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Marine planktonic ciliates that prey on macroalgae and enslave their chloroplasts

Abstract—We found two tide-pool ciliates, *Strombidium oculatum* and *Strombidium stylifer,* that ingest ulvaceous green macroalgae and retain their chloroplasts. Sequences of the form I ribulose bisphosphate carboxylase/oxygenase (rubisco) large subunit gene (*rbcL*1) from chloroplasts found in the ciliates cluster with the Ulvophyceae sequences on GenBank and with those of ulvaceous macroalgae from pools in which the ciliates were collected. In addition, we have cultivated *S. stylifer* in vitro on filtered seawater supplemented with pieces of *Enteromorpha* thalli that had been treated with light and temperature shock to maximize production of reproductive unicells (swarmers). An average growth rate of 1.08 ± 0.07 SE [standard error] d^{-1} was measured when *S. stylifer* was grown this way. Because both ciliates and the algal swarmers contain eyespots while vegetative cells in the Ulvophyceae do not, we speculate that these reproductive cells are the source of both the chloroplasts and the pigments used in the ciliates' eyespots. This ciliate will not grow in the dark and is required to ingest fresh chloroplasts every few days, making it an obligate mixotroph. Ingestion of macroalgae by ciliates constitutes an upside-down trophic link, contrary to the usual pattern for planktonic food webs, in which production passes from very small organisms to successively larger ones. Our finding suggests that macroalgal production, heretofore believed to contribute predominantly to detrital or macroherbivore food chains, may be an important food source in the nearshore plankton and that ciliates may play an important role in this trophic pathway.

Planktonic ciliates are often the dominant herbivores in coastal waters and members of the genus *Strombidium* usually comprise the most abundant taxon within this group (Heinbokel and Beers 1979; Capriulo and Carpenter 1983; Montagnes et al. 1988; Pierce and Turner 1992). Some of the Strombidiidae have a mixotrophic nutritional mode, retaining the chloroplasts of ingested algae and using them to supplement their nutrition with photosynthesis (McManus and Fuhrman 1986; Stoecker et al. 1987; Stoecker 1991; Bernard and Rassoulzadegan 1994). These ciliates are thus both producers and consumers, and models of marine food webs suggest that mixotrophy results in increased trophic efficiency in the sea (Stoecker 1998; Stickney et al. 2000). Until now, ciliate mixotrophy has only been found to result from ingestion of unicellular microalgae. Grazing of ciliates on macroalgae has not been reported.

We studied the ingested chloroplasts of two ciliate species, *Strombidium oculatum* and *Strombidium stylifer,* isolated from tide pools in Europe and North America. Both are small (≈50–60 μm in length) and planktonic. *S. oculatum* has the interesting habit of encysting and excysting on an endogenous circatidal rhythm such that it is active and swimming in tide pools during low tide and encysted during high tide (Faure-Fremiet 1948; Jonsson 1994; Montagnes et al. 2002*b*). Both ciliates contain chloroplasts that are grass-green in color, and partially digested green unicells can be seen within food vacuoles (Fig. 1), suggesting a chlorophyte origin. Both ciliates also have a prominent eyespot at the oral end and show strong phototactic behavior.

Methods—Cloning and phylogenetic analysis: We collected *S. oculatum* from tide pools on the Irish Sea (Isle of Man) and Dublin Bay and Galway Bay (Ireland) and *S. stylifer* from pools on Long Island Sound (USA). Samples of green macroalgae (*Enteromorpha*-like members of the Ulvales) were collected from Dublin Bay and Galway Bay pools. Samples for ciliate DNA analysis were collected from pools isolated from the open sea at low tide. Several 10s of ciliates (typically 40–60) were picked with a drawn capillary pipette into microcentrifuge tubes containing DNA extraction buffer (100 mmol L^{-1} NaCl, 50 mmol L^{-1} TRIS-HCl [pH 8.0], 25 mmol L^{-1} EDTA, 0.5% sodium dodecyl sulfate [SDS]). After treatment with proteinase K, DNA was extracted and the form I ribulose bisphosphate carboxylase/oxygenase (rubisco) large subunit gene (*rbcL*1), which in all eukaryotic algae is encoded in the chloroplast genome (Tabita 1999), was amplified by PCR. Primer sequences were designed based on a conserved region of form I rubisco large subunits from various organisms: RubI-forward: GCT-GCATTCCGTWTBACWCCWCAACCAGG; RubI-reverse: GTGRATACCRCCWGAAGCWACWGG. Polymerase chain reaction (PCR) was performed as follows: 35 cycles of 25 s of denaturation at 94°C, 30 s of annealing at 58°C, and 40 s of elongation at 72°C. PCR products of the expected size (1,053 base pairs) were cloned into the TA vector and transformed into competent cells (Invitrogen). For each PCR product we obtained from the ciliate samples, 17–26 bacterial clones were randomly picked up and plasmid DNA was isolated. Macroalgal samples from Dublin Bay and Galway Bay were treated in a similar manner. All ciliate and green macroalgal clones were subjected to restriction fragment length polymorphism (RFLP) analysis using the restriction enzymes *Eco*RI and *Hind*III. We then selected 27 clones for sequencing based on differences observed in RFLP patterns (17 identical and 4 different clones for ciliates, 6 identical clones for green macroalgae) in order to maximize coverage of different clones. Sequencing was carried out using the BigDye Terminator Cycle Sequencing Kit (PE Applied Biosystems) and an ABI Prism 377 automated sequencer. Sequences were analyzed using the Basic Local Alignment Search Tool (BLAST) program against gene sequences in GenBank for similarity to known gene sequences. Alignment of sequences and subsequent phylogenetic analyses were carried out using CLUSTAL X1.8 (Thompson et al. 1994). The deduced amino acid (aa) sequences of *rbcL*1 from the ciliate and algal samples were analyzed with those published in GenBank. A phylogenetic tree was constructed using the neighbor-joining method (Saitou and Nei 1987) and was corrected using the Kimura method (Kimura 1980). The reli-

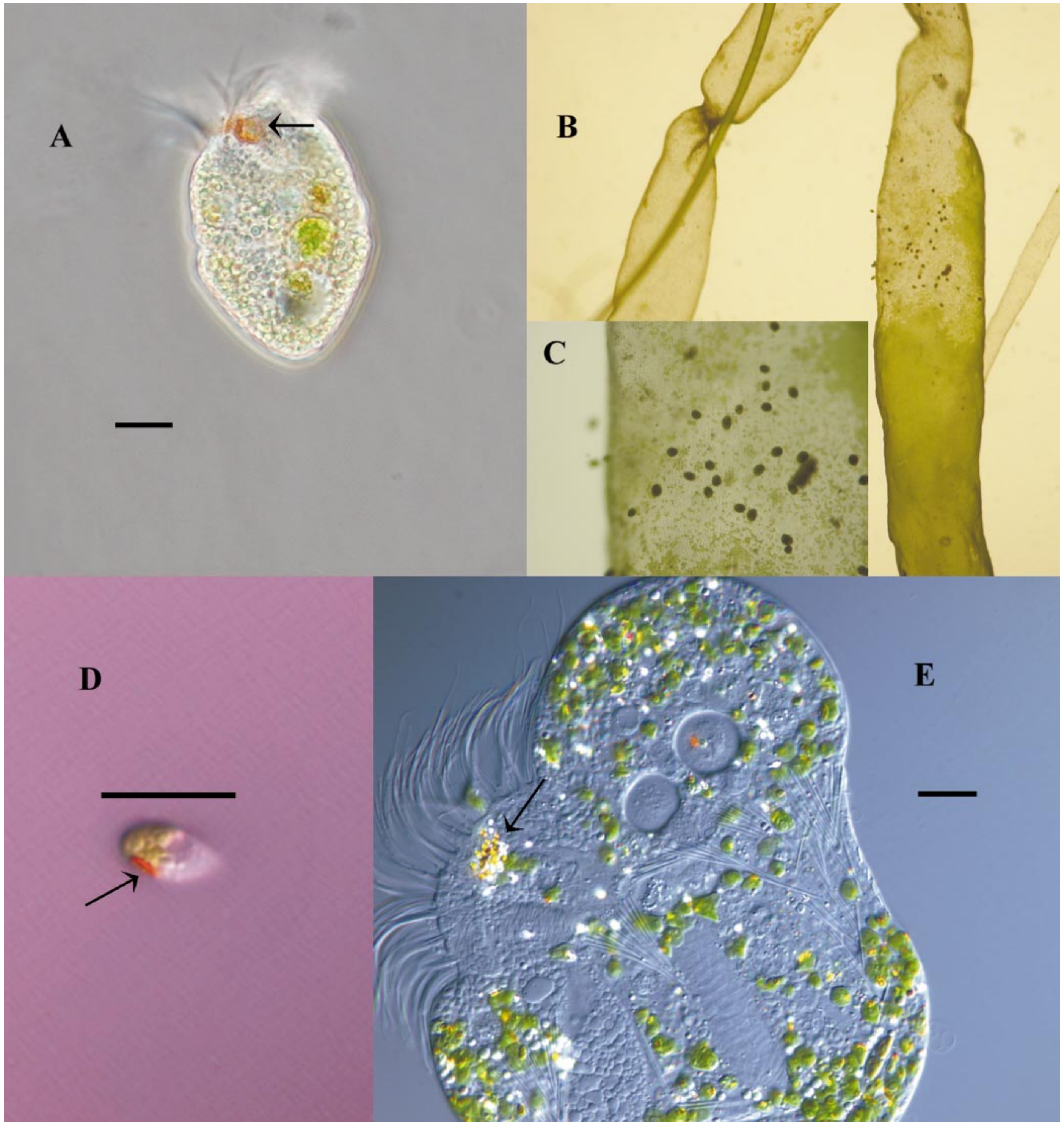

Fig. 1. Composite photo of the organisms: (A) *Strombidium oculatum*, (B) *Enteromorpha* sp. with *S. oculatum* cells swarming near reproductive portion of thallus, (C) higher magnification of ciliates on the *Enteromorpha*, (D) a swarmer cell from *Enteromorpha* with orange-red eyespot embedded in the chloroplast (arrow), (E) *S. stylifer.* All photos are of live specimens. The *S. stylifer* cell is distorted (flattened) because it is held still between coverslips. Both ciliates show the characteristic pigmented eyespot (arrows), and food vacuoles can also be seen. All scale bars = 10 μm.

ability of the tree topology was evaluated by bootstrap analyses with 1,000 replicates.

Culture observations: To evaluate whether algal thalli could sustain *S. stylifer,* we collected *Enteromorpha* sp. from tide pools on Long Island Sound. Whole thalli were placed between moist paper towels and held over night at 4°C in darkness. Thalli so treated will usually produce large numbers of swarmer cells when subsequently placed in the light. We cut pieces of thallus (≃1 cm), wiped them with sterile cotton swabs to remove attached diatoms, and placed them in multiwell plates with 10 ml of filtered autoclaved seawater medium (F/2 [Guillard 1975], plus 1 mg L^{-1} GeO_2 to inhibit growth of any diatoms that may have been transferred with the *Enteromorpha*). Salinity of the medium was 20. We added *S. stylifer* (10 ciliates per well) and incubated at 18°C in the light (≈200 μmol photons m^{-2} s^{-1}). Two such experiments were performed, with six replicate wells each time. After 3 d (first experiment) or 4 d (second experiment), ciliates were preserved with 10% acid Lugol's solution and enumerated. Growth rates of the ciliates were calculated assuming exponential growth of the original population of 10 individuals.

We compared survival of *S. stylifer* in the presence or absence of light. Ten ciliates were added to each of 12 wells containing filtered, autoclaved seawater medium (as above). One plate (6 wells) was incubated in darkness (two layers of heavy-duty aluminum foil). The other plate was incubated in the light (≈200 μmol photons m^{-2} s^{-1}). After 48 h, well contents were preserved with 10% acid Lugol's and surviving ciliates were counted. The null hypothesis of no treatment effect was evaluated with the nonparametric Kruskal–Wallis test (Sokal and Rohlf 1995).

For comparison with growth rates achieved with macroalgal food, we also measured growth of *S. stylifer* using the microalga *Tetraselmis* sp. (strain PLY 429). Populations of the algae in late exponential phase were diluted to a range of concentrations (80, 160, 310, 620, 1.25×10^3, 2.5×10^3, 5×10^3, 1×10^4, and 5×10^4 cells ml^{-1}) and added to well plates as food. Ciliates were added to each well and the plates were incubated at 18°C in the light (≈200 μmol photons m^{-2} s^{-1}). The ciliates were allowed to acclimate over night, then 10 ciliates each were added to fresh wells at the various food concentrations. After 3 d, incubations were terminated with 10% Lugol's solution and growth rates were estimated assuming exponential growth. The growth rate data were fit to a modified Michaelis–Menten equation using SigmaPlot,

$$y = \mu_{max}(x - t)/(k + x - t)$$

where y is growth rate (d^{-1}), x is food concentration (cells ml^{-1}) and μ_{max}, t, and k are parameters specifying maximum growth rate, x-intercept (concentration of food at which growth is 0), and half-saturation, respectively (Montagnes 1996).

Results and discussion—Twenty of 21 *rbcL*1 clones from the ciliates clustered into the green-like clade of the *rbcL*1 tree (Tabita 1999). This clade contains the green plastids (e.g., prasinophytes, euglenophytes, chlorophytes) and a cyanobacterium (Fig. 2). Seventeen of those 21 clones (all of which showed an identical RFLP pattern), along with the *rbcL*1 clones of the green macroalgae we collected in Dublin Bay and Galway Bay tide pools, and the *rbcL*1 sequences of Ulvales obtained from GenBank, form a tight cluster (similarity of the aa sequences in this cluster is 97–100%). Among these, one *rbcL*1 sequence from *S. stylifer* was identical to that of *Enteromorpha clathrata* collected from Long Island Sound and sequenced previously (unpublished; GenBank accession No. AF525939). Three of the clones (all of which showed an RFLP pattern different from that of the 17 clones) were slightly outside the Ulvales cluster but still within the Ulvophycean group. The aa sequences of all 20 of the green-like ciliate *rbcL*1 clones were at least 5% different from the nearest microalgal clone available on GenBank (i.e., *Chlorella* in Fig. 2). One clone from the ciliate samples clustered on the red-like clade of the form I rubisco tree, which includes all of the nongreen algal lineages, and appeared to be a diatom *rbcL*1. This likely resulted from a diatom contaminating the ciliate samples, which were picked manually from natural assemblages, or from diatoms in a food vacuole of the ciliate. These results strongly suggest that the chloroplasts in *S. oculatum* and *S. stylifer* were mainly obtained from green macroalgae in the Ulvophyceae.

The results of the two experiments in which *S. stylifer* was fed only whole algal thalli were not significantly different from each other ($P > 0.05$; t-test). The mean per capita growth rate for the pooled results was 1.08 d^{-1} (±0.07 SE [standard error]), equivalent to 1.56 doublings per day. Release of swarmers during these experiments was verified by the observation that large areas of the thalli had been converted to spent reproductive tissue (empty cell walls). We were also able to grow *S. stylifer* this way using *Ulva* instead of *Enteromorpha,* but growth rates were not as high (data not shown).

The experiment comparing growth in light versus dark showed better survival of *S. stylifer* in the light ($P < 0.005$). This ciliate survived for a very short time in darkness without food (e.g., only 1 of 60 ciliates in the dark wells survived the 48-h incubation). When starved in the light, ciliate survival was better (50% or higher in all wells), but only one well showed net positive growth.

When fed the prasinophyte microalga *Tetraselmis* sp., *S. stylifer* grew well (Fig. 3), with estimated $\mu_{max} > 1.8$ d^{-1}, even faster than it grew on *Enteromorpha.* In this experiment, the estimated half-saturation constant, *k,* was only 201 cells ml^{-1}. The high growth rate of this ciliate in the light at low food concentrations and its poor survival in the dark suggest that *S. stylifer* is an obligate mixotroph, as shown in another strombidiid, *Laboea strobila* (Stoecker et al. 1988). If given a modest supply of food with which to replenish its chloroplasts, it grows rapidly. But it cannot survive long in either darkness or without any food. Our estimate of *t,* the parameter indicating the concentration at which growth is zero, was virtually zero ($<10^{-5}$ cells ml^{-1}), supporting the idea that this ciliate only needs a small amount of food to replenish its supply of chloroplasts and achieve net positive growth.

The manner in which *S. oculatum* and *S. stylifer* obtain chloroplasts from *Enteromorpha* or other green macroalgae

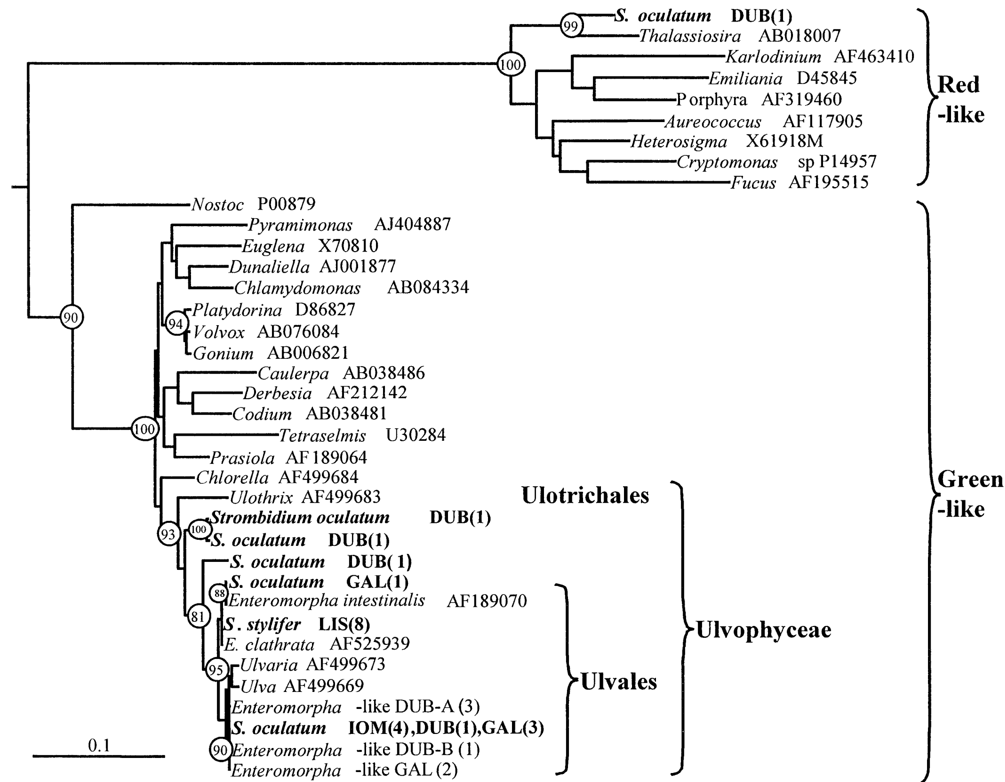

Fig. 2. Rooted neighbor-joining tree of algal form I rubisco based on amino acid sequences. The tree was rooted by Proteobacteria *Allochromatium* Rubisco (GenBank accession No. P22849). All *S. oculatum* and *S. stylifer* sequences are from this study, as are the *Enteromorpha* sequences with site designations (GenBank accession Nos. AY257104–AY257124). Circled numbers at nodes are bootstrap confidence values based on 1,000 replicates (only values higher than 80% are shown), while the numbers in parentheses indicate number of identical sequences. Sequences from *Strombidium* are in bold. The scale bar denotes aa substitutions per site. DUB, Dublin Bay; GAL, Galway Bay; IOM, Isle of Man; LIS, Long Island Sound.

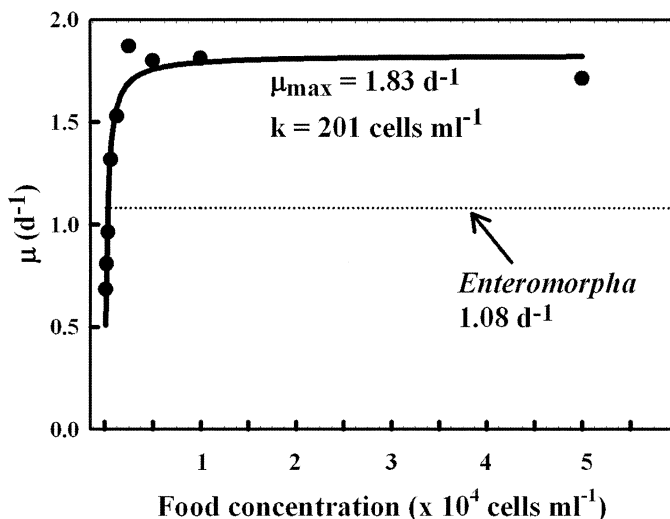

Fig. 3. Numerical response of *S. stylifer* grown in the light on *Tetraselmis* sp. (PLY429). For reference, ciliate growth rate when pieces of *Enteromorpha* were added as the sole food is shown by the dotted line (1.08 d^{-1} ± 0.07 SE; n = 12).

is not known. In the laboratory, the ciliates can sometimes be observed to aggregate near green algal reproductive tissues (Fig. 1B,C). This suggests that they are ingesting gametes or zoospores as they are shed by the thallus. Supporting this idea is the fact that both ciliates contain a prominent eyespot, which they probably also obtain from ingested algae (Montagnes et al. 2002*a*). The vegetative cells of the Ulvales, like those of other green macroalgae, do not contain eyespots, but their swarmers do. The latter are unicellular, flagellated dispersal stages that may be haploid or diploid. They are close to the optimal size for ingestion by ciliates (8–10 μm; Fig. 1D). In the laboratory, we have cultivated *S. stylifer* through multiple generations in filtered seawater containing pieces of *Enteromorpha* that had been treated to maximize swarmer production (temperature and light shock), further supporting the idea that the swarmers are the source of ciliate chloroplasts and eyespot pigments.

The use of swarmer chloroplasts and eyespots by these ciliates raises interesting questions about how the ciliates' behavior is connected to that of their food. For example, green macroalgae have been shown to release swarmers periodically in response to cycles of exposure/inundation (cued to temperature and/or desiccation), light, and spring/neap tidal variations (Haxo and Clendenning 1953; Christie and

Evans 1962; Pringle 1986). Thus, the ciliates' food supply is pulsed in response to a variety of forcings. For *S. oculatum,* one of those forcings (the semidiurnal tidal elevation cycle) also sets a limit on the period during which the ciliates are active. The growth rates of both ciliates must also be dependent on the diurnal cycle of light availability for photosynthesis of their chloroplasts, as suggested by the better survival we measured in the light. Thus, both macroalga and ciliate need to be adapted to a variety of environmental and biotic cycles, some of which may be in harmony at various times. Another interesting aspect of this interaction is how the photobehavior of the ciliates may be cued to that of their swarmer cell prey. The gamete swarmer cells of some green algae show positive phototaxis prior to syngamy and negative phototaxis (for settlement) thereafter (Haxo and Clendenning 1953). *S. oculatum* has been shown to shift from positive to negative phototaxis depending on tidal stage (Jonsson 1994). It moves toward a light source during the first half of the low tide period, presumably to maximize light exposure for its chloroplasts. Prior to high tide and encystment, however, it becomes negatively phototactic, swimming to the bottom of pools. Whether this behavior is physiologically related to the ingested swarmer chloroplasts is not known. However, if the ciliates have a photobehavior that is similar to that of their prey, they might increase their ability to find food in the environment.

Ingestion of macroalgal production by ciliates represents an example of an upside-down trophic link. Contrary to the usual marine trophic paradigm, which consigns macroalgal production to detrital or benthic macrograzer (e.g., urchin) pathways, the link we have shown funnels macroalgal production into the plankton. This pathway is potentially very large. The green macroalga *Ulva lactuca* releases 20–60% of its biomass as swarmers during the growing season (Niesenbaum 1988). Because they do not have specialized reproductive structures for swarmer production, as brown algae do, green algae can convert vegetative tissue to massive reproductive output quickly, allowing them to respond to stress or other conditions that favor dispersal. For plankton that live in tide pools or very nearshore waters, this may provide a cyclical (hence predictable) and substantial food source.

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