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The Effects of Salinity on Dimethylsulfoniopropionate Production in the Green Alga *Ulva fenestrata* Postels et Ruprecht (Chlorophyta)¹

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Dimethylsulfoniopropionate (DMSP) in marine algae has been hypothesized to serve as a compatible solute which functions in osmotic acclimation or cryoprotection. However, many macroalgae that produce large quantities of DMSP live in habitats where they are unlikely to experience large fluctuations in salinities or freezing temperatures. We hypothesized that DMSP has other functions in these algae and that they should not show large changes in DMSP concentrations in response to salinity changes. We tested this hypothesis by placing 1.5 cm² diameter disks of the chlorophyte *Ulva fenestrata* in artificial seawater (ASW) at salinities from 10 ‰ ASW to 300 ‰ ASW. Over the next 24 h, DMSP concentrations tended to be lower in the algae in the higher salinity media. After 4 weeks, the final DMSP concentrations tended to be highest in the algae grown at the highest salinities, but the mean DMSP concentrations were only 23 % higher or 12 % lower in algae grown in the high and low salinity media, respectively, relative to algae in 100 ‰ ASW. This suggests that osmotic acclimation is not a primary function of DMSP in *U. fenestrata*. Disks acclimated in 25 ‰ ASW, 100 ‰ ASW, or 200 ‰ ASW then transferred to a higher or lower salinity did not generate measurable amounts of dimethylsulfide (DMS), demonstrating that *U. fenestrata* is not using DMSP cleavage as a short-term mechanism for reducing internal DMSP stores, as occurs in some phytoplankton. Survival, as measured by the absence of bleaching, was highest in intermediate to high salinities. Growth was highest in low to intermediate salinities and reproduction only occurred in intermediate salinities. These results suggest that *U. fenestrata* can tolerate salinity changes, but uses metabolites other than DMSP for osmotic acclimation. Based on the results of this and previous studies, we propose that DMSP has other functions in *U. fenestrata* such as acting as an herbivore deterrent or antioxidant.

Keywords: DMS; DMSP; osmotic acclimation; salinity; *Ulva fenestrata*.

Abbreviations: ASW, artificial seawater; DMS, dimethylsulfide; DMSP, dimethylsulfoniopropionate; SNK, Student-Newman-Keuls.

Introduction

The tertiary sulphonium compound DMSP is a metabolite that is found in many groups of algae including the chlorophytes, rhodophytes, prymnesiophytes, dinophytes, diatoms, chrysophytes, and prasinophytes (Malin and Kirst 1997). DMSP is important to global sulfur cycling and climate because it is a precursor to DMS. DMS and acrylic acid are produced when DMSP is enzymatically cleaved by DMSP lyase (Cantoni and Anderson 1956). Once released from algal cells, DMS reacts to form sulfur dioxides, which can serve as cloud condensation nuclei and affect local climate (Charlson *et al.* 1987). DMS produced by algae accounts for about half the biogenic sulfur released into the atmosphere annually (Andreae 1986, Bates *et al.* 1992).

Although DMSP's function in many algal groups has not been experimentally determined, several

roles have been suggested. DMSP has been hypothesized to be an osmogulator (Reed 1983a,b, Edwards *et al.* 1987, 1988, Kirst 1996), a cryoprotectant (Karsten *et al.* 1996), a precursor to an antiherbivore chemical defense (Wolfe and Steinke 1996, Wolfe *et al.* 1997, Van Alstyne *et al.* 2001, Van Alstyne and Houser 2003), an antioxidant (Sunda *et al.* 2002), a precursor to a potentially allelopathic compound, acrylic acid (Sieburth 1960), and as part of a system to expel excess sulfur and energy (Stefels 2000). The widespread distribution of DMSP suggests that it has a common function in these algae or that it has multiple functions.

One of the more prevalent suggestions for DMSP's function is that it serves as a compatible solute, allowing algae to cope with osmotic changes. Most of the evidence for DMSP's role as an osmoregulator in algae comes from studies of the high intertidal alga *Enteromorpha intestinalis* (L.) Nees (Edwards *et al.* 1987, 1988), the intertidal epiphyte *Polysiphonia lanosa* (L.) Tandy (Reed 1983a) and Antarctic

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species, which may use DMSP for cryoprotection (Karsten *et al.* 1992, 1996). *E. intestinalis* and *P. lanosa* live in intertidal habitats where they will experience large short-term fluctuations in salinities with changes in tidal levels. These species are also common in estuarine environments where salinities may fluctuate over longer time periods. However, many macroalgae produce comparable quantities of DMSP but live in environments where salinity fluctuations are not as extreme. In these species, we would expect that DMSP has a different function in the alga's physiology or ecology and that DMSP concentrations will not respond to salinity changes as they do in high intertidal species.

The green alga *Ulva fenestrata* Postels *et* Ruprecht belongs to the same family as *Enteromorpha intestinalis* and the two species often co-occur on north-eastern Pacific coasts; however, they differ in their vertical distribution. *E. intestinalis* is generally found in high to mid intertidal habitats and is common in freshwater seeps whereas *U. fenestrata* typically occurs in the mid to low intertidal zone (O'Clair and Lindstrom 2000), where salinity fluctuations are less extreme. In Washington, USA, both species produce DMSP; however, DMSP concentrations are about 3 times higher in *U. fenestrata* than in *E. intestinalis* (Van Alstyne *et al.* 2001). Herbivores that avoid consuming *U. fenestrata* and a related green alga *Enteromorpha linza* (L.) J. Ag. also avoid agar-based foods that contain DMS and acrylic acid (Van Alstyne *et al.* 2001, Van Alstyne and Houser 2003). Thus, in *U. fenestrata*, the primary function of DMSP may be herbivore deterrence rather than osmotic acclimation.

Because *U. fenestrata* is found in environments where salinity fluctuations tend to be low, we hypothesize that DMSP concentrations in this alga will not be strongly affected by salinity changes. To test this hypothesis, we conducted both short-term and long-term experiments in the laboratory to determine the effects of salinity on DMSP concentrations. We also determined whether DMS would be produced if *U. fenestrata* were subjected to rapid salinity fluctuations.

Material and Methods

Culture methods

Ulva fenestrata was collected during low tides from the beach in front of the Shannon Point Marine Center in Anacortes, WA, USA. Before being used in experiments, the algae were maintained in an incubator (16:8 light:dark, 12 °C, 43 $\mu\text{mol photons m}^{-2}\text{s}^{-1}$) in 100% ASW that was based on the ESAW recipe of Harrison *et al.* (1980) to which the following modifications were made: (1) molar equivalents of MnCl_2 and CoCl_2 were used in place of MnSO_4 and CoSO_4 respectively, (2) equimolar amounts of KF were used in place of NaF, and (3) nutrient enrichment stock # 3

($\text{Na}_2\text{SiO}_2 \cdot 9\text{H}_2\text{O}$) was omitted. Six additional media were prepared by adjusting the amounts of the salts used. To ensure that any differences in responses were due to salinity differences rather than differences in nutrient concentrations, we added the same amount of nutrient enrichment stock solutions (trace metals and vitamins) to each medium, regardless of the salinity. The salinities of the experimental media were 5 PSU (10% ASW), 10 PSU (25% ASW), 19 PSU (50% ASW), 35 PSU (100% ASW), 53 PSU (150% ASW), 65 PSU (200% ASW), and 95 PSU (300% ASW).

Short-term acclimation experiments

To determine if salinity changes could cause short-term DMSP changes, we cut 1.5 cm² diameter disks of *U. fenestrata* and placed them in 8 cm bowls containing 30 ml of medium. Ten replicate bowls were prepared for each of the seven media types and each contained 10 to 14 disks of *U. fenestrata*. One disk of *U. fenestrata* was removed from each of the bowls after 0, 3, 6, 12, and 24 h and weighed to obtain a fresh mass. The disks were dried overnight at 60 °C then reweighed to obtain a dry mass and placed in either 30 or 50 ml gas-tight vials that each contained 4 ml of 4N NaOH. Previous studies have shown that drying *U. fenestrata* (K. Van Alstyne unpubl. data) and other green algae (Karsten *et al.* 1994) can increase the amount of DMSP extracted. The vials were stored at 4 °C in the dark and the concentration of DMS in the headspace was measured on the next day by direct injection onto an SRI gas chromatograph (Chromasil 330 column, flame-photometric detector) as described in Van Alstyne *et al.* (2001). Vials containing known amounts of DMSP were used to generate a standard curve. After checking the data for equality of covariance matrices with a Box's test and equality of error variances with a Levene's test, a repeated measures ANOVA (SPSS 10.0) was conducted with salinity as a fixed effect. Individual bowls were treated as experimental units that were sampled over time. A SNK *post hoc* test was then used to test for differences in DMSP concentrations across salinities.

One mechanism that algae could use to rapidly reduce internal concentrations of DMSP is to cleave the molecule into DMS and acrylic acid (Vairavamurthy *et al.* 1985). If this mechanism is used to rapidly lower DMSP concentrations when algae experience reduced salinities, then DMS concentrations should increase as algae are moved to media with lower salinities but not higher salinities. To test this hypothesis, we placed 10 disks of *U. fenestrata* in 25%, 100%, and 200% ASW (10 replicate bowls for each treatment). The algae were allowed to acclimate to these salinities for two weeks. We then removed three disks from each bowl and placed each into a 25 ml gas-tight flask that contained either 5 ml of

25%, 100%, or 200% ASW. After 3 h, we measured the DMS concentration in the headspace of the flask as described above. Flasks containing known amounts of DMS were used as standards.

Bacteria associated with *U. fenestrata* could have taken up the DMS produced during the acclimation experiments, making any DMS production resulting from osmoacclimation difficult to detect. To determine if *U. fenestrata* and its associated bacterial flora caused significant losses of DMS, we incubated 40 gas-tight 30 ml vials containing 100% ASW and 0, 5, 10, or 20 μg of DMS with and without disks of *U. fenestrata* ($N = 5$). After 3 h, the DMS concentrations in the vials' headspaces were measured with gas chromatography. An analysis of covariance was used to test for a difference in vials with and without *U. fenestrata* after the data were checked for homogeneity of variances with a Levene's test (SPSS 10.0).

Long-term acclimation experiments

To determine if salinity changes cause longer-term changes in the growth, survivorship, reproduction, and DMSP concentrations of *U. fenestrata*, we placed 10 to 14 disks of *U. fenestrata* in 8 cm bowls as described above. The medium was replaced twice a week. At weekly intervals for 4 weeks, we determined the number of disks in each bowl that were bleached and the number of bowls containing spores. A disk was considered bleached if more than half its surface area was noticeably white to very light green in color. Although spore release caused localized bleaching in some disks, it was never greater than 50% of the surface area; therefore, disks releasing spores were never tallied as bleached. At each sampling interval, we randomly selected one disk from each bowl, photographed it with a digital camera, and measured its surface area on a personal computer with ImageJ software (NIH: <http://rsb.info.nih.gov/ij/index.html>). The disk was then weighed to obtain a fresh mass, dried, then reweighed to obtain a dry mass and placed in a gas-tight vial with 4N NaOH. Headspace DMS concentrations were measured as described above.

The DMSP concentration, dry mass, and fresh mass data were analyzed with a repeated measures multivariate ANOVA that was similar to the analysis described for the short-term experiments. Variances were significantly different among treatment groups (Box's Test, $p < 0.05$) and tended to increase over time. Therefore, the Pillai-Bartlett V statistic was used because it is robust to moderate heteroscedasticity when the design is balanced or nearly so (Johnson and Field 1993). The surface area data were analyzed with a two-way ANOVA with salinity and time as fixed factors. A Levene's test for homoscedasticity (SPSS 10.0) was significant ($p < 0.05$) and we were unable to transform the data so that the variances were equal. However, ANOVA is robust to het-

eroscedasticity for relatively large balanced data sets (Underwood 1997).

Results

In the short-term experiment, DMSP concentrations decreased significantly among *Ulva fenestrata* disks over time (Fig. 1; repeated measures ANOVA: Pillai-Bartlett $V = 0.855$, $F = 82.233$, $df = 4$, error $df = 56$, $p < 0.001$) and among disks grown in different salinities (repeated measures ANOVA: salinity effect $F = 20.091$, $df = 6$, error $df = 59$, $p < 0.001$). DMSP concen-

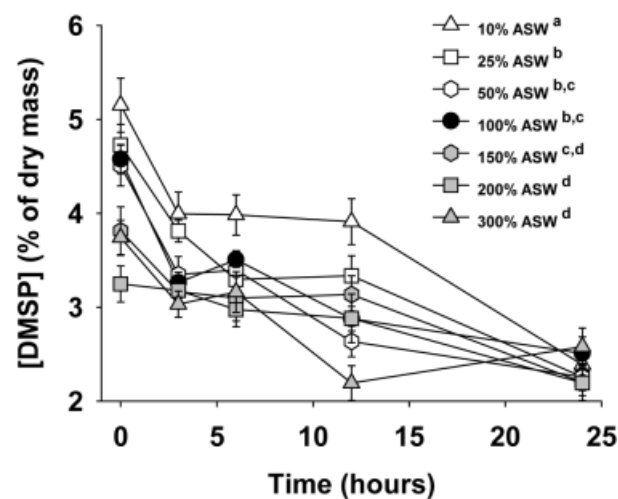


Fig. 1. Short-term changes in DMSP concentrations in *Ulva fenestrata* maintained in artificial seawater (ASW) at seven different salinities. Data are means \pm 1 SE ($N = 10$). DMSP concentrations in disks grown in media with the same subscripts are not significantly different (SNK *post hoc* test, $\alpha = 0.05$).

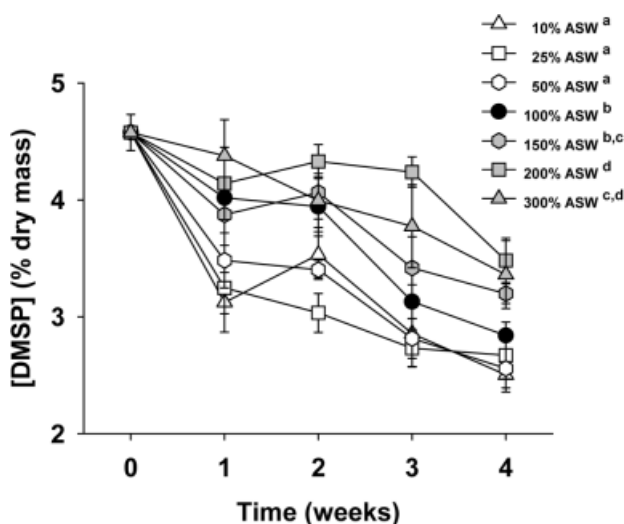


Fig. 2. Long-term changes in DMSP concentrations in *Ulva fenestrata* maintained in artificial seawater (ASW) at seven different salinities. Data are means \pm 1 SE ($N = 10$). DMSP concentrations in disks grown in media with the same subscripts are not significantly different (SNK *post hoc* test, $\alpha = 0.05$).

trations tended to decrease with increasing salinity (Fig. 1). Thus, the directions of the DMSP changes were the opposite of those predicted.

U. fenestrata showed no evidence of cleaving DMSP into DMS and acrylic acid to reduce internal DMSP stores over short periods. None of the algae that were acclimated to 25% ASW, 100% ASW, and 200% ASW and transferred to the same or another medium produced measurable amounts of DMS. The minimum amount of DMS that would be detectable using our methods was 2.5 μg or approximately 2.25% of the amount of DMS that could be produced by the alga. The lack of an acclimation effect did not appear to be caused by bacteria associated with *U. fenestrata*. When algae were incubated in the presence of DMS, there was a significant DMS loss, relative to controls without algae (analysis of covariance: algal effect $F = 194.00$, $p < 0.001$, $df = 1$, error $df = 37$), suggesting that bacteria associated with

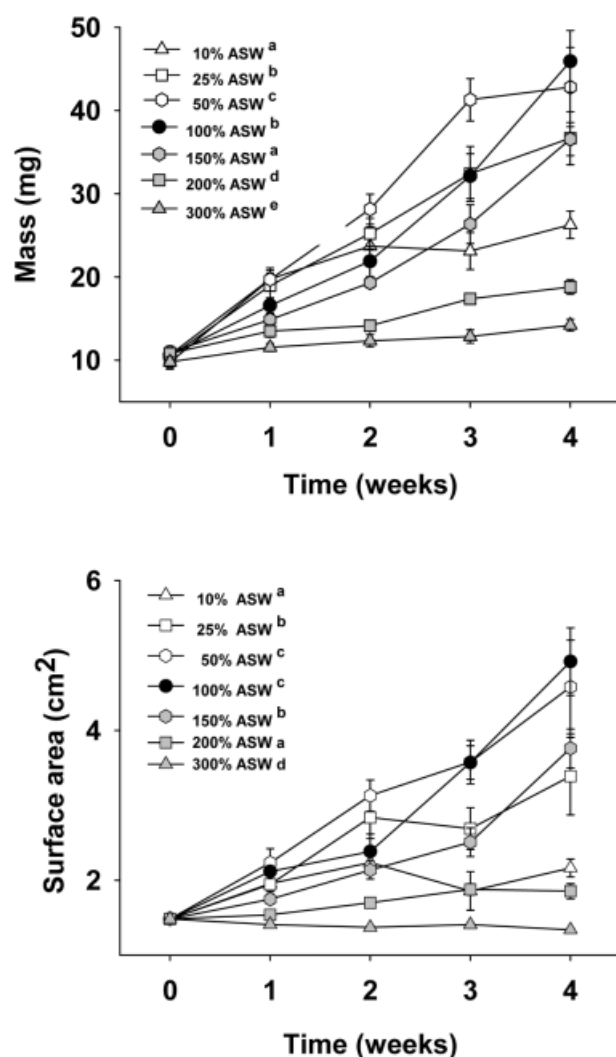


Fig. 3. Fresh masses and surface areas of *Ulva fenestrata* maintained in artificial seawater (ASW) at seven different salinities. Data are means \pm 1 SE ($N = 10$). Masses or surface areas in disks grown in media with the same subscripts are not significantly different (SNK *post hoc* test, $\alpha = 0.05$).

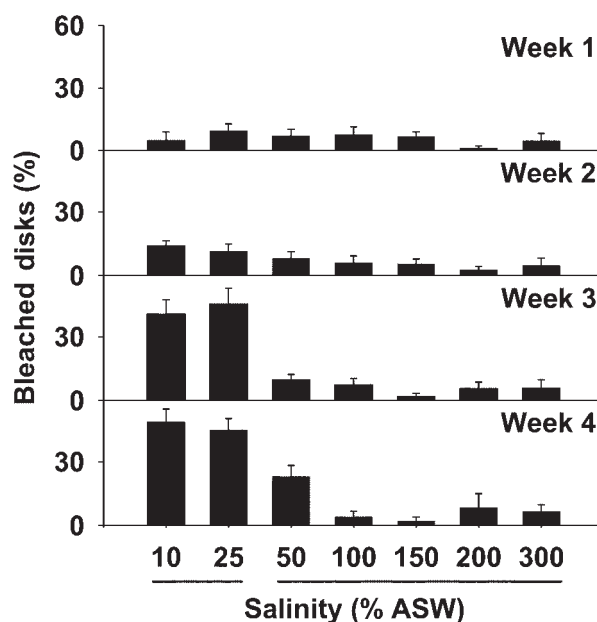


Fig. 4. Bleaching of *Ulva fenestrata* disks maintained in artificial seawater (ASW) at seven different salinities over a 4-week period. Data are means of percentages of disks in each dish that were visibly bleached over half their surface area (\pm 1 SE). Lines below the x-axis indicate treatments in which the percentages of bleached disks are not significantly different (SNK *post hoc* test: $p > 0.05$).

U. fenestrata may be metabolizing some DMS. The mean amounts of DMS (\pm 1 SE, $N = 5$) in the vials containing *U. fenestrata* were $46.1 \pm 24.2\%$, $44.7 \pm 8.6\%$ and $53.8 \pm 4.3\%$ lower than in vials lacking algae when 5, 10, and 20 μg , respectively, of DMS were added. However, even when only 5 μg was added to the vials, the DMS concentration after 3 h was still large enough to be measurable with gas chromatography, suggesting the lack of measurable DMS in the acclimation experiment was due to a lack of DMS production.

In the long-term acclimation experiments, salinity had a significant effect on algal growth and DMSP production (Figs 2, 3; repeated measures MANOVA: Pillai-Bartlett $V = 1.713$, $F = 13.981$, $df = 18$, error $df = 189$, $p < 0.001$). At the end of four weeks, mean DMSP concentrations were positively correlated with salinity (Pearson correlation coefficient = 0.911, $P = 0.004$). However, the lowest mean DMSP concentrations occurred in algae from 10% ASW and were only about 12% lower than DMSP concentrations than in algae grown in 100% ASW. Likewise, the highest increases occurred in the algae grown in 200% ASW medium and were only about 23% higher than concentrations in the 100% ASW controls.

Bleaching rates differed among algae grown in different salinities (Fig. 4; repeated measures ANOVA: $F = 14.434$, $df = 6$, error $df = 63$, $p < 0.001$) and increased over time (repeated measures ANOVA: Pillai-Bartlett $V = 0.489$, $F = 19.419$, $df = 3$, error $df = 61$,

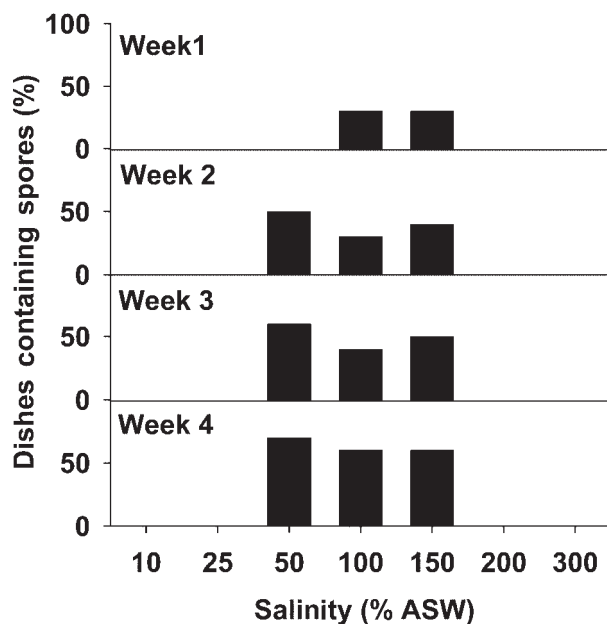


Fig. 5. Spore production by *Ulva fenestrata* disks maintained in artificial seawater (ASW) at seven different salinities over a 4-week period. Data are percentages of dishes that contained visible spores.

$p < 0.001$). Bleaching was significantly higher in the 10% ASW and 25% ASW media than in all others (SNK test: $p < 0.05$) and increased rapidly between weeks 2 and 3 (Fig. 4). Growth, measured as both surface area and fresh mass, was highest in the intermediate salinities (Fig. 3). Growth rates were significantly lower in the 10% ASW, 200% ASW and 300% ASW media than in the 50% and 100% ASW media (SNK test: $p < 0.05$). Reproduction was limited to algae in intermediate salinities (Fig. 5). Only algae in 50% ASW, 100% ASW, and 150% SW media produced visible spores and the percentage of dishes in which spores were produced were comparable across the three media. Spores were detectable in some dishes within a week and continued to be produced until the end of the experiment.

Discussion

DMSP concentrations in *Ulva fenestrata* were positively correlated with increased salinity in the long-term acclimation experiments but showed the opposite pattern in the short-term experiments (Figs 1, 2). Because the magnitude of the effect was small relative to the salinity change in the long-term experiment, we question whether DMSP has a significant role in osmotic acclimation in this alga. In previous studies with *Enteromorpha intestinalis*, DMSP concentrations were over twice as high in algae grown in hypersaline media relative to control media (Edwards *et al.* 1988). In studies with polar macrophytes, internal DMSP concentrations increased by as much

as two-fold within 72 h of salinity increases (Karsten *et al.* 1992). However DMSP concentrations in *U. fenestrata* only increased by 23% in the hypersaline medium relative to the control medium in the long-term experiments (Fig. 2). The lower DMSP concentrations in the algae grown in 10% and 25% ASW, particularly during the 3rd and 4th weeks, may have been due to some of the algae being very stressed. Several of the algae in each of these samples were pale green in color over enough of their surface areas to be categorized as bleached.

The lack of a measurable DMS release when acclimated algae were transferred from a high to a low salinity medium provides further evidence that short-term osmotic acclimation is not an important function of DMSP in *U. fenestrata*. Although some osmolytes can remain within the apoplast or cell wall compartment and be difficult to detect, this is unlikely to happen with DMS because of its high volatility. In the planktonic prymnesiophyte *Hymenomonas carterae* (Braarud and Fagerland) Manton *et Peterfi*, a species that is thought to use DMSP for osmotic acclimation, DMSP concentrations increased when algae were acclimated to higher salinities (Vairavamurthy *et al.* 1985). When the cells were then transferred to a lower salinity medium, DMS was released to lower internal stores of DMSP. When we transferred *U. fenestrata* to a lower salinity medium, no such release occurred.

In both the long-term and short-term experiments, DMSP concentrations decreased over time, independently of the salinity treatments (Figs 1, 2). These decreases may have resulted from moving field-collected algae into incubators with lower light levels. DMSP concentrations in macroalgae can be affected by light (Karsten *et al.* 1991, 1992). In *Ulva rigida* C. Ag., DMSP concentrations can be 60% lower in algae maintained at low light intensities relative to those grown in high intensities (Karsten *et al.* 1991). This range is comparable to the decrease we observed in our experiments.

In its natural environment, some *U. fenestrata* individuals may experience gradual or abrupt changes in salinity. Our data show that it can persist in hyposaline or hypersaline waters for up to a month with reproduction occurring within a more limited range of salt concentrations. Consequently, *U. fenestrata* must have a physiological mechanism for coping with hyposaline or hypersaline conditions. However, our results suggest that DMSP is not the major metabolite used for osmoacclimation. Many other osmolytes are known from marine plants, including alcohols, simple sugars, amino acids, and methylated compounds such as methylamines and betaines (summarized in Edwards *et al.* 1987). In *Enteromorpha intestinalis*, proline increases dramatically during both short-term (48 h) and long-term (35 days) acclimation (Edwards *et al.* 1987). Sucrose is also used to osmoregulate over short time scales, but not over longer ones in *E. in-*

intestinalis, whereas DMSP is used to adapt to long-term, but not short-term, salinity changes (Edwards *et al.* 1987). Thus, *U. fenestrata* may be using other osmolytes, such as proline and sucrose, to deal with osmotic stresses.

DMSP concentrations in *U. fenestrata* are typically three to five percent of the alga's dry mass (Van Alstyne *et al.* 2001, Van Alstyne and Houser 2003). This large investment in a single compound suggests that DMSP plays an important role in the physiology or ecology of the alga. However, the lack of a significant increase in DMSP during large changes in salinity suggests that osmotic acclimation in *U. fenestrata* is, at best, a minor function of DMSP. There is more support for other roles for DMSP in *U. fenestrata*, particularly herbivore deterrence or protection from oxidation. In multiple choice feeding preference assays, *U. fenestrata* was among the least preferred foods of several herbivores including the green sea urchin *Strongylocentrotus droebachiensis* [Müller] (Van Alstyne and Houser 2003), the purple sea urchin *Strongylocentrotus purpuratus* [Stimpson] (Kirby and Van Alstyne unpubl. data), and the gastropod snail *Lacuna vincta* [Montagu] (Kirby and Van Alstyne unpubl. data). Two of the products of DMSP cleavage, DMS and acrylic acid, have been shown to deter feeding by *S. droebachiensis*, and acrylic acid is also a feeding inhibitor to *S. purpuratus* (Van Alstyne *et al.* 2001, Van Alstyne and Houser 2003). It should be noted that many herbivores prefer to eat *U. fenestrata* (Kirby and Van Alstyne unpubl. data) and that feeding by one of these herbivores, the isopod *Idotea wosnesenskii* [Brandt], is not affected by acrylic acid (Van Alstyne *et al.* 2001). Thus, DMSP only seems to be an effective defense against specific herbivores. DMSP concentrations are also higher in *U. fenestrata* (Van Alstyne and Nelson in prep.) and other

macroalgae grown in higher light levels (Karsten *et al.* 1991, 1992), suggesting that it could be functioning as a sunscreen, possibly by scavenging hydroxyl radicals generated during photosynthesis (Sunda *et al.* 2002).

DMSP occurs in many diverse groups of algae and is likely to have multiple physiological and ecological functions. In many marine macrophytes, DMSP functions in osmotic acclimation (Kirst 1996); however, our data demonstrate that this is not true for all DMSP-producing algae. Evidence from our experiments and others suggest that in *U. fenestrata* alternative functions of DMSP may include being a precursor for the production of DMS and acrylic acid, which deter feeding by some herbivores (Van Alstyne *et al.* 2001, Van Alstyne and Houser 2003) or providing protection from photooxidation (Sunda *et al.* 2002). In other algae, such as *Enteromorpha intestinalis*, which is commonly found in freshwater seeps, osmotic acclimation is likely to be the primary function. In yet other species, such as ice algae, the primary function may be cryoprotection (Karsten *et al.* 1990, 1996). The combined results of these studies suggest that even closely related species may use individual compounds for different functions and caution against a one-function-fits-all approach to the study of algal metabolites.

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