations (Edwards et al. 1988).

DMSP was first identified by Challenger & Simpson (1948) as a substrate for transmethylation and also as the precursor for dimethyl sulfide (DMS) production, as noted by Haas (1935) in *Polysiphonia fastigiata*. The cleavage pathway producing DMS and acrylic acid (Fig. 1) was also detected in *Enteromorpha intestinalis* by Bywood & Challenger (1953). Cantoni & Anderson (1956) showed that the reaction was catalyzed by the enzyme DMSP lyase. The DMS produced by this reaction is important to the global sulfur cycle (e.g., Lovelock et al. 1972, Andreae & Raemdonck 1983, Liss et al. 1994, Groene 1995, Malin & Kirst 1997) and possibly to climate (Bates et al. 1987, Charlson et al. 1987). Although the cleavage reaction has been the subject of intense study, its biological function is still poorly understood.

In oceanic systems, DMS production is often associated with grazing on phytoplankton containing DMSP (Dacey & Wakeham 1986, Daly & DiTullio 1993). Recently, the DMSP-cleavage reaction was proposed as an activated defense system in microalgae (Wolfe & Steinke 1996, Wolfe et al. 1997), producing concentrated acrylate inside protozoan food vacuoles. We hypothesized that DMSP may also function as part of an herbivore-activated defense system in marine macroalgae. To test this hypothesis, we first examined

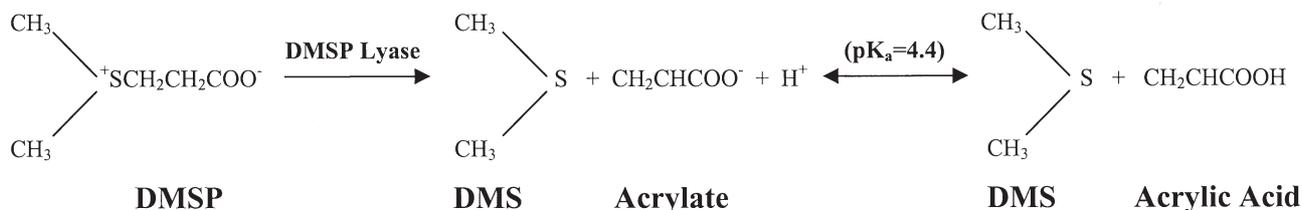


Fig. 1. Conversion of DMSP by DMSP lyase to DMS and acrylate or acrylic acid

local northeastern Pacific macroalgae to determine whether they produced DMSP and DMSP lyase. We then conducted experiments to determine if acrylic acid and DMSP deter feeding by local generalist grazers. The results of our study provide strong evidence that DMSP can function in an activated defense system in some macroalgal species.

## MATERIALS AND METHODS

**Collection sites and study organisms.** Purple sea urchins *Strongylocentrotus purpuratus* were collected from low- to mid-intertidal pools at Boiler Bay, Oregon, USA (44° 40' N, 124° 03' W). All sea urchins were kept in flow-through seawater tables at the Hatfield Marine Science Center (HMSC) in Newport, Oregon, and fed a mixture of red, green and brown macroalgae. Green sea urchins *S. droebachiensis* were collected with SCUBA from ~5 m depth from Burrows Channel at the north end of Fidalgo Island, Washington, USA (48° 29' N, 122° 41' W). Isopods *Idotea wosnesenskii* were collected from the low intertidal zone at the Fort Ebey boat launch on Whidbey Island, Washington (48° 15' N, 122° 45' W). *S. droebachiensis* and *I. wosnesenskii* were maintained in flow-through seawater tables at the Shannon Point Marine Center and were fed a mixed-species diet of macroalgae. Macroalgae for DMSP and DMSP lyase analysis were collected from a number of intertidal sites including Boiler Bay, Oregon, the beach in front of the Shannon Point Marine Center in Anacortes, Washington (48° 31' N, 122° 41' W), the Friday Harbor Laboratories (48° 33' N, 123° 01' W) and Jackell's Lagoon (48° 31' N, 123° 01' W) on San Juan Island, Washington, and from Allan Island Washington (48° 28' N, 122° 42' W).

**Analysis of DMSP concentrations and DMSP lyase activity.** DMSP was analyzed as DMS following alkaline cleavage with methods similar to those described in Wolfe et al. (1994). Freshly collected algal samples were weighed and placed in 5 ml of 10 N NaOH in 100 ml serum vials and sealed with Teflon-coated septa. Bottles were incubated in the dark for a minimum of 12 h to allow complete conversion to DMS, and shaken to hasten equilibration with headspace. Ten to 100  $\mu$ l of DMS was sampled from the headspace by gas-tight syringe and injected to a gas chromatograph equipped with a Chromosil 330 column and flame photometric detector. DMSP standard additions to equal volumes of NaOH were used for calibration.

We measured DMSP lyase concentrations in 2 of the macroalgal species that contained measurable amounts of DMSP, *Ulva fenestrata* (Chlorophyta) and *Polysiphonia hendryi* (Rhodophyta). Algae were collected and epiphytic algae were removed from their

surfaces. They were then rinsed briefly in ice-cold deionized water and blotted dry. Because DMSP lyase activity may be pH-sensitive, algal tissues (0.5 to 2.0 g) were ground using a chilled mortar and pestle in each of 3 buffers of differing pH: (1) 100 mM sodium citrate, 1 M NaCl, 10% (v/v) glycerol, and 0.5% (v/v) polyoxyethylenesorbitan (Tween-80), pH 5.2; (2) 100 mM 2-[N-morpholino]ethanesulfonic acid (MES), 1 M NaCl, 10% (v/v) glycerol, and 0.5% (v/v) Tween-80, pH 6.2; and (3) 100 mM 2-[N-morpholino]ethanesulfonic acid (MES), 1 M NaCl, 10% (v/v) glycerol, and 0.5% (v/v) Tween-80, pH 7.2.

The homogenate was transferred to a microcentrifuge tube and incubated on ice with occasional stirring for 30 min, then spun in a centrifuge at 4500 rpm (3000  $\times$  g) for 5 min. The pellet was collected and resuspended in 1 ml of buffer then centrifuged again. The supernatants from both centrifugations were pooled and diluted 1:100 with deionized water. Extract (270  $\mu$ l) and 10  $\mu$ l of 30 mM dithiothreitol (DTT) were added to 2 ml gas-tight vials and equilibrated to room temperature. DMS concentrations were determined from headspace samples (10  $\mu$ l) collected from the vials and used to establish a baseline of DMS production. Once the baseline was established, the reaction was initiated by adding DMSP-Cl (University of Gronigen Chemical Laboratory, The Netherlands) to a final concentration of 10 mM. DMS concentrations in the headspace were quantified over 20 to 50 min after the addition of DMSP.

**Feeding bioassays with DMSP and acrylic acid.** The effectiveness of acrylic acid and DMSP as feeding deterrents and attractants was tested with 2-choice laboratory bioassays in which compounds were incorporated into diets and feeding rates on those diets were compared. Diets for assays involving *Strongylocentrotus purpuratus* were made by homogenizing 30 g of sushi nori (*Porphyra* sp.) in a blender with 600 ml of tap water. The solid materials were filtered out through several layers of cheesecloth and the remaining water extract was stored in darkness at 4°C. An anchovy homogenate was made by grinding 57 g of anchovies packaged in olive oil and 200 ml of tap water in a blender. The homogenate was also stored at 4°C in darkness prior to use. Diets were made by combining 4.5 g of agar with 75 ml of the nori extract, heating the mixture in a microwave at highest power for 60 s, and then adding 3 ml of anchovy extract. DMSP-Cl (University of Gronigen Chemical Laboratory, The Netherlands) or acrylic acid (Sigma) were added to the diet after it had cooled but before it gelled to avoid thermal decomposition. The diet was then poured into shallow trays and cooled. After the diets had gelled, ~3 cm diameter pieces were cut and weighed. Distilled water without acrylic acid was added to control diets.

In feeding experiments at HMSC, urchins *Strongylocentrotus purpuratus* were offered 2 pieces of diet in 20 cm diameter round plastic containers. To start the experiments, the diets were placed in the center of the containers and the urchins were placed on top of the diets so they had equal access to each piece of food. The containers were filled almost to the top with seawater and were placed in a seawater table with seawater flowing around, but not into the containers, to cool the water in the containers. Urchins were allowed to feed on the diets until about half of the food in the containers had been consumed. Replicates were discarded if the urchins did not consume at least half the food in the containers in a 6 h period. Preliminary experiments showed that in the absence of herbivores mass loss of the diets over a 6 h period was generally less than 2% and did not differ in control diets versus diets containing acrylic acid; therefore, control arenas for autogenic losses were not used in these experiments. Urchins were starved for 2 wk before being used in feeding experiments in order to ensure they would eat the diets rapidly. Some herbivores have been shown to be less selective when starved (Cronin & Hay 1996c). If this is the case for the herbivores we used, then these assays should be conservative in determining the effectiveness of acrylic acid and DMSP. After the urchins were removed from the containers, the remaining pieces of diet were blotted dry and weighed. The change in mass of the treated food in each replicate was subtracted from the change in mass of the control food and the differences were compared to zero ( $\alpha = 0.05$ ) with a 1-sample Student's *t*-test as described in Peterson & Renaud (1989).

Feeding experiments with *Strongylocentrotus droebachiensis* were conducted in a similar manner at the Shannon Point Marine Center (SPMC) in Anacortes, Washington. The diets were prepared as described above except that they contained 40 g of *Laminaria saccharina* homogenate (300 g *L. saccharina* homogenized with 300 g water), 3.5 g agar, 50 ml deionized water, and 3 ml of anchovy homogenate. The heated mixture was poured onto a 25 × 25 cm glass plate with 4 mm plastic spacers at each corner. A second glass plate was placed on the spacers and served to flatten the agar mixture to a uniform width. Three grams of filtered beach sand was spread in a thin layer on the bottom plate before pouring the agar; this acted as a weight to keep the food from floating.

Diets for experiments involving isopods *Idotea wosnesenskii* were made as described for *Strongylocentrotus purpuratus* diets, except that the diet was made with 2.5 g agar, 100 ml water, and 2 g ground, oven-dried (60°C for 48 h) *Ulva fenestrata*. Two-choice experiments were conducted in 20 cm diameter plastic

containers to which 1 mm mesh fiberglass screens were added to allow water flow through the containers. Isopods were allowed to feed until approximately half the food in a container had been removed. Arenas in which isopods did not consume at least half the food in 24 h were not included in the analyses. Statistical analyses were conducted as described above.

## RESULTS

### DMSP concentrations

Detectable concentrations of DMSP were found in 7 of 8 green algal species examined: *Acrosiphonia coalita*, *Codium fragile*, *Enteromorpha intestinalis*, *E. linza*, *Ulva californica*, *U. fenestrata*, and *U. taeniata* (Table 1). Concentrations of DMSP in chlorophytes ranged from 0.04% FM in *C. fragile* to 1.68% FM in *E. linza*. Only 1 species of red algae, *Polysiphonia hendryi*, had detectable concentrations of DMSP (Table 1). None of the brown algae examined had detectable concentrations of DMSP.

Several of the algae were sampled across more than one site and on more than one date. Concentrations of DMSP in *Ulva californica* and *Polysiphonia hendryi* were similar ( $p > 0.05$ , Student's *t*-test) in tissues collected in January and February from Boiler Bay, Oregon; however, concentrations differed significantly between samples collected in winter at Boiler Bay and summer at Anacortes, Washington (Table 1). Concentrations of DMSP in *P. hendryi* were also significantly higher in the summer San Juan Island collection than the winter Boiler Bay collection (Table 1; 1-way ANOVA:  $F = 6.28$ ,  $p = 0.009$ , Tukey's test,  $p < 0.05$ ), indicating that among-site or seasonal variation in DMSP levels was occurring. Concentrations of DMSP in *Ulva taeniata* were significantly higher in algae collected from Anacortes than from San Juan Island in the summer of 1997 (Table 1; 1-way ANOVA:  $F = 7.57$ ,  $p = 0.031$ , Tukey's test,  $p < 0.05$ ). Concentrations were significantly higher in the winter Boiler Bay collection of *Enteromorpha intestinalis* than in the summer Anacortes collection (Table 1; Student's *t*-test:  $t = 5.56$ ,  $p = 0.011$ ). DMSP concentrations in *Codium fragile* did not differ significantly between the winter Boiler Bay collections and the summer Anacortes collections (Table 1; Student's *t*-test,  $t = -1.91$ ,  $p = 0.31$ ).

### DMSP lyase activity

We found significant DMSP lyase activity in both *Ulva fenestrata* and *Polysiphonia hendryi* (Fig. 2).

Table 1. Mean DMSP ( $\pm 1$  SD) concentrations in marine macroalgae from Washington and Oregon, USA, sites. nd: DMSP was not detectable. FM: fresh mass

Species	Site	Date	[DMSP] (% FM)	n
<b>Phylum (division) Chlorophyta</b>				
<i>Acrosiphonia coalita</i>	Anacortes, WA	Jul 98	0.08 $\pm$ 0.02	2
<i>Cladophora</i> sp.	Boiler Bay, OR	Feb 97	nd	3
<i>Codium fragile</i>	Boiler Bay, OR	Feb 97	0.04 $\pm$ 0.02	3
<i>Enteromorpha intestinalis</i>	Anacortes, WA	Jul 98	0.20 $\pm$ 0.11	2
	Boiler Bay, OR	Feb 97	0.33 $\pm$ 0.04	3
<i>Enteromorpha linza</i>	San Juan Island, WA	Jul 98	0.18 $\pm$ 0.03	3
	Anacortes, WA	Jul 98	1.68 $\pm$ 0.00	1
<i>Ulva californica</i>	Boiler Bay, OR	Jan 97	1.04 $\pm$ 0.14	3
	Boiler Bay, OR	Feb 97	0.84 $\pm$ 0.10	3
	Anacortes, WA	Jul 98	1.58 $\pm$ 0.31	2
<i>Ulva fenestrata</i>	San Juan Island, WA	Jul 98	0.99 $\pm$ 0.39	3
<i>Ulva taeniata</i>	San Juan Island, WA	Jul 98	0.49 $\pm$ 0.22	3
	Anacortes, WA	Jul 97	1.08 $\pm$ 0.21	3
	Anacortes, WA	Jul 98	0.90 $\pm$ 0.08	2
<b>Phylum Rhodophyta</b>				
<i>Calliarthron tuberosum</i>	Boiler Bay, OR	Feb 97	nd	3
<i>Callithamnion</i> sp.	San Juan Island, WA	Jul 97	nd	3
<i>Ceramium</i> sp.	San Juan Island, WA	Jul 97	nd	3
<i>Colpomenia bullosa</i>	Anacortes, WA	Jul 98	nd	2
<i>Corallina officianalis</i>	Boiler Bay, OR	Feb 97	nd	3
<i>Corallina vancouveriensis</i>	Boiler Bay, OR	Feb 97	nd	3
<i>Endocladia muricata</i>	Boiler Bay, OR	Jan 97, Feb 97	nd	3,3
<i>Mastocarpus papillatus</i>	Boiler Bay, OR	Jan 97, Feb 97	nd	3,3
<i>Mazzeaella cornucopiae</i>	Boiler Bay, OR	Feb 97	nd	3
<i>Membranoptera multiramosa</i>	Boiler Bay, OR	Feb 97	nd	3
<i>Microcladia borealis</i>	San Juan Island, WA	Jul 97	nd	3
<i>Neorhodomela larix</i>	Boiler Bay, OR	Feb 97	nd	3
<i>Odonthalia floccosa</i>	Boiler Bay, OR	Jan 97, Feb 97	nd	3,6
	San Juan Island, WA	Jul 97	nd	3
' <i>Petrocelis</i> '	Anacortes, WA	Jul 99	nd	2
<i>Plocamium cartilaginum</i>	Boiler Bay, OR	Feb 97	nd	1
<i>Plocamium violaceum</i>	Boiler Bay, OR	Feb 97	nd	3
<i>Polysiphonia hendryi</i>	Boiler Bay, OR	Feb 97	0.10 $\pm$ 0.08	3
	San Juan Island, WA	Jul 97	0.40 $\pm$ 0.14	15
	Anacortes, WA	Jul 98	0.30 $\pm$ 0.10	2
<i>Porphyra</i> sp. 1	Boiler Bay, OR	Feb 97	nd	3
<i>Porphyra</i> sp. 2	Anacortes, WA	Jul 98	nd	2
<i>Porphyra</i> sp. 3	Boiler Bay, OR	Feb 97	nd	3
<i>Prionitis lanceolata</i>	Boiler Bay, OR	Feb 97	nd	3
<i>Ptilota filicina</i>	Boiler Bay, OR	Jan 97	nd	3
	San Juan Island, WA	Jul 97	nd	3
<b>Phylum Phaeophyta</b>				
<i>Egregia menziesii</i>	Boiler Bay, OR	Feb 97	nd	3
<i>Fucus gardneri</i>	Boiler Bay, OR	Feb 97	nd	3
<i>Fucus spiralis</i>	Boiler Bay, OR	Feb 97	nd	3
<i>Leathesia difformis</i>	Anacortes, WA	Jul 98	nd	2
<i>Pelvetiopsis limitata</i>	Boiler Bay, OR	Feb 97	nd	3
<i>Phaeostrophion irregulare</i>	Boiler Bay, OR	Feb 97	nd	3
<i>Scytosiphon lomentaria</i>	Boiler Bay, OR	Feb 97	nd	3

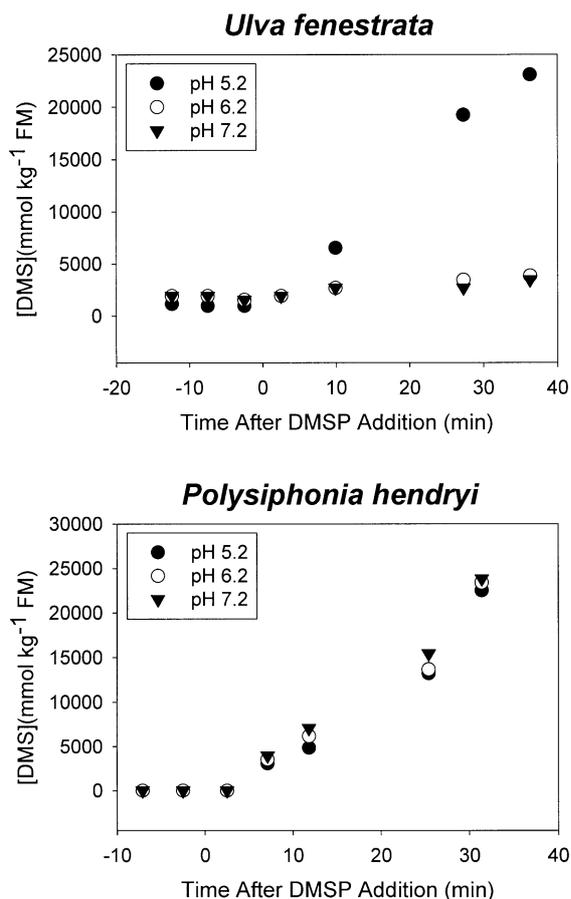


Fig. 2. *Ulva fenestrata* (Chlorophyta) and *Polysiphonia hendryi* (Phodophyta). DMSP lyase activity at 3 pH levels. Measurements at times less than zero were taken before addition of DMSP to the algal extract. FM: fresh mass

Enzyme activities were  $320 \text{ mmol kg}^{-1} \text{ min}^{-1}$  at a pH of 5.2 in *U. fenestrata* and 260 to  $270 \text{ mmol kg}^{-1} \text{ min}^{-1}$  in *P. hendryi*. DMSP lyase in *U. fenestrata* was sensitive to the pH of the buffer used, whereas DMSP lyase in *P. hendryi* was not, within the range of buffers used in the analyses. High DMSP lyase activity was seen at pH 5.2, 6.2, and 7.2 in *P. hendryi*. In *U. fenestrata*, enzyme activity was highest at a pH of 5.2; little activity was seen at pH 6.2 or 7.2.

Although we did not quantify cleavage of DMSP following tissue damage, we compared concentrations of DMSP in whole tissues to tissues that were crushed using a mortar and pestle. Grinding tissues of both *Ulva californica* and *Polysiphonia hendryi* resulted in a significant decrease in DMSP levels (Table 2; 2-way ANOVA: grinding effect,  $F = 7.86$ ,  $p = 0.023$ , species effect,  $F = 203.27$ ,  $p < 0.001$ , grinding  $\times$  species effect,  $F = 1.52$ ,  $p = 0.253$ ), and *P. hendryi* gave off a strong odor of DMS when crushed.

Table 2. *Ulva californica* and *Polysiphonia hendryi*. Mean ( $\pm 1$  SE) DMSP concentrations (% fresh mass, FM) in crushed and uncrushed tissues

Species	Uncrushed tissues [DMSP] (% FM)	Crushed tissues [DMSP] (% FM)
<i>Polysiphonia hendryi</i>	$0.10 \pm 0.05$ (n = 3)	$0.03 \pm 0.01$ (n = 3)
<i>Ulva californica</i>	$0.84 \pm 0.05$ (n = 3)	$0.65 \pm 0.06$ (n = 3)

### Bioassays with herbivores

The addition of acrylic acid to diets significantly reduced feeding by *Strongylocentrotus purpuratus* at concentrations from 2 to 0.25% FM, but not at 0.1% FM (Fig. 3, 1-sample *t*-test,  $p < 0.05$ ). In contrast, DMSP acted as a significant feeding attractant at concentrations from 4 to 0.5% FM, but not at 0.25% FM (Fig. 3, 1-sample *t*-test,  $p < 0.05$ ). When urchins were offered choices of diets containing DMSP and diets containing acrylic acid at equimolar concentrations, they significantly preferred the DMSP-containing diet (Fig. 3, 1-sample *t*-test,  $p < 0.05$ ), except at the lowest concentration (0.2% FM DMSP vs 0.1% acrylic acid), in which there was no significant preference (Fig. 3, 1-sample *t*-test,  $p > 0.05$ ).

Assays with green sea urchins *Strongylocentrotus droebachiensis* produced similar results. The addition of acrylic acid to the diets reduced feeding at all concentrations tested from 0.1 to 2% FM (Fig. 4, 1-sample *t*-test,  $p < 0.05$ ). The addition of DMSP acted as a feeding attractant at concentrations of 0.5 to 2% FM (Fig. 4, 1-sample *t*-test,  $p < 0.05$ ), but was not significantly attractant at concentrations of 0.2 or 4% FM (Fig. 4, 1-sample *t*-test,  $p > 0.05$ ). Diets containing DMSP were preferred over diets containing equimolar amounts of acrylic acid at all concentrations tested (Fig. 4, 1-sample *t*-test,  $p < 0.05$ ).

Feeding by the isopods *Idotea wosnesenskii* was not deterred by the addition of acrylic acid to the diets (Fig. 5). Acrylic acid was a significant feeding attractant to isopods at concentrations of 0.1 and 1% FM (1-sample *t*-test,  $p < 0.05$ ). At all other concentrations, up to 8% FM, there were no significant differences in feeding rates on control diets versus diets containing acrylic acid (1-sample *t*-test,  $p > 0.05$ ).

## DISCUSSION

### Evidence for activated defense

The occurrence and concentrations of DMSP we observed are similar to previous observations of temper-

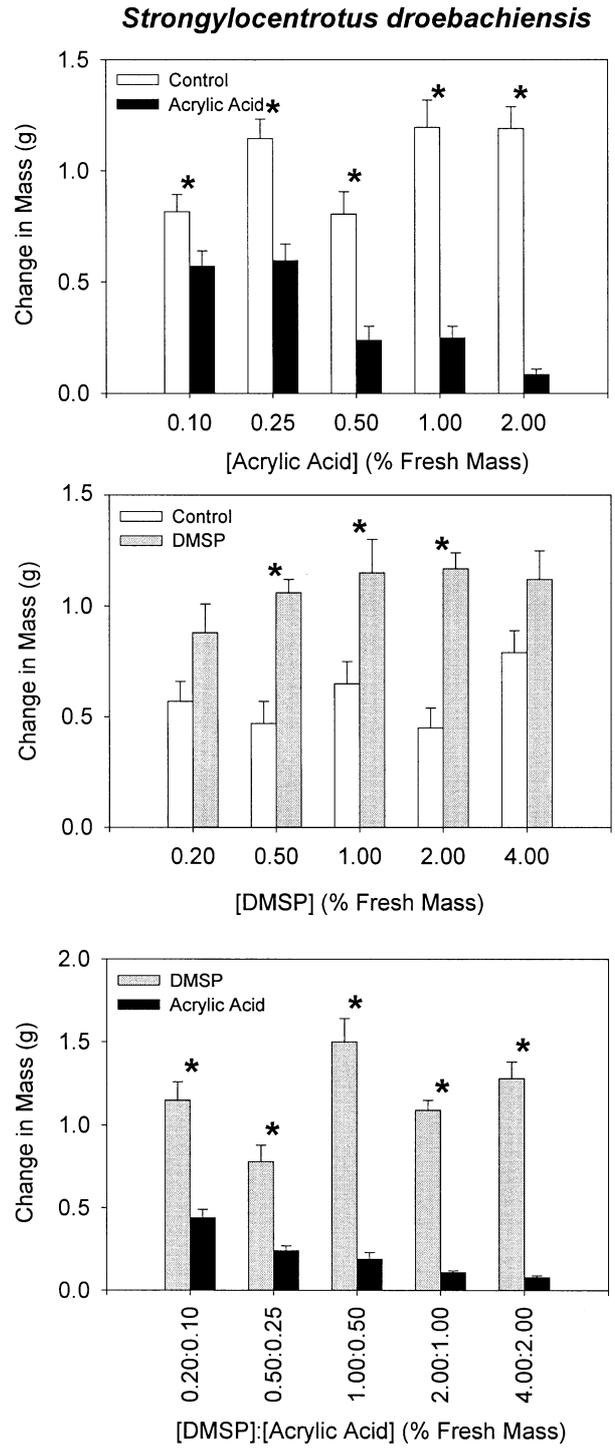
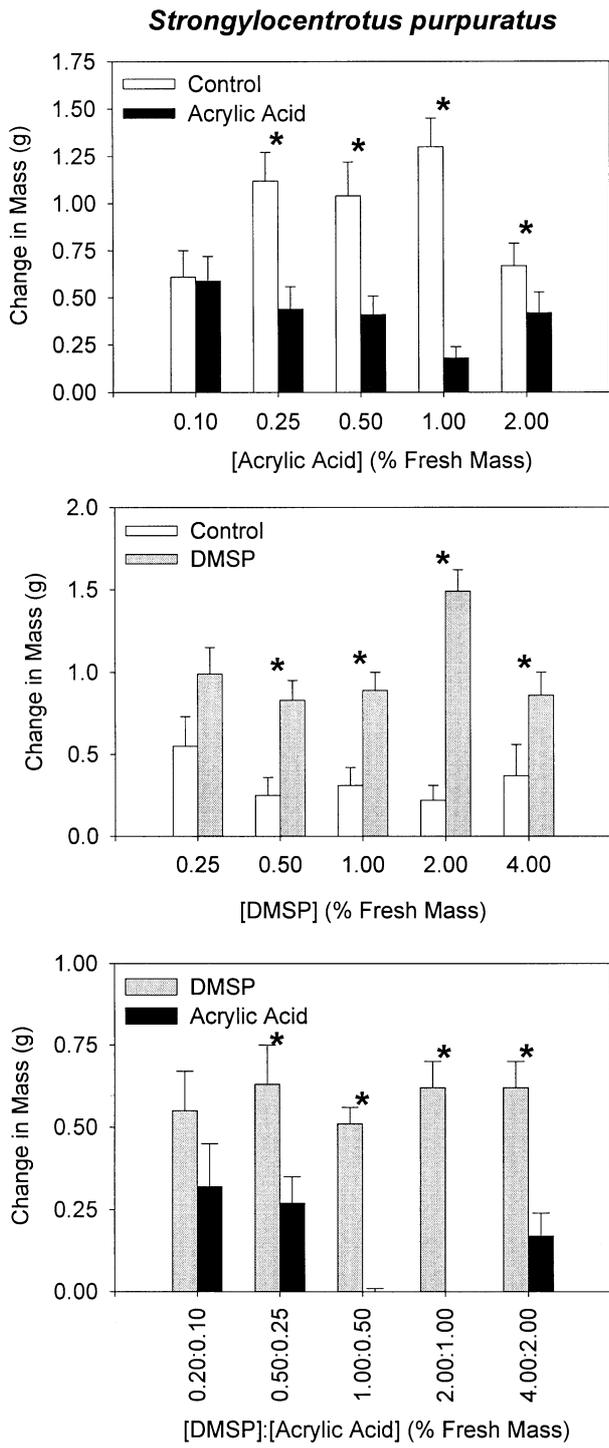


Fig. 3. *Strongylocentrotus purpuratus*. Results of feeding bioassays. Urchins were offered choices of 2 of the following diets in laboratory feeding experiments: control diets, diets containing acrylic acid, or diets containing DMSP. Concentrations of compounds added to diets are given below each set of bars. Data are means  $\pm$  1 SE. Asterisks indicate experiments in which consumption rates differed significantly between treatments ( $p < 0.05$ , 1-sample *t*-test)

Fig. 4. *Strongylocentrotus droebachiensis*. Results of feeding bioassays. Urchins were offered choices of 2 of the following diets in laboratory feeding experiments: control diets, diets containing acrylic acid, or diets containing DMSP. Concentrations of compounds added to diets are given below each set of bars. Data are means  $\pm$  1 SE. Asterisks indicate experiments in which consumption rates differed significantly between treatments ( $p < 0.05$ , 1-sample *t*-test)

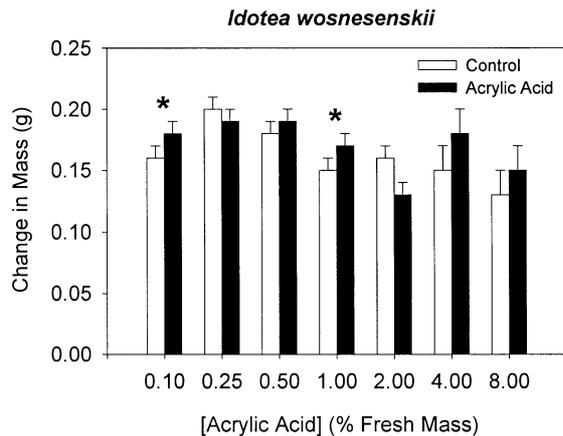


Fig. 5. *Idotea wosnesenskii*. Results of feeding bioassays. Isopods were offered choices of control diets or diets containing acrylic acid in laboratory feeding experiments. Concentrations of compounds added to diets are given below each set of bars. Data are means  $\pm$  1 SE. Asterisks indicate experiments in which consumption rates differed significantly between treatments ( $p < 0.05$ , 1-sample  $t$ -test)

ate macroalgae (White 1982, Reed 1983a, Karsten et al. 1990). Concentrations of DMSP in *Enteromorpha linza* and *Ulva californica* reached  $125 \text{ mmol kg}^{-1}$  FM or 1.8% FM (Table 1), while other species and *Polysiphonia hendryi* had somewhat lower levels. Several of the species with detectable levels of DMSP, including *E. linza*, *U. fenestrata*, and *P. hendryi*, are low-preference foods for green sea urchins (Van Alstyne unpubl. data). The algae containing high levels of DMSP are not known to contain other types of chemical defenses. Many of them had thin, delicate tissues, suggesting that physical defenses were not a cause of the low feeding preferences. Many of the species producing DMSP are fast-growing ephemeral species, similar to some of the glucosinolate-producing crucifers that produce activated defenses (Louda & Mole 1991).

Extracts of the DMSP-containing species *Ulva fenestrata* and *Polysiphonia hendryi* also contained highly active DMSP lyase (Table 2), with DMSP cleavage rates approaching  $300 \text{ mmol kg}^{-1} \text{ FM min}^{-1}$ . These rates were probably underestimates: we only measured soluble DMSP lyase activity, although most algal DMSP lyase enzymes appear to be at least partially membrane-bound (Steinke et al. 1998); we did not optimize the assay for salt, pH, or other chemical requirements (Steinke et al. 1996, 1998); and we used only 10 mM DMSP in our assays, while internal pools may reach 100 mM or more.

Our DMSP lyase measurements and tissue-crushing experiments provide indirect evidence that herbivore grazing may cause damage to the tissues that could result in the rapid conversion of DMSP to DMS and

acrylate or acrylic acid. When *Ulva californica* or *Polysiphonia hendryi* tissues were crushed, DMSP tissue concentrations decreased (Table 2). We also noted a strong odor of DMS when *P. hendryi* was crushed. Macroalgal DMSP lyase enzymes are probably active at both physiological (acid) or seawater (neutral/alkaline) pH. Although we found an acid pH optimum for DMSP lyase in crude extracts of *U. fenestrata*, de Souza et al. (1996) found a pH optimum of 8 in a partially purified DMSP lyase in *U. curvata*. Cleavage of about  $100 \text{ mmol kg}^{-1}$  FM DMSP during tissue damage by feeding could therefore potentially occur at  $300 \text{ mmol DMS kg}^{-1} \text{ FM min}^{-1}$ , producing local acrylic acid concentrations of up to 0.9% FM in tens of seconds or less. We found that such concentrations were highly effective feeding deterrents to *Strongylocentrotus* spp. Acrylic acid was effective against *S. droebachiensis* at concentrations as low as 0.1% FM (Fig. 4), and against *S. purpuratus* at concentrations of 0.25% FM (Fig. 3). Therefore, although further work is needed to determine whether the physical damage resulting from herbivore grazing is sufficient to cause the cleavage of DMSP, it seems likely that DMSP cleavage may occur during grazing injury, and the resultant production of concentrated acrylic acid may act as a feeding deterrent against at least some herbivores. Our data provides the strongest evidence yet that DMSP can function as a substrate for an activated defense system in marine algae.

#### Acrylic acid as the active ingredient

The very high internal concentrations of DMSP in algae, combined with high potential DMSP lyase activity, present a mechanism for generating localized, concentrated acrylic acid, which is bioactive (Sieburth 1960) and may affect gut microbes in large herbivores and higher-order predators (Sieburth 1961). Both DMSP and acrylic acid are highly water-soluble, and above pH 4.4, acrylic acid is largely deprotonated to acrylate. If these compounds become diluted in seawater, diluted acrylate at low (micromolar) concentrations is non-inhibitory and a good bacterial substrate (Slezak et al. 1994). Therefore, the key to the deterrence hypothesis is that concentrated acrylic acid or acrylate is produced locally at the site of tissue injury. Acrylic acid is not very toxic (Rydzynski 1997); rather, its deterrent properties may be due to reactivity, acidity, or unpleasant taste, features common to other concentrated, volatile organic acids such as formic acid, widely used for defense among insects (Bennett et al. 1996, Rossini et al. 1997).

Acidity is also used by other macroalgae for defense, such as the sulfuric acid producer *Desmarestia munda* (Pelletreau 1999). However, we believe that acidity

alone is not the mechanism responsible, since the DMSP-Cl used in the bioassays is also highly acidic, yet this compound was in contrast a feeding attractant at concentrations of 0.5 to 4% FM for *Strongylocentrotus purpuratus* (Fig. 3) and at 0.5 to 2.0% FM for *S. droebachiensis* (Fig. 4). Other studies have also found positive chemosensory responses to DMSP-Cl by bacteria (Zimmer-Faust et al. 1996), protozoa (Hauser et al. 1975), and fishes (Nakajima et al. 1990), although sub-micromolar concentrations may inhibit feeding by some protozoa (S. Strom pers. comm.).

Although 2 species of sea urchins were deterred by the presence of acrylic acid in our laboratory bioassays, the addition of acrylic acid to foods had no impact on feeding by the isopod *Idotea wosnesenskii*, even at concentrations that were 4 to 8 times greater than the maximum amount that would be found in most macroalgae (Fig. 5). It is not unusual for algal secondary metabolites to have different effects on different herbivores (Hay et al. 1987, 1988a,b, Paul et al. 1987). Mesograzers, herbivores that have limited mobility and are very small relative to the plants they consume, have been hypothesized to be mostly unaffected by their host plants' defenses, whereas larger grazers are expected to be more susceptible to the effects of these compounds (Hay et al. 1989, 1990a,b, Duffy & Hay 1994). Many tropical species of mesograzers have been shown to be unaffected by compounds that are deterrent towards larger, more mobile grazers such as fishes and urchins. Although *I. wosnesenskii* is larger, more mobile, and more of a generalist than many of the mesograzers species previously studied, it is smaller than many generalist herbivores such as urchins and fishes, which are typically strongly deterred by algal secondary metabolites. It is also possible that differences in feeding mechanisms among herbivores may alter the conversion of DMSP to acrylic acid: the amount of mechanical grinding, or the presence of enzymes which might denature or inactivate DMSP lyase.

#### Activated chemical defense systems in tropical and temperate macroalgae

The DMSP-activated defense system shares some similarities to the activated production of halimedatrial in the tropical Pacific green algae *Halimeda* spp. (Paul & Van Alstyne 1992), in which a less potent diterpenoid metabolite, halimedatetraacetate, is rapidly converted to a more potent feeding deterrent, halimedatrial, when tissues are crushed. In both systems, the conversion from the precursor to the product happens extremely rapidly, usually on the scale of seconds, and the products of the reaction are significantly more deterrent towards grazers than the compounds stored

in the algae. However, in the temperate system the precursor is a feeding attractant, while in the tropical system the precursor is also a feeding deterrent, but a less effective one than the product halimedatrial.

One advantage of producing an herbivore-activated defense system is that molecules that are biologically active, presumably towards both the alga and grazers, need not be stored in the algal thallus. In activated defense systems, the precursor molecules and the enzymes are likely to be compartmentalized (Paul & Van Alstyne 1992). The precursors and enzymes would only react upon physical damage to the tissue such as crushing or maceration by grazers. In an activated defense system, the active product is only produced in tissues that are removed by grazers; the living tissue left behind by the grazer is unaffected. Such tactics are also widely used by terrestrial vascular plants employing glucosinolate hydrolysis (Grob & Matile 1979, Lüthy & Matile 1984, Chew 1988, Louda & Mole 1991, Newman et al. 1992) or cyanogenesis (Poulton 1990, Wajant et al. 1994).

Because activated defenses allow algae to produce a highly bioactive product very rapidly while avoiding autotoxicity, they have clear advantages in tropical systems, where the grazers are typically large, rapidly-moving, visually foraging fishes that travel in schools. The advantages of activated defenses are less obvious in cold-water temperate systems, where the grazers are more likely to be slow-moving sea urchins, molluscs, or arthropods that are not visual foragers. Visual foragers would quickly learn to avoid algae containing activated defenses once they discovered the algae were distasteful; non-visual foragers would presumably have to sample an alga each time it encountered it to determine if the alga was a distasteful food unless the grazer was capable of determining the quality of the food from surface characteristics of the alga. The behavioral mechanisms by which temperate grazers chose their food in the field are not well known. Littorinid snails climb up on algae to forage during low tide and return to the substrate before the tide comes in (Van Alstyne 1988). Snails tend to leave lower-preference foods more rapidly than they leave higher-preference foods. Consuming a small portion of an alga with an activated defense may provide a grazer with a signal to move off the alga in search of a better quality food.

The main DMSP-containing macroalgal genera *Enteromorpha*, *Ulva*, and *Polysiphonia* are opportunistic species of the temperate eulittoral or upper sublittoral zones. They can tolerate desiccation and wide ranges of salinity, and rapidly colonize disturbed areas. Nutrification of coasts in northern Europe is leading to 'green tides' of *Enteromorpha* and *Ulva*, which are becoming major nuisances (Fletcher et al. 1990, Valeila et al. 1997). These foliose taxa are minimally protected phys-

ically against grazers, and tropical species are sometimes used as control (preferred) foods in feeding studies (e.g., Paul & Van Alstyne 1987, 1988, Van Alstyne & Paul 1990), under the assumption they are not chemically defended. Our results suggest that some of the green macroalgae may in fact possess activated chemical defenses against at least some herbivores, which may possibly contribute to their widespread success.

However, deterrence ability due to DMSP cleavage probably varies geographically, and even within a population. DMSP concentrations in tropical genera are typically low, ranging from 0.5 to 1.4 mmol kg<sup>-1</sup> FM (Dacey et al. 1994, Karsten et al. 1994), a trend which parallels levels of brown algal phlorotannins, typically found in higher concentrations in temperate Pacific than tropical Pacific macroalgae (Steinberg 1986, Van Alstyne & Paul 1990). In tropical fishes, acrylic acid appeared not to affect gut bacteria (Dacey et al. 1994). There is also great variation in DMSP lyase activity among algae that contain high concentrations of DMSP, even within a species (Steinke et al. 1996). Little is known about the genetics and biosynthesis of DMSP and DMSP lyase. In *Trifolium repens*, the genes for the cyanogenic precursors and the  $\beta$ -glucosidase system reside on different alleles (Nass 1972), and polymorphisms in both alleles lead to differing defense abilities (Compton & Jones 1985, Hughes 1991). We hypothesize that the DMSP cleavage reaction will only function for defense when algae possess both high concentrations of DMSP and high DMSP lyase activities, and further work is clearly needed to determine the contribution of this reaction to the ecological success of macroalgae from diverse environments.

#### Activated chemical defense systems in macroalgae and unicellular phytoplankton

The DMSP cleavage reaction has also been hypothesized to function in deterrence in microalgae, thus presenting the unusual opportunity to compare and contrast the ecological function of a conserved chemical reaction between micro- and macroorganisms. Although DMSP occurs primarily in the green macroalgae, especially the Ulvophyceae, it is rare in unicellular Chlorophyta; rather, it is found primarily among the prymnesiophytes and dinoflagellates (Keller et al. 1989), including many notorious bloom-forming taxa (e.g., species of *Emiliana*, *Phaeocystis* and *Alexandrium*; Wolfe 2000).

DMSP lyase in phytoplankton appears to be constitutive, but the cleavage reaction is activated by physical stress or cell disruption (Wolfe & Steinke 1996). Differences in DMSP lyase concentrations in unicellular algae have been used to test the function-

ing of DMSP as part of a feeding deterrence system (Wolfe & Steinke 1996, Wolfe et al. 1997). Some omnivorous protozoa (e.g., the dinoflagellate *Oxyrrhis marina*) eat microalgae polymorphic in the DMSP lyase enzyme, and DMS production following grazing suggests that the reaction occurs inside protozoan food vacuoles after ingestion, when the algae are degraded enzymatically. However, there is no evidence to date that these grazers suffer any harm from the reaction, although they may prefer prey with low-activity forms of the DMSP lyase enzyme. Other dinoflagellates are much more selective than *O. marina* and feed poorly or not at all on high-activity DMSP lyase prey, but it is still unclear whether this reaction plays a role in food selection (S. Strom & G. Wolfe unpubl. data).

Obviously, the sacrifice of tissue by a macrophyte to deliver a deterrent signal may be a successful strategy to prevent loss of the whole organism, while in a microbe, deterrence is more likely to be all-or-nothing. Among unicellular organisms growing asexually, the reaction may act as a 'suicide' defense which might benefit the clonal prey population. An interesting intermediate case is the colonial prymnesiophyte *Phaeocystis* sp., which, though unicellular, forms multicelled macroscopic colonies within a mucilaginous spherical shell. *Phaeocystis* sp. has very active DMSP lyase and produces DMS actively during growth (Stefels & van Boekel 1993). The result is that colonies contain concentrated acrylate (Noordkamp et al. 1998), which may function in defense against grazers or bacteria (Liss et al. 1994).

Macroalgae have several advantages over microalgae as model organisms for examining the role of DMSP in ecological systems. The grazers are larger and bioassays can be conducted with diets that differ only in acrylic acid levels. Macroalgae can also be manipulated in the field more easily than microalgae, making it easier to study the effects of environmental change on the system in a more realistic setting. We suggest this reaction presents an opportunity to compare chemical defense across size scales and at different levels of cellular organization, a relatively unexplored area in the comparative ecology of micro- and macroorganisms (Andrews 1991).

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