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ARE "GREEN TIDES" HARMFUL ALGAL BLOOMS? TOXIC PROPERTIES OF WATER-SOLUBLE EXTRACTS FROM TWO BLOOM-FORMING MACROALGAE, *ULVA FENESTRATA* AND *ULVARIA OBSCURA* (ULVOPHYCEAE)[1]

Timothy A. Nelson,[2] *Dejah S. Lee*

Department of Biology, Seattle Pacific University, Seattle, Washington 98119-1997, USA

and

Bridget C. Smith

School of Aquatic and Fishery Science, Box 355020, University of Washington, Seattle, Washington 98195-5020, USA

Toxic properties are more often associated with microalgal blooms than with macroalgal blooms, although herbivore deterrents are well known in macroalgae, and inhibition of invertebrate larval development by extracts from *Ulva* spp. has been suggested. This study was prompted from our observation that substantial discoloration of seawater occurred in small bays after mass desiccation-induced mortality of *Ulvaria obscura* (Kützing) Gayral. We examined the effects of extracts from *Ulva fenestrata* Postels et Ruprecht and *Ulvaria obscura* on *Fucus gardneri* Silva zygote development, growth of *Ulva* and *Ulvaria*, epiphytic algal accumulation, and oyster larval development. *Fucus* zygote development was inhibited by extracts from both species, although the effects of *Ulvaria* extracts were significantly greater. Epiphytic algal accumulation and the growth of *Ulva* and *Ulvaria* were inhibited by extracts from both species. Oyster larval development was arrested by the presence of extracts from each species. We conclude that extracts from both *Ulva fenestrata* and *Ulvaria obscura* have allelopathic properties. The effects are more widespread and occur at lower concentrations for extracts from *Ulvaria* than *Ulva*. These properties could alter competitive interactions by inhibiting germination or development of algae and invertebrates.

Key index words: **allelochemicals; *Crassostrea gigas*; diatom; epiphytes; *Fucus gardneri*; green tide; microalgae; Pacific oyster**

This study examines the toxic properties of two common "green tide" seaweeds from the northeastern Pacific, *Ulva fenestrata* Postels et Ruprecht and *Ulvaria obscura* (Kützing) Gayral (Nelson et al. 2003). Allelopathic properties are not usually associated with green tides (i.e. blooms of ulvoid macroalgae) or other macroalgal blooms (Valiela et al. 1997), although some bloom-forming macroalgae are chemically defended against herbivory (Paul et al. 2001). A handful of studies have shown or suggested that extracts from *Ulva* spp. inhibit larval development in barnacles, crab, and flounder (Magre 1974, Johnson 1980, Johnson and Welsh 1985). Further, extracts from *Ulva pertusa* Kjellman and other macroalgae have been shown to have inhibitory effects on dinoflagellates (Jeong et al. 2000).

Motivation to consider the toxic properties of *Ulvaria obscura* comes from three observations. First, *Ulvaria* is avoided by a suite of mesograzers (Vadas 1977, Nelson 2001). Second, at death the thallus of *Ulvaria obscura* var. *blyttii* (Areschoug) Bliding turns dark brown. This darkening has been associated with dopamine oxidase activity (reported as *Monostroma fuscum*, an outdated synonym for *Ulvaria obscura*, in Tocher and Meeuse 1966, Tocher and Craigie 1966). If immersed after death by desiccation, the water around the thallus turns tawny-orange within minutes, then reddish-brown, and finally (after 24–48 h) dark brown or black. After several days, a black precipitate forms. Third, we observed a large natural desiccation event in Armitage Bay, Blakely Island, Washington state, USA (Nelson and Lee 2001) that caused substantial discoloration of the water around *Ulvaria* specimens as the tide rose.

We tested for toxic properties of extracts from *Ulvaria* and *Ulva* on several model systems, including *Fucus gardneri* Silva zygote germination, epiphytic algal accumulation on seagrass blade segments, growth of *Ulva* and *Ulvaria* thalli, and Pacific oyster (*Crassostrea gigas* Thunberg) larval development. Overall, we addressed six hypotheses (designated H-1 to H-6) that included tests comparing the toxicity of *Ulva* versus *Ulvaria* extracts, determination of dose–response relationships, and an examination of the effect of algal desiccation time before extraction on efficacy of the extract.

MATERIALS AND METHODS

General method for extract preparation. Algae were collected from Parks Bay, Shaw Island, San Juan County, Washington, USA (48°33.5′N, 122°58.4′W). Putative toxins were extracted by drying 9-cm disks of algae (approximately 0.7 g fresh mass per disk) for 30 min outdoors in direct sunlight or, on cloudy days, for 45–60 min in the laboratory under halogen work lamps. This treatment effectively kills the alga. Dried disks were placed in an extraction medium for 90 min at a ratio of one disk per 50 mL of medium. The medium was either microwave-sterilized

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[2]Author for correspondence: e-mail tnelson@spu.edu.

seawater or a buffer solution, as described below. This yielded what we estimated to be the maximum concentration of extract that organisms encounter in the field based on our observations of algal density and the degree of discoloration caused by *Ulvaria obscura in situ*. Specifically, this is approximately the concentration found when a layer of seawater 5 cm deep covered a heavy bloom of ulvoid algae (i.e. six "layers" of algae thick). This preparation is referred to as our standard extract or as a concentration of 100%.

Fucus gardneri *germination success. Fucus gardneri* zygotes were grown in culture to examine the effects of algal extracts on germination success. Culture techniques were consistent for all *Fucus* experiments. Receptacles were collected from Cattle Point, San Juan Island, San Juan County, Washington, USA (48°27.3′N, 122°57.2′W) and allowed to dry on a laboratory bench overnight. The following day 2 × 2-cm squares of tissue were cut from the receptacles and placed in a 500-mL cylindrical culture vessel (approximately 9 cm diameter by approximately 7.5 cm depth) containing 150 mL of sterile seawater. The vessel was placed in an outdoor seawater table (i.e. with water flowing around, but not into, the vessel) for 24 h to induce gamete release. For each replicate, cultures were gently mixed to evenly distribute the fertilized eggs, and 0.5–1.0 mL of the mixture was transferred to a Petri dish containing 20 mL of either algal extract or sterile seawater as a control. Petri dishes containing zygotes were cultured at 14° C at 3 μmol photons·m^{-2}·s^{-1} in a 16:8-h light:dark photoperiod. Light was provided by a mix of cool white and warm white fluorescent bulbs. After 4 days, 25 zygotes in each Petri dish were scored as either germinated or not and germ tube lengths measured.

An initial experiment compared *Fucus* germination success in seawater extracts from *Ulva* and *Ulvaria* to a sterile seawater control (H-1). Ten replicate Petri dishes were prepared for each of the three treatments (*Ulva* extract, *Ulvaria* extract, and sterile seawater). This experiment was followed by a test of the effect of varying *Ulvaria* extract concentration on germination success (H-2). Five concentrations were tested (0%, 1%, 10%, 50%, and 100% of the standard extract), and each concentration was replicated in five Petri dishes. We also tested the effect of desiccation on extract potency (H-3) by drying *Ulvaria* for 0, 5, 15, and 30 min before extraction. Five replicate Petri dishes were used for each drying time treatment.

Other organismal assays. The effects of algal extracts on the growth of the algae from which they were extracted were measured in culture experiments (H-4). We cut circular disks of *Ulvaria* and *Ulva* with an initial area of 4.8 cm^2 and grew them in individual 500-mL cylindrical culture dishes. Each dish contained one algal disk, supplemental nutrients (Guillard's f/2), and 200 mL of *Ulva* extracts, *Ulvaria* extracts, or sterile seawater. The algae were cultured at 14° C at 80 μmol photons·m^{-2}·s^{-1} (16:8-h light:dark, with a mix of warm white and cool white fluorescent bulbs) for 7 days. After this incubation period, the area of each disk was measured using an LI 3000A area meter (LI-COR, Lincoln, NE, USA) and the change in area calculated.

Inhibition of periphyton accumulation by algal extracts (H-5) was tested using 8-cm eelgrass (*Zostera marina* L.) blade segments as a substratum for epiphytes. Eelgrass was collected from Argyle Lagoon, San Juan Island, San Juan County, Washington, USA (48°31.5′N, 123°00.7′W). Eelgrass blade segments were placed in 600-mL tissue culture flasks containing *Ulva* extract, *Ulvaria* extract, or seawater as a control. Extracts were prepared with unfiltered unsterilized seawater to allow for a natural inoculum of microalgae. The flasks were aerated and placed in an outdoor seawater table for cooling. After 2 weeks, the epiphytic community was quantified as [chl *a*] by scraping the microalgae from the eelgrass blade onto filter paper and extracting pigments in 90% acetone (Nelson 1997).

We conducted a series of oyster embryo larval bioassays (ASTM 1989) to test the effects of algal extracts on an animal model (H-6). This study was conducted during July and August 2001 to correspond with the natural gamete maturation in oysters. To collect the eggs from the broodstock, each female was removed from its shell and held in a beaker containing 25° C filtered seawater. To aid egg release, pressure was applied across the gonads. Subsamples of eggs deposited in the water were removed from the beaker and assessed under a microscope to determine egg quality and maturation. Eggs from two oysters were selected, sieved through an 80-μm mesh screen to remove excess debris, and rinsed into a sterile beaker. To obtain the sperm, the gonads of several individual male oysters were cut and rinsed with filtered seawater into a beaker. Samples of sperm from each male were observed under a microscope for motility. Active sperm from three males were selected and sieved through an 80-μm mesh screen into a sterile beaker. Aliquots of sperm were added to egg suspension to achieve a ratio of 10–20 sperm per egg. The beaker was stirred for 10 min to facilitate fertilization.

Within 1 h of fertilization, the embryo suspension was transferred into test chambers (polystyrene Coulter counter vials). Approximately 500 embryos (0.5 mL) were delivered into 19.5-mL solutions of either *Ulva* extracts or *Ulvaria* extracts. For each extract, we tested concentrations of 0%, 26%, 36%, 51%, 71%, and 100% of the standard extract. Each treatment dilution had five replicates. Filtered seawater (0.5-μm filter) from Friday Harbor, Washington, USA, was used for seawater controls, stock solutions, and dilutions for each treatment. The test chambers were maintained at 25 ± 1° C for 24 h. Temperature and $[O_2]$ of a separate container of seawater were checked every 4–6 h as a proxy for the incubation vials containing oyster larvae. Direct examination of $[O_2]$ in the vials with oyster larvae would have created an unacceptable risk of contamination from the probe. After 24 h, 1 mL of formaldehyde was added to each vial to preserve the oyster larvae for assessment.

Using an inverted scope, 100 randomly selected larvae in each test chamber were identified as either normal or abnormal based on their development to the D-hinge stage (ASTM 1989). In some cases, there were fewer than 100 total larvae available for counting due to the drastic effects of algal extracts on the embryos. In these cases, no larvae were observed to have developed normally so the replicate was scored as zero normal development.

Statistical analyses. The effects of extract source (i.e. *Ulvaria*, *Ulva*, or seawater controls) on *Fucus* germination frequency (H-1), epiphytic accumulation (H-5), and oyster larval development (H-6) were tested with one-factor analysis of variance (ANOVA) generally followed by post-hoc Tukey's tests. Similar procedures were used to examine the effect of drying time on extract potency (H-3). For the oyster toxicity assay, we used Dunnett's post-hoc test (instead of Tukey's test) to compare each concentration of extract with the seawater control. A nested ANOVA (Zar 1999) was used to examine the effects of extracts on germ tube length (H-1 and H-3). Individual germ tube lengths were nested within each Petri dish. The Petri dishes, in turn, were replicates of the three extract sources (H-1) or drying times (H-3). If a significant difference was found, Tukey's post-hoc tests (Zar 1999) were conducted to compare individual treatments.

Concentration effects on *Fucus* germination success (H-2) were tested by regressing germination frequency and average germ tube length separately against extract concentration and using Student's *t*-test to determine whether the slope of the regression line was equal to zero. Germination frequencies and germ tube lengths were $\log_e$ transformed before regression to homogenize variability, increase the coefficient of correlation, and provide a more biologically meaningful model. The latter goal was achieved by an asymptotic, rather than linear, approach to zero germination success as extract concentration increases. EC_{50} values were calculated for each of these dose–response functions.

The effect of extracts on growth of *Ulva* and *Ulvaria* (H-4) was tested using a two-way ANOVA with extract source (or control) and species tested treated as fixed factors. Post-hoc Tukey's tests were conducted on extract source treatment means (e.g. control vs. *Ulva* grown in *Ulva* extract; Neter et al. 1985).

RESULTS

Fucus *assays.* *Ulva* and *Ulvaria* extract treatments had a significant impact on germination frequency and germ tube length of *Fucus* zygotes (H-1, Fig. 1, $F_{2,27} = 144.34$, $P < 0.001$ and $F_{2,27} = 4063$, $P < 0.001$, respectively). *Ulvaria* extracts significantly reduced zygote germination frequency and germ tube length when compared with controls or *Ulva* extracts ($P < 0.001$ for both germination frequency and germ tube length when compared with both *Ulva* extracts and controls). *Ulva* extracts significantly reduced germination frequency and germ tube length relative to controls ($P = 0.041$ and $P < 0.001$, respectively). In a separate trial (H-2), germination frequency and germ tube length were negatively correlated with *Ulvaria* extract concentration (Fig. 2, $F_{1,23} = 62.27$, $P < 0.0001$ and $F_{1,23} = 122.5$, $P < 0.0001$, respectively). The EC_{50} for germ tube length was 66.1% of the standard extract. The standard *Ulvaria* extract (=100%) was approximately the EC_{50} for germination success. *Fucus* zygote germination frequency and germ tube length were lower in extracts prepared from *Ulvaria* dried 15 or 30 min relative to controls and extracts prepared from material dried 5 min or less (H-3, Fig. 3, $F_{4,20} = 58.4$, $P < 0.001$ and $F_{4,20} = 77.96$, $P < 0.001$, respectively).

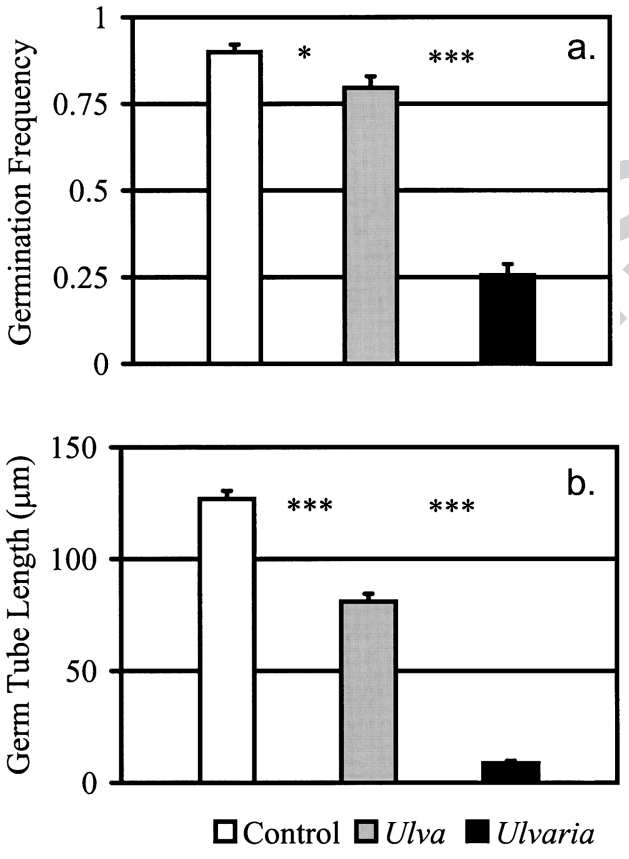

FIG. 1. Inhibition of germination in *Fucus gardneri* by extracts from *Ulva* and *Ulvaria*. (a) Germination frequency; (b) mean germ tube lengths. Error bars are ± 1.0 SE. For germ tube length, all measurements within a treatment were pooled to calculate the SE. Asterisks indicate the magnitude of significant differences in post-hoc tests (*$0.01 < P < 0.05$, ***$P < 0.001$).

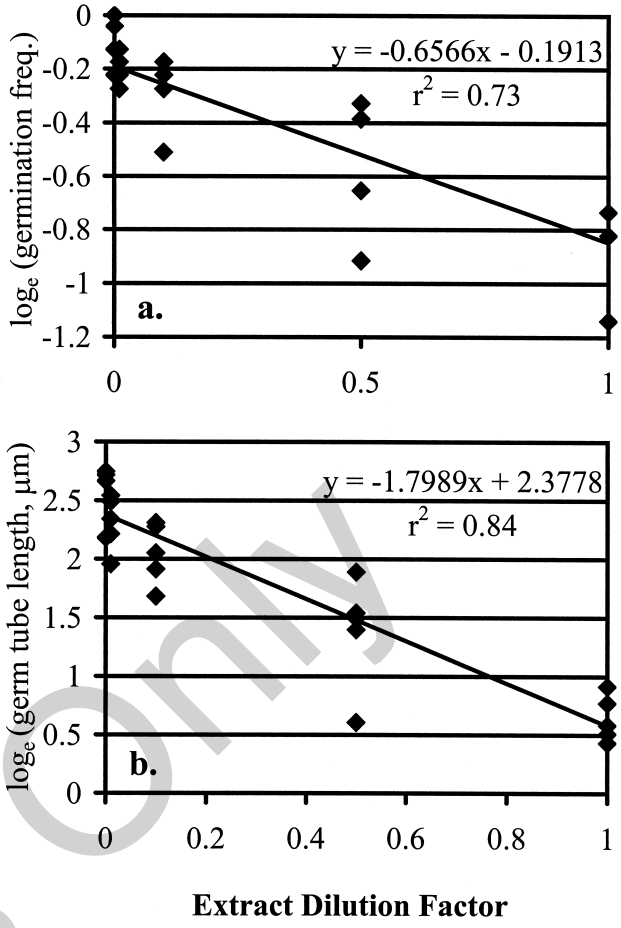

FIG. 2. Effect of *Ulvaria* extract concentration on germination success of *Fucus gardneri* zygotes. (a) Germination frequency; (b) mean germ tube lengths.

Other organismal assays. Extracts from *Ulva* and *Ulvaria* significantly affected the growth of *Ulva* and *Ulvaria* disks (H-4, Fig. 4, $F_{2,24} = 32.18$, $P < 0.001$). Extracts from both species resulted in significantly reduced growth in disks of *Ulva* and *Ulvaria* relative to seawater controls ($P < 0.001$). There was no significant difference between the two extracts ($P = 0.547$). In all treatments, *Ulvaria* grew more slowly on average than *Ulva* ($F_{1,24} = 14.73$, $P < 0.001$). Despite the reduction in growth rate, all specimens grown in algal extracts appeared healthy.

Extracts from *Ulva* and *Ulvaria* significantly affected epiphytic algal accumulation on eelgrass blade segments (H-5, $F_{2,9} = 9.44$, $P = 0.006$). Control communities averaged 0.46 ± 0.09 µg chl $a \cdot cm^{-2}$ (mean ± SE) and appeared healthy, with the typical golden-brown color of diatoms. Communities grown in *Ulva* extracts averaged 0.10 ± 0.03 µg chl $a \cdot cm^{-2}$ and ap-

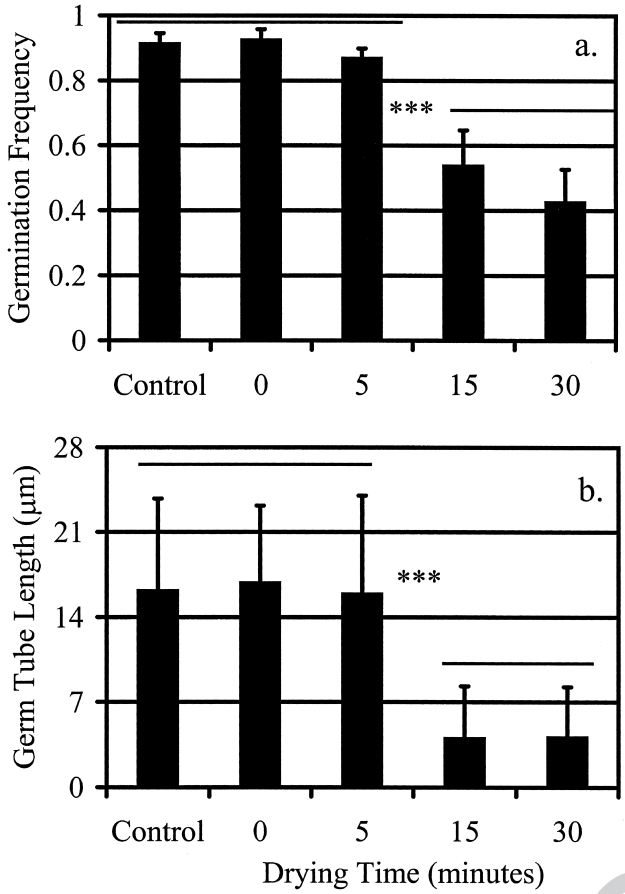

FIG. 3. Effect of *Ulvaria* drying time before extraction on *Fucus gardneri* zygote germination success. (a) Germination frequency; (b) mean germ tube lengths. Continuous lines over columns indicate means that are not significantly different. Error bars and asterisks are as in Figure 1.

peared to be covered by a white debris. Communities grown in *Ulvaria* extracts averaged 0.02 ± 0.01 μg chl $a \cdot cm^{-2}$ and were covered by black debris. The difference between both extracts and the control was significant ($P \leq 0.002$), but the difference between the two algal extracts was not significant ($P = 0.629$).

ANOVA results revealed significant effects of *Ulva* and *Ulvaria* extract on oyster larval development ($F_{10,45} = 5349.9$, $P < 0.001$). No oyster larvae developed normally to the D-hinge stage in any tested extract concentration (H-6). In controls, 92.8% of larvae developed successfully to the D-hinge stage. All extract treatments were significantly different from the control ($P < 0.001$) but were not significantly different from each other ($P = 1.000$). Interestingly, no embryos were found in the highest concentrations (71% and 100%) of the *Ulvaria* extract treatments; only embryo debris remained. In contrast, the lowest *Ulva* concentrations (26% and 36%) allowed many embryos to survive, although they all developed abnormally. Higher concentrations of *Ulva* extracts and lower concentrations of *Ulvaria* extracts had some embryonic development (albeit abnormal), but most of the embryos were obliterated by these treatments.

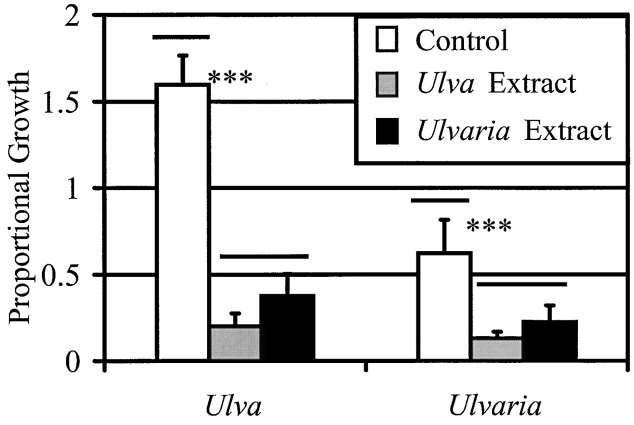

FIG. 4. Growth of *Ulva* and *Ulvaria* in seawater controls, *Ulva* extracts, and *Ulvaria* extracts. Continuous lines over the columns are as in Figure 3. Error bars and asterisks are as in Figure 1.

DISCUSSION

Community and ecosystem impacts. The toxic impacts of ulvoid algal blooms may be more important than are generally recognized. Every model system examined in this study was inhibited by extracts from *Ulvaria* and several were inhibited by extracts of *Ulva*. Several other studies have attributed inhibition of animal larvae to extracts from macroalgae, including *Ulva* (Johnson 1980, Johnson and Welsh 1985). For example, Johnson and Welsh (1985) found that extracts prepared from fresh *Ulva lactuca* L. inhibited the development of crab larvae. Though they used fresh algae (rather than dried), a much longer extraction period (24 vs. 1.5 h), a greater quantity of algal material (25–100 vs. approximately 3.6–14 g fresh mass·L^{-1}), and a longer exposure period (up to 20 days, vs. 1 day), their results were ultimately similar, in that all tested concentrations resulted in zero larval success. These data, combined with results from this study, suggest that toxicity of ulvoid extracts is a general phenomenon and is not restricted to the species considered in this study.

Exudates from diverse algae have been shown to inhibit animal development. The brown alga *Pilayella littoralis* (L.) Kjellman causes high mortality of Baltic herring (*Clupea harengus* L.) eggs (Aneer 1987). "Fresh" extracts from *Ulva reticulata* Forsskål inhibit larval settlement of the bryozoan *Bugula neritina* L. and the polychaete tube worm *Hydroides elegans* Haswell, whereas extracts from *Ulva fasciata* Delile increase larval settlement by *Hydroides* (Walters et al. 1996). Extracts of either alga aged 24 h had no effect on larval settlement. *Plocamium hamatum* J. Agardh fronds release a terpene capable of causing necroses in a potentially competing octocoral (de Nys et al. 1991). Compounds used by *Dictyota menstrualis* (Hoyt) Schnet-

ter et al. as defenses against grazers also reduce larval survival in potentially fouling invertebrate species (Schmitt et al. 1995, 1998).

Impacts like these could substantially alter community structure in the vicinity of ulvoid blooms. Specifically, the success of invertebrates, microalgae, and macroalgae could be limited by algal extracts. Substantial blooms of ulvoid algae, followed by desiccation events, could lead to reduced primary productivity (as micro- and macroalgal growth is limited) and reduced colonization success by both algal and animal propagules. Reduced settlement success and growth will have negative economic impacts, especially when larvae of harvested shellfish are harmed. These effects may not be as readily associated with the macroalgal bloom as the more obvious impacts of anoxia or direct shading.

Extract concentration is key to the magnitude of any potentially toxic impact. Inhibition of *Fucus* zygotes occurred at higher concentrations than inhibition of oyster larvae; this suggests that animal larvae may be more sensitive to the toxic properties of ulvoid blooms. Several factors will affect the extract concentration that any organism experiences, including tidal movement, wave action, and proximity to a source. Microbial communities beneath a bloom are most likely to be affected, because they are close to the source, diffusion is limited, and the effects of currents and waves are ameliorated by the macroalga. Inhibition of these communities could alter rates of nutrient cycling and other key processes at the sediment–water interface. Extracts may also remain (or become) concentrated in tide pools. Brown algae have been observed to release a burst of phlorotannins after desiccation, and these compounds accumulate in tide pools where planktonic animal survival is limited (Conover and Sieburth 1966, Carlson and Carlson 1984). Similarly, *Ulvaria* in or near such a pool may desiccate, releasing toxins into the pool. There is one report that extracts from *Ulva* sp. induce spawning in *Crassostrea gigas* (Miyazaki 1938). This is difficult to reconcile with our observations of larval mortality induced by *Ulva* extracts in this species.

Implications for the seaweed. The release of toxic compounds from *Ulvaria* was tied to desiccation for periods of time that ordinarily cause death in this species (Nelson 2000). The extracts released interfere with the success of plant and animal competitors for space, nutrients, and light. Inhibition of these competitors could enhance the reinvasion of rapidly growing ulvoid algae. Death of the entire alga may not be required for the release of toxic compounds. We have observed the distal portions of *Ulvaria* dying due to desiccation in a tide pool at low tide. The basal portions of the plant remained healthy. Thus, competitors could be inhibited without the death of the whole thallus. The reduction of growth noted in *Ulva* and *Ulvaria* in response to extracts from their own species is of particular interest. The compounds released may inhibit the growth of conspecific algae to reduce intraspecific competition or as a signal of less than optimal conditions, indicating a need for slower growth.

Many marine plants produce compounds thought to inhibit the growth of epiphytic microalgae and other fouling organisms, but reports of marine macrophytes producing compounds that inhibit the growth of other macrophytes, as we have observed in the present study, are rare. Antimicrobial compounds are produced by *Zostera marina* L., but *Zostera* detritus enhances the growth of *Ulva fenestrata* (Harrison 1977, 1982). Compounds produced by *Delisea pulchra* (Greville) Montagne inhibit the germination success of *Ulva lactuca* spores and barnacle larval settlement (de Nys et al. 1995).

Nature of the toxin(s). Extracts from *Ulva* and *Ulvaria* behave differently in both qualitative and quantitative ways. In some model systems, *Ulvaria* extracts caused greater inhibition of growth and development than did *Ulva* extracts, whereas in others the effect of the two extracts was similar. This suggests that either each alga has a unique set of toxic compounds, or at least that *Ulvaria* produces a toxin not found in *Ulva*.

Several of our observations regarding toxicity of *Ulvaria* extracts are consistent with the presence of dopamine oxidase and dopamine in this species (Tocher and Meeuse 1966, Tocher and Craigie 1966). Dopamine hydrochloride has recently been demonstrated to function as a grazer deterrent in *Ulvaria* (Nelson 2003). The reddish-brown extract that darkens to black and precipitates (as was seen in the present study) is consistent with polymerization of oxidized dopamine and binding of these compounds to organic matter. Effects of *Ulvaria* extracts on organismal systems could be caused by polymers blocking membrane proteins or direct effects of dopamine and its derivatives.

Several alternative explanations are also possible. Seaweeds contain a large variety of biologically active compounds (Paul et al. 2001). For example, both *Ulva fenestrata* and *Ulvaria obscura* contain dimethylsulfoniopropionate, which, together with its activation products, functions as an antiherbivore activated defense system (van Alstyne et al. 2001, K. van Alstyne, personal communication). Bacteria could play a role as well, either by producing a toxin or by creating anaerobic conditions in the extracts. The former would require bacteria specific in taxonomy and number on each species of alga, because the effects of each extract were quite different. The latter seems unlikely, given that extracts from *Ulvaria* have a much larger effect (in some cases) than extracts from *Ulva* and the toxic effect was seen in an aerated test (H-5). Bioassays after biochemical fractionation will be necessary to determine the compounds involved.

Evolutionary implications. It is of interest to speculate about the evolutionary origins of the toxic properties of these algal extracts. Selective pressures from a suite of grazers, pathogens, fouling organisms, and competitors may shape the evolution of any alga (Rausher 1996). *Ulva* and *Ulvaria* produce compounds

used in chemical defenses against grazers (Borowsky and Borowsky 1990, Nelson 2003) that might also cause the toxic effects seen here. If so, grazer defense seems a more likely explanation as the selective force behind the origin of toxicity than inhibition of competitors. *Ulvaria*, for example, is exposed to grazers more regularly than it is desiccated. In a tidal situation, substantial mixing will dilute the impact of allelochemicals on competitors. In contrast, a grazer will concentrate the compounds in its gut and will still be digesting the alga for some time after feeding. We cannot conclude, however, that grazing pressure is the main selective force behind toxicity. The relative importance of selection for compounds that reduce competition should be explored further.

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