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Chemical defense in the microplankton I: Feeding and growth rates of heterotrophic protists on the DMS-producing phytoplankter *Emiliania huxleyi*

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Abstract

In this study, the hypothesis that *Emiliania huxleyi,* a cosmopolitan, bloom-forming coccolithophorid, produces chemical defenses against protist grazers was tested using four axenic strains of the alga. The putative chemical defense involves the cleavage of dimethylsulfoniopropionate (DMSP) by the enzyme DMSP lyase to yield dimethylsulfide (DMS) and acrylate. Out of six tested protist grazer species, five (including ciliates and heterotrophic dinoflagellates) showed lower feeding rates on *E. huxleyi* strains with high DMPS lyase activity than on low-lyase strains. Reductions in population growth rate were consistent with feeding reductions. These results suggest that high levels of DMSP lyase activity somehow promote reduced palatability, although covariation of *E. huxleyi* protein and carbohydrate content with DMSP lyase activity meant that prey ''nutritional quality'' could not be ruled out as a contributing factor. Additional experiments with the heterotrophic dinoflagellate *Amphidinium longum* demonstrated that (1) individuals need not ingest *E. huxleyi* cells to receive the ''don't eat me'' cue and (2) exposure to high-lyase *E. huxleyi* cells confers no harmful consequences in terms of the ability to feed and grow on alternate phytoplankton prey. Thus, factors promoting reduced grazing on high-lyase *E. huxleyi* are hypothesized to constitute signals rather than acute toxins. Furthermore, although cell surface carbohydrates (as assayed by lectin binding) showed few differences among strains, results of feeding studies indicate that the deterrent signal resides on the cell surface or in the near-cell dissolved phase. Behavior responses to such cues might play an important role in the outcome of encounters between protist grazers and their prey, with profound consequences for planktonic community structure.

Chemical defenses—chemicals used by individuals to reduce or eliminate agents of mortality—are widespread throughout the natural world. Best studied in terrestrial systems, chemical defense compounds are highly diverse (Berenbaum 1995), ranging from the tannins in oaks to the mustard oil bomb of the bombardier beetle. Similarly, marine macroalgae and invertebrates utilize chemical defenses in systems ranging from kelp forests to coral reefs (Hay 1996). Chemical defenses are employed by bacteria, fungi, plants, and animals, and can defend against attack and mortality due to herbivory, carnivory, and parasitic or microbial infections. The myriad ways in which chemical defenses can be deployed, countermined, co-opted, and mimicked provide not only a rich source of ecological insight, but appear to be fundamental in structuring terrestrial and marine benthic ecosystems.

Examples of chemical defenses are known from marine planktonic ecosystems as well, although the significance of the phenomenon for ecosystem structure and function has not been systematically explored. One group of phytoplankton that has received attention historically is the toxic red tide dinoflagellates. *Alexandrium* spp., a well-studied example, produce toxins inducing paralytic shellfish poisoning and can be eaten without apparent ill effects by some copepods, whereas others experience impaired swimming, feeding, and reproduction rates (Turiff et al. 1995; Teegarden 1999; Frangopulos et al. 2000). A putative chemical defense receiving considerable recent attention is the potential toxicity of certain diatom species (Ban et al. 1997). Diatom fatty acids and their aldehyde reaction products, formed in response to cell breakage and other types of stress, can be acutely toxic to crustacean zooplankton (Pohnert 2000; Juttner 2001). Aldehydes extracted from bloom-forming diatoms have also been shown to reduce copepod egg hatching rates (Miralto et al. 1999). However, diatoms in mixed natural assemblages do not necessarily have deleterious effects on copepod reproduction (Irigoien et al. 2002), so the source and significance of this potential defense interaction is not yet known (Jonasdottir and Kiorboe 1996).

The hypothesis that protist grazers can chemically defend against phytoplankton, though recognized as potentially important (Verity and Smetacek 1996; Turner and Tester 1997), has not been widely explored, either experimentally or conceptually (McClintock et al. 2001). As for copepods, the available evidence suggests that ''toxic'' species (*Heterosigma akashiwo, Gymnodinium catenatum, Alexandrium* spp., and others) can be eaten with impunity by some protist grazers, whereas others experience behavioral changes, reduced population growth rates, and in some instances, death (Verity and Stoecker 1982; Nielsen et al. 1990; Hansen et al. 1992; Jeong and Latz 1994; Kamiyama and Arima 1997; Jeong et

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Table 1. Diameter, biochemical content, and DMSP lyase activity of high-lyase (HL) and low-lyase (LL) *Emiliania huxleyi* strains used in protist feeding and growth experiments. CCMP strain number shown in parentheses. All data from batch cultures in exponential growth phase. Average values ($n = 3$; $n = 6$ for DMSP), with 1 SD in parentheses.

	LL-1 (370)	LL-2 (374)	HL-1 (373)	HL-2 (379)
Diameter (μm)	3.8(0.3)	3.9(0.4)	4.7(0.1)	4.1(0.3)
$C_{organic}$ (pg cell^{-1})	5.77(0.01)	8.09(0.03)	7.99(0.09)	8.05(0.03)
$N_{organic}$ (pg cell^{-1})	1.19(0.01)	1.58(0.03)	1.54(0.05)	1.49(0.01)
Lipid (pg cell^{-1})	3.11(0.57)	2.00(0.58)	4.11(0.79)	2.29(0.54)
Carbohydrate (pg cell^{-1})	1.05(0.06)	1.21(0.03)	2.15(0.19)	1.89(0.28)
Protein (pg cell^{-1})	2.49(0.52)	2.83(0.37)	2.76(0.19)	2.31(0.20)
DMSP (pg cell^{-1})	0.50(0.01)	0.52(0.03)	0.85(0.10)	0.68(0.02)
DMSP lyase* (fmol cell^{-1} min^{-1})	0.01–0.02	0.01–0.08	2.1–5.9	0.1–3.0
DMSP lyase** (fmol cell^{-1} min^{-1})	0.008	0.002	12	6

* Range of values measured in this study; $n = 2$ (LL strains), $n = 7$ (HL strains).
** From Steinke et al. (1998), their fig. 1.

al. 1999) The degree to which protist behavioral responses to potentially toxic species shape their diet in nature is unknown. Ciliates and heterotrophic flagellates can alter their behavior in response to chemical cues, both dissolved and in association with cell surfaces (Sibbald et al. 1987; Snyder 1991; Verity 1991). Such behavioral alterations can potentially influence the detection and selection of prey particles (Stoecker 1988; Hansen and Calado 1999). We know from other fields of investigation, in particular immunology, that intercellular interactions are strongly influenced by sensing of cell surface properties, so the potential for free-living single-celled organisms to utilize this information pathway seems high.

For marine phytoplankton communities, chemical defenses against protist grazers could be particularly important in structuring phytoplankton communities. This is because grazing by protists is by far the largest loss process affecting marine phytoplankton, both in the open ocean and in coastal waters (Banse 1992). Small reductions in the magnitude of protist grazing relative to phytoplankton growth can lead to major shifts in species composition and, given sufficient resource availability, can set the stage for a phytoplankton bloom. Furthermore, the importance of grazing losses as a source of mortality for phytoplankton cells suggests an intense selective pressure for the evolution of defenses against grazers (Strom in press).

Wolfe et al. (1997) hypothesized that the cleavage of DMSP to yield DMS and acrylate constitutes a grazing-activated chemical defense for some marine phytoplankton species. DMSP, present at high intracellular concentrations in some marine dinoflagellates, prymnesiophytes, and other taxa, has also been shown to act as an osmolyte and cryoprotectant (Vairavamurthy et al. 1985; Karsten et al. 1996). Cleavage of DMSP by the constitutive phytoplankton enzyme DMSP lyase (dimethylpropiothetin dethiomethylase 4.4.1.3) is triggered by physiological stress, as induced by pH variation, shear, and prolonged darkness (Steinke et al. 1998; Wolfe et al. 2002). Preliminary evidence (Wolfe and Steinke 1996; Wolfe et al. 1997) indicated that grazing by the heterotrophic dinoflagellate *Oxyrrhis marina* was also associated with DMSP lyase activation, DMSP cleavage, and production of DMS and acrylate.

In this study, we compared the grazing and growth responses of six heterotrophic protists fed the prymnesiophyte *Emiliania huxleyi* using four different *E. huxleyi* strains as our experimental system. Although all four have similar intracellular DMSP concentrations, strains HL-1 (CCMP 373) and HL-2 (CCMP 379) exhibit high DMSP lyase activity, whereas LL-1 (CCMP 370) and LL-2 (CCMP 374) exhibit low DMSP lyase activity. By contrasting protist feeding and growth responses to high-lyase (HL) and low-lyase (LL) strains, we were able to gain insight into the potential for DMSP cleavage to act as a chemical defense. Related components of this work are presented separately, including algal initiation of DMSP cleavage (Wolfe et al. 2002), the effects of DMSP and related compounds on grazers (Strom et al. 2003), and a detailed study of feeding mechanisms and ultrastructure in one grazer, *Amphidinium longum* (Jacobson et al. pers. comm.). Olson and Strom (2002) present evidence for suppressed microzooplankton grazing in a Bering Sea coccolithophorid bloom.

Materials and methods

Isolation and maintenance of protists—Axenic strains of *E. huxleyi* (Table 1) were obtained from the Center for the Collection of Marine Phytoplankton (CCMP, Bigelow Laboratories) and maintained on f/2 medium (without added Si) at 15°C on a 14 : 10 light : dark cycle (LD). Irradiance levels (cool white fluorescent bulbs) were approximately 100 μmol photons m^{-2} s^{-1}. Cultures were checked for bacterial contamination at regular intervals by staining subsamples with 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI) and examining them using epifluorescence microscopy. Cultures used in experiments (*see below*) were axenic.

Ciliates and heterotrophic dinoflagellates (Fig. 1) were isolated from local marine waters and maintained in sterile filtered seawater with dilute trace metal additions (henceforth ''ciliate medium,'' Gifford 1985). These protists were fed a mixed diet of phytoplankton as optimized in growth trials (Strom and Morello 1998) and maintained at 12°C in dim light on a 14 : 10 LD cycle. Maintenance cultures were fed once or twice per week and transferred weekly. Collec-

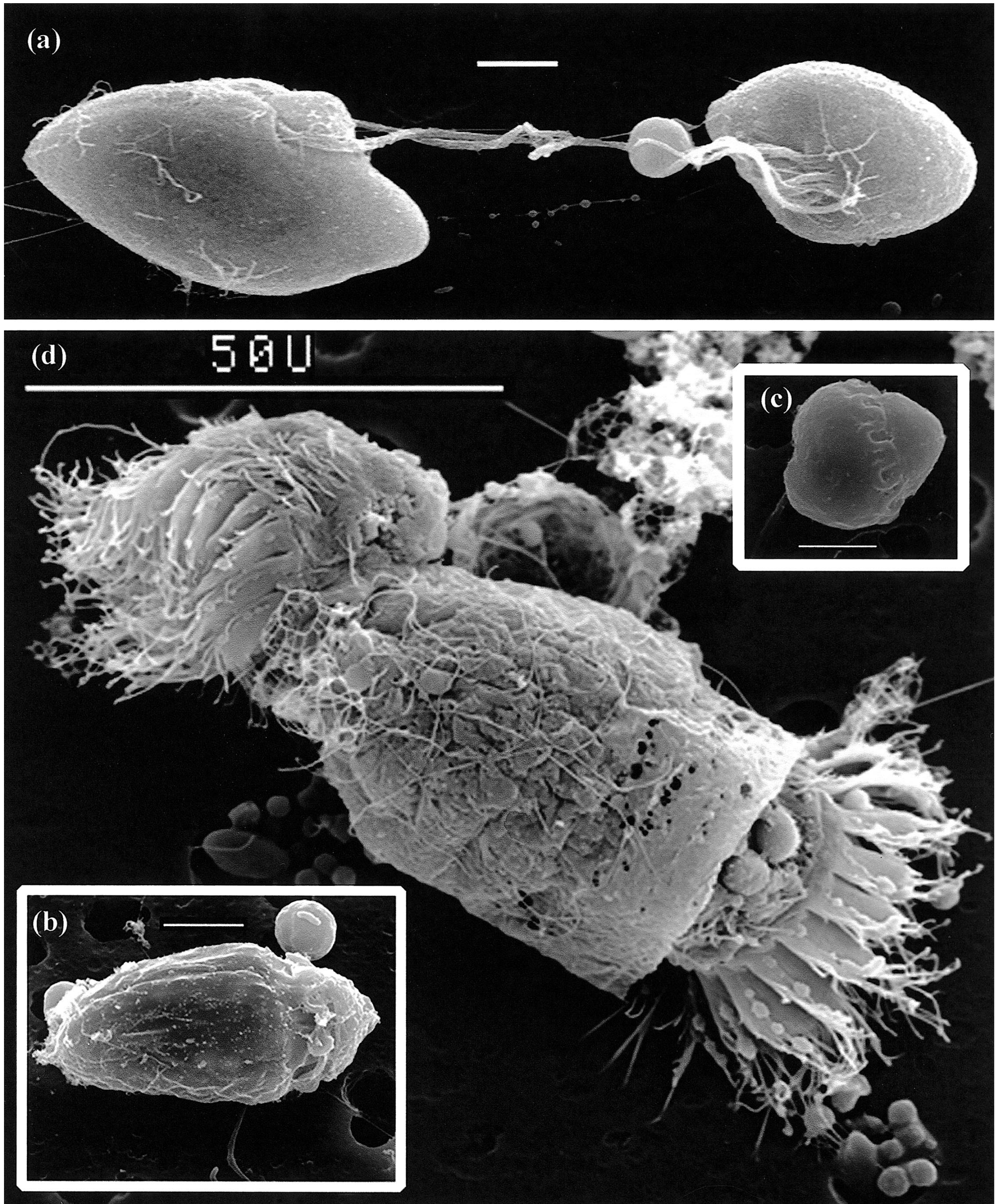

Fig. 1. Scanning electron micrographs of the four grazer taxa used most extensively in these experiments. Dinoflagellates (a–c) are scaled approximately to comparative sizes, whereas the ciliate (d) is about twice as large as illustrated relative to the dinoflagellates. (a) *Oxyrrhis marina,* showing two cells with entangled longitudinal flagella holding an *Emiliania huxleyi* prey cell. (b, inset) *Amphidinium longum* feeding on *E. huxleyi.* The dinoflagellate peduncle can be seen touching the prey cell, while a second prey cell is visible behind the hypocone. (c, inset) *Gymnodinium* sp. (d) *Coxliella* sp. (two individuals shown) feeding on *E. huxleyi* and cryptophytes. All dinoflagellate scale bars = 5 μm.

Table 2. *Emiliania huxleyi* cell surface carbohydrates as determined by lectin binding. Scoring of fluorescence binding/agglutination observations: − (none), + (weak), ++ (moderate), +++ (strong). ND, not determined.

Carbohydrate group	Lectin source species	Binding specificity*	*Emiliania huxleyi* strain results			
			LL-1	LL-2	HL-1	HL-2
Galactose	PNA *Arachis hypogaea*	β-D-galactose, β-gal (1 → 3)galNAc	−/+	−/+	−/−	ND/−
	ECA *Erythrina cristagalli*	β-gal(1 → 4)glcNAc	ND/−	−/ND	−/−	−/ND
N-acetyl galactosamine	SBA *Glycine maxima*	galNAc	−/−	−/−	−/−	−/−
Mannose	ConA *Canavalia ensiformis*	α-mannose, α-D-glucose	+/+	ND/+	+/ND	ND/−
Fucose	Lotus *Tetragonolobus purpureas*	α-L-fucose	−/−	−/−	−/−	−/−
Sialic acid	LPA *Limulus polyphemus*	NeuNAc, galNAc, glcNAc	−/−	−/−	−/−	−/−
	MAA *Maackia amurensis*	NeuNAc	−/−	−/−	+/−	−/−
Oligo-*N*-acetyl glucosamine	WGA *Triticum vulgaris*	$(glcNAc)_2$, NeuNAc	+++/++	+/++	+/++	+/++
	DSA *Datura stratimonium*	$(glcNAc)_2$	+/+	+/++	+/++	+/+
	STA *Solanum tuberosum*	$(glcNAc)_3$	+/+	+/++	+/++	+/+

* Sugar abbreviations: β-gal(1 → 3 or 4)galNAc = D-galactose-β(1 → 3 or 4)-*N*-acetyl-α-D-galactosamine; glcNAc = *N*-acetyl-β-D-glucosamine; galNAc = *N*-acetyl-β-D-galactosamine; NeuNAc = *N*-acetylneuraminic acid (sialic acid).

tively, these taxa represent a wide range of size, taxonomy, feeding methods (raptorial vs. filter-feeders, phagocytosis vs. myzocytosis), and prey selectivity.

Biochemical and size analyses—All *E. huxleyi* strains were noncalcifying in laboratory culture. *E. huxleyi* cell numbers and diameters were determined using a Coulter Counter model TA-II, calibrated with 3.46 μm polystyrene microspheres (PolySciences). Cultures of *E. huxleyi* were grown to midexponential phase in triplicate and sampled for bulk biochemical analyses by filtration onto pre-ashed glass fiber filters (effective pore size 0.7 μm).

Bulk lipid was measured gravimetrically according to Mayzaud and Martin (1975). Carbohydrates were extracted by the method of Fisher and Harrison (1996) and assayed by a modified Dubois et al. (1956) protocol using fructose as the standard. Samples for total protein were digested with 5 ml 1 M NaOH for 30 min at 80°C, neutralized with 1 M HCl, and analyzed with a commercial Coomassie Blue dye-binding assay (Pierce Chemical) standardized against bovine serum albumin. Total organic carbon and nitrogen contents were measured using a Leeman Labs model CEC440 elemental analyzer (acetanilide standard) according to Hedges and Stern (1984). *E. huxleyi* cell diameters were determined as equivalent spherical diameter using a Coulter Counter model TA-II. DMS and DMSP measurement techniques are described in Wolfe et al. (2002).

Cell surface carbohydrates were assessed qualitatively by lectin binding assays using a variety of fluorescein isothiocyanate (FITC)-labeled lectins (Sigma or EY Laboratories), recognizing the sugars mannose, fucose, and galactose; oligomers of *N*-acetyl glucosamine; and sialic acid (Table 2). Lectins were used as working solutions of 0.5–1 mg ml^{-1} in 0.01 M phosphate-buffered saline (0.1 M NaCl). To observe binding by green fluorescence, we filtered 1–5 ml of glutaraldehyde-preserved cells onto polycarbonate membrane filters (Osmonics), rinsed the filters with filtered seawater to remove excess glutaraldehyde, then applied the lectins (final conc. 20–50 μg ml^{-1}) to the filter for 3–5 min. The lectin solution was subsequently filtered, and the sample was rinsed again before mounting in oil and examining by epifluorescence microscopy with excitation at 450–480 nm. Cell agglutination was assayed by centrifuging 50 ml of unfixed exponential-phase *E. huxleyi* culture for 5 min, removing the upper 45 ml, and briefly vortexing the concentrated cells in the lower 5 ml. Of this concentrate, 400 μl was pipetted into cleaned 4-ml microtiter plate wells and lectins were added to 20 μg ml^{-1}; filtered seawater was added to control samples. Cultures were incubated in the dark at 16°C or at room temperature for several hours and examined by dissecting scope for agglutination. In both assays, binding was scored qualitatively as none, low, moderate, or high.

Rates of ingestion and growth on E. huxleyi—Ingestion rates of protist grazers were estimated from rates of *E. huxleyi* accumulation in grazer food vacuoles. Maintenance phytoplankton prey was removed from grazer stock cultures either by sieving grazers and resuspending them in ciliate medium for 12–24 h (*Coxliella* sp., *Metacylis* sp.) or by allowing grazers to remove prey until levels were sufficiently low that >90% of protist cells had empty food vacuoles (all other species).

Experiments were initiated by combining grazers and ciliate medium in polycarbonate bottles (duplicate or triplicate)

to reach desired concentrations (5–15 ciliates ml^{-1}, 25 to several 100 dinoflagellates ml^{-1}). *E. huxleyi* stock cultures used for experiments were in midexponential phase (densities 1–5 × 10^5 cells ml^{-1}), and cells were added to experiment bottles to achieve initial concentrations of 5 × 10^4 cells ml^{-1} (~400 μg C L^{-1}). Bottles were incubated at 15°C in the dark. For ingestion rate determinations, experimental bottles were sampled at regular intervals, ranging from 5 min for voracious feeders such as the tintinnids to several hours for slower feeders such as *Gymnodinium* sp. Subsamples were preserved in glutaraldehyde (final conc. 0.5%), stained with DAPI, allowed to sit overnight in darkness at 4°C, then filtered (1.0-μm pore size polycarbonate filters), slide-mounted, and frozen (−20°C) for later microscopic analysis. At least 100 individual grazers per slide were examined under blue light excitation, either for presence or absence of ingested *E. huxleyi* cells or for number of ingested *E. huxleyi* cells per grazer (*see below*).

To estimate growth rates of protist grazers, additional samples were removed from experimental bottles initially and every 24 h for at least 3 d. In some cases, a control treatment consisting of grazers with no added food was also included in growth experiments. Samples were preserved in acid Lugol's (final conc. 10% for ciliates and 2% for dinoflagellates) and settled in 10-ml chambers, and grazer cells were enumerated in their entirety using an inverted microscope. For ciliates, >50 and, for dinoflagellates, >100 cells per settled sample were counted. *Coxliella* sp. had to be preconcentrated using a sieving–back rinse method in order to achieve >50 cells in each 10-ml sample. Preliminary tests showed that this preconcentration method did not result in ciliate losses, provided 10% acid Lugol's was used as the rinse solution.

Ingestion rates were calculated in one of two ways. For grazer species that did not contain individually countable *E. huxleyi* cells (including *A. longum,* which ingests cell contents using a peduncle, and *Coxliella* sp., which ingests large numbers of *E. huxleyi* per minute), grazers were simply scored as "feeding" (i.e., containing *E. huxleyi*) or "nonfeeding" (i.e., food vacuoles empty). Ingestion rates were estimated from the slope of the regression of "percent population with ingested prey" versus time (units: percent induced h^{-1}). The percentage of the grazer population feeding at saturation was also estimated. For all other species, the number of *E. huxleyi* cells in each grazer was determined, and feeding rates were estimated as the slope of the regression of (average number *E. huxleyi* per grazer) versus time (units: cells $grazer^{-1}$ h^{-1}). Similarly, the average number of *E. huxleyi* per grazer at feeding saturation was also estimated. Note that all ingestion rates reported here are more precisely considered rates of feeding induction, as all grazer populations were starved before prey addition.

Population growth rates of ciliates and heterotrophic dinoflagellates were estimated from the slope of the regression of ln(grazer concentration) versus time (units: d^{-1}). Variations in size of individual grazer cells over the incubation time course were not accounted for in growth rate estimates.

Dual-prey experiment—An experiment was conducted to determine whether the presence of a high-lyase *E. huxleyi* strain would prevent ingestion of a "high-quality" prey species. The cryptomonad *Rhodomonas salina,* always ingested readily by the dinoflagellate *A. longum,* was combined in varying proportions (in duplicate) with strain HL-2, and the mixture was offered to *A. longum.* Control treatments consisted of each phytoplankton species alone. Because *R. salina* has a biovolume approximately four times that of *E. huxleyi,* mixtures were offered as "*E. huxleyi* equivalents"; that is, a 50 : 50 mixture of the two consisted of 1 : 4 parts *R. salina* : *E. huxleyi* by cell number, but equal parts by cell volume. Total cell density in each bottle was 5 × 10^4 *E huxleyi* equivalents ml^{-1}. Bottles were sampled at 0, 30, 60, and 90 min for epifluorescent observation of *A. longum* food vacuole contents, as described above. Appearance of *R. salina* and *E. huxleyi* in food vacuoles, including mixtures of the two prey types in a single vacuole, could be identified readily based on their characteristic autofluorescence (orange and red, respectively).

Amphidinium longum *growth inhibition experiment*—To test whether exposure to high-lyase *E. huxleyi* strains would inhibit subsequent growth on a high-quality phytoplankton diet, we conducted a growth rate experiment with *A. longum.* Duplicate 200-ml bottles were established for each of five diet treatments: the four *E. huxleyi* strains singly (at 5 × 10^4 cells ml^{-1}, equivalent to ~400 μg C L^{-1}) as well as a mixture of the high-quality prey species *Isochrysis galbana* and *R. salina* (at 2.5 × 10^4 and 1 × 10^4 cells ml^{-1}, respectively, so that each contributed ~200 μg C L^{-1}). *A. longum* was added, and each bottle was sampled initially and every 24 h for three subsequent days to establish growth rates (as described above) and to allow adaptation to the five diet treatments. After 3 d incubation, 32-ml volumes from each bottle were transferred to 320-ml bottles, the mixed *I. galbana*/*R. salina* diet (as above) was added to each bottle, and the bottles were filled to the brim with ciliate media so that *A. longum* were diluted to 10% of their original concentration and all treatments now had the high-quality diet at 400 μg C L^{-1} total. Samples for *A. longum* concentration were taken immediately and at 24-h intervals for another 4 d to establish growth rates on the high-quality diet following exposure to the various initial diet treatments. Population growth rates were calculated as described above for three time intervals: (i) days 0–3 (growth on the experimental diet), (ii) days 3–5 (growth during acclimation to the *I. galbana*/*R. salina* mixture), and (iii) days 5–7 (acclimated growth on *I. galbana*/*R. salina*).

Results

E. huxleyi *size and biochemical composition*—All four *E. huxleyi* strains were noncalcifying in our laboratory cultures, motile, and similar in size except for HL-1, whose cell diameter averaged 19% greater than the others (Table 1). Strains grew at nearly identical rates under saturating light and nutrient conditions (Wolfe et al. 2002), although HL-1 consistently exhibited a longer lag phase after transfer.

Lipid, carbohydrate, and DMSP content of HL-1 were higher than those of the other strains, as expected, given the larger volume of these cells (Table 1); however, cell volume–normalized content (fg μm^{-3}) of major biochemicals was

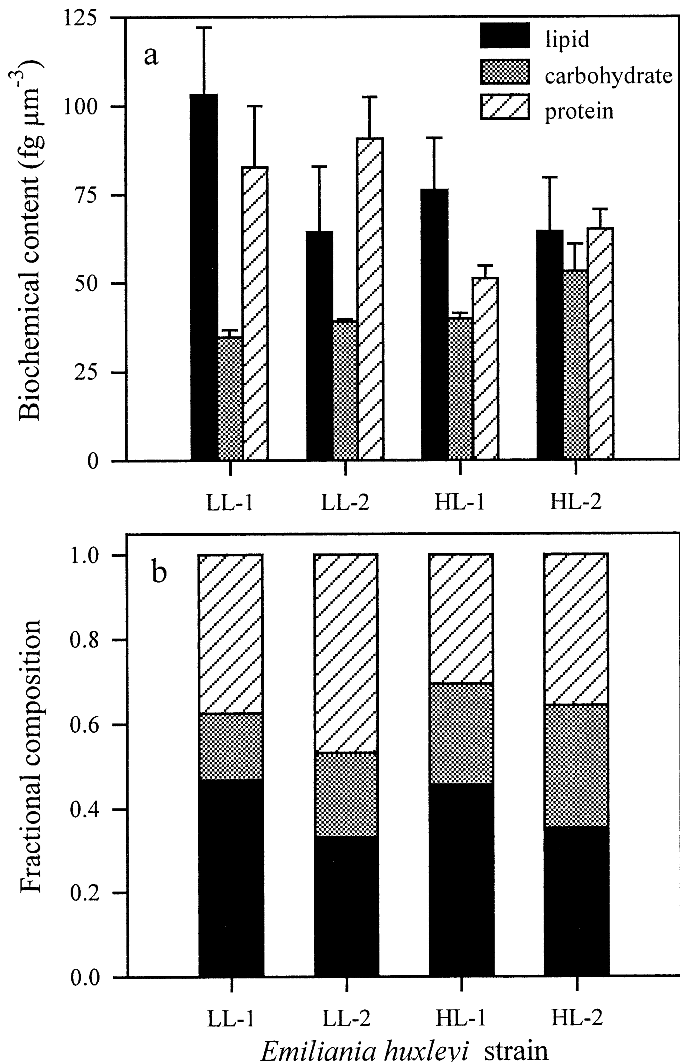

Fig. 2. Bulk biochemical content as (a) biochemical density (fg μm^{-3} cell volume) and (b) fraction of total lipids + carbohydrates + proteins in four strains of *Emiliania huxleyi.*

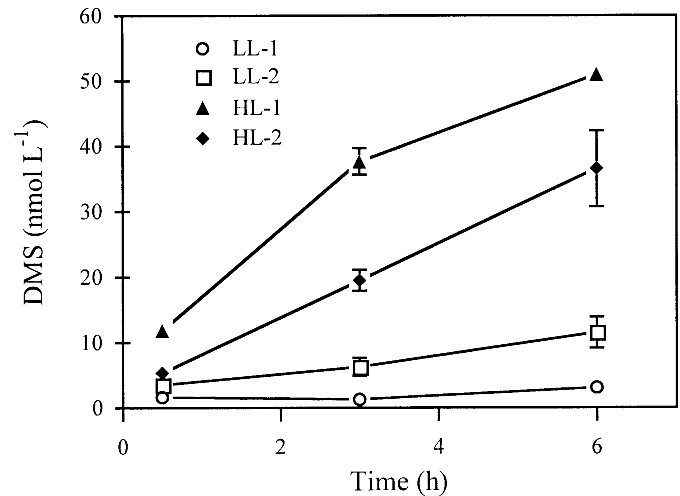

Fig. 3. Production of DMS during feeding by *Oxyrrhis marina* on four strains of *Emiliania huxleyi. O. marina* fed at approximately equal rates on all four *E. huxleyi* strains. *E. huxleyi* strains with low DMSP lyase activity (open symbols) and high DMSP lyase activity (filled symbols).

higher in some of the other strains (Fig. 2a). Although LL-1 had somewhat lower organic C and N content, all had similar C : N ratios (average 5.14 by weight, range 4.84–5.40). DMSP per unit cell volume was also similar (16–19 fg μm^{-3}) for all four strains. Comparison (one-way ANOVA) of HL versus LL strains showed that HL strains had more carbohydrate ($p = 0.028$) and less protein ($p = 0.001$) per unit cell volume than LL strains. Differences in protein content were larger than differences in carbohydrate content (Fig. 2b): HL strains averaged 33% lower in protein, and 26% higher in carbohydrate. Volume-specific lipid content varied among strains but was not related to DMSP lyase activity ($p = 0.452$).

In vitro DMSP lyase activity was consistently low in LL strains, whereas activity in HL strains was variable but always 10- to 100-fold higher (Table 1). These results are qualitatively similar to those found previously with different batches of the same cultures (Table 1, *see also* Wolfe et al. 1997; Steinke et al. 1998). Differences between our lyase activity measurements and those reported previously might be due in part to calibration differences and to suboptimal buffer conditions. In addition to directly measuring DMSP lyase activity, we confirmed production of equimolar DMS and acrylate by strain HL-1 (Wolfe et al. 2002). In further agreement with previous findings (Wolfe et al. 1997), *O. marina,* the only studied grazer that ingests all strains equally, activated DMS production when fed *E. huxleyi* (Fig. 3). Production of DMS during grazing was proportional to prey DMSP lyase activity, providing additional confirmation of strain lyase activity differences.

All *E. huxleyi* strains had similar cell surface carbohydrates, as indicated by lectin binding (Table 2). All strains reacted positively with the lectins WGA, DSA, and STA (Table 2), which bind *N*-acetyl glucosamine oligomers. Other lectins generally failed to react, except for very weak binding of ConA (strains LL-1, LL-2, HL-1), PNA (both LL strains), and MAA (strain HL-1). FITC labeling of glutaraldehyde-fixed cells gave results similar to agglutination of live cells, suggesting no preservation artifacts.

Feeding on E. huxleyi *alone*—Five out of the six protist grazer species tested showed significantly reduced feeding levels on HL relative to LL *E. huxleyi* strains (Table 3). This response was observed for three ciliate species (two tintinnids and a naked choreotrich) and two heterotrophic dinoflagellate species, one a phagocytotic feeder (*Gymnodinium* sp.) and one a myzocytotic (peduncle) feeder (*A. longum*). *O. marina,* a phagocytotic dinoflagellate, fed equally on all four *E. huxleyi* strains.

For affected grazer species, feeding reductions were observed both as reduced rates of particle uptake during the initial stages of the incubations and as reduced feeding levels (percentage of grazer population feeding or average number of prey cells per grazer) once prey uptake was saturated (Table 3). We emphasize that these reduced feeding rates

Table 3. Feeding rates of heterotrophic protists on four strains of *Emiliania huxleyi,* with date of experiment initiation. Feeding was estimated two ways: method a, from the increase in *E. huxleyi* cells per grazer over time (induction: cells ingested individual^{-1} h^{-1}) or the average number of *E. huxleyi* per grazer at feeding saturation (cells individual^{-1}), and method b, from the percentage of population with ingested cells. For method b, rates of induction (increase in percentage of population feeding over time) are as reported % induced hr^{-1}, whereas saturation feeding levels are reported as % feeding. All values are averages (1 SD) of duplicate treatments (triplicate for all *Metacylis* and *Coxliella* Jun 00 experiments) nd, not determined. % reduction is the average percent decrease in feeding on high-lyase strains relative to low-lyase strains for experiments in which reductions were significant (na, not applicable); *p*-values are for one-way ANOVA tests of feeding on low- versus high-lyase strains.

				Feeding rate					
Grazer	Date	Method	Mode	LL-1	LL-2	HL-1	HL-2	% red.	*p*
Ciliates									
Coxliella sp.	Jun 00	a	Induction	nd	81.2(12.4)	63.3(14.8)	nd	na	0.184
	Jun 00	a	Saturation	nd	14.2(1.4)	11.1(0.4)	nd	22	0.019
	Jul 99	b	Saturation	87.0(0.0)	89.5(0.7)	79.0(1.4)	79.0(2.8)	11	0.004
Strombidinopsis sp.	Mar 99	a	Saturation	19.8(0.8)	20.1(0.2)	13.5(0.3)	15.6(0.4)	27	<0.001
Metacylis sp.	Jul 01	a	Induction	nd	83.4(3.3)	29.6(3.4)	nd	65	<0.001
	Sep 01	a	Saturation	8.5(0.4)	nd	3.1(0.8)	nd	64	<0.001
	Nov 01	a	Saturation	nd	7.9(0.3)	nd	6.5(0.3)	18	0.007
Dinoflagellates									
Amphidinium longum	Dec 98	b	Induction	3.3(0.3)	5.6(0.2)*	0.3(0.1)	0.1(0.03)	96	<0.001
			Saturation	73.8(0.5)	91.9(2.0)	5.8(0.1)	2.1(0.7)	95	<0.001
Oxyrrhis marina	Aug 99	a	Induction	12.7(0.5)	14.1(3.1)	10.1(0.7)	13.7(1.6)	na	0.368
			Saturation	13.4(0.8)	13.9(0.2)	14.3(0.4)	14.3(0.1)	5†	0.089
Gymnodinium sp.	Feb 99	a	Induction(i)‡	0.31(0.02)	0.31(0.08)	0.29(0.07)	0.11(0.14)	na	0.177
			Induction(ii)‡	0.19(0.03)	0.14(0.02)	0.02(0.09)	−0.07(0.06)	94	0.004
			Saturation	1.28(0.17)	1.19(0.20)	1.29(0.16)	0.60(0.03)	na	0.241
	Aug 99	a	Induction	0.15(0.00)	0.12(0.05)	0.07(0.11)	0.03(0.02)	63	0.078

* Minimum estimate (vacuoles saturated during first sampling interval).
† In this instance, food vacuoles contained more high-lyase than low-lyase prey, on average.
‡ Induction(i), feeding rates 0–3 h after *E. huxleyi* addition; induction(ii), feeding rates 3–7.5 h after addition.

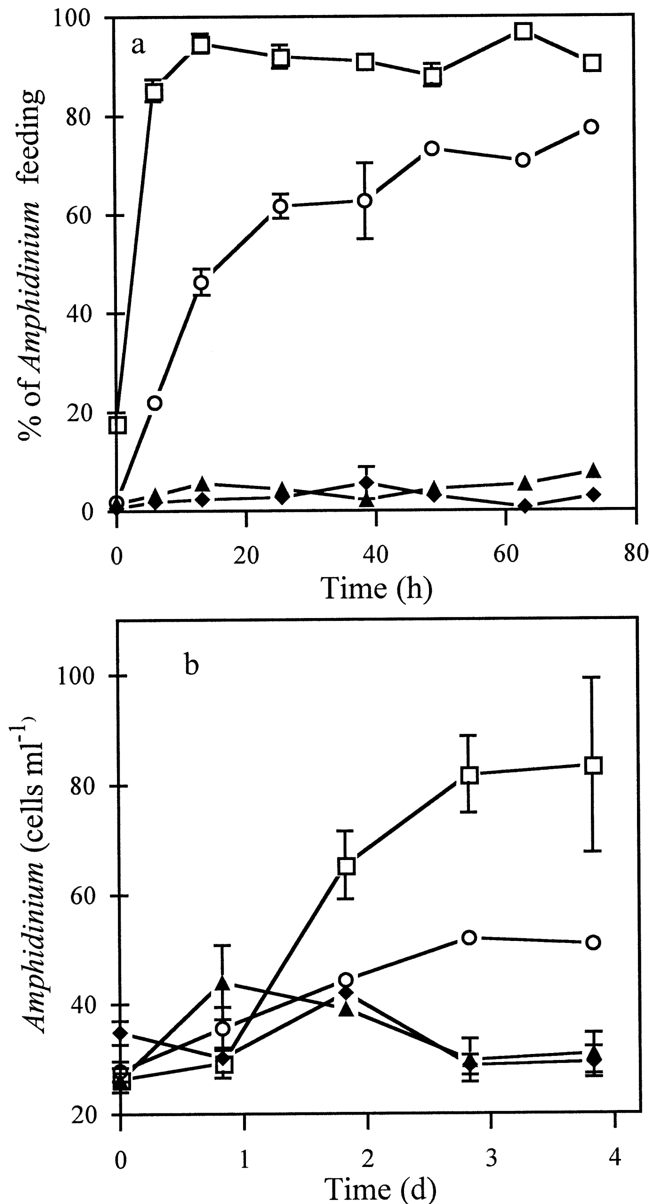

Fig. 4. Responses of (a) *Amphidinium longum* feeding activity and (b) population density to four strains of *Emiliania huxleyi* (all offered at 50,000 cells ml^{-1}). Data points are averages of duplicate flasks, with error bars showing range of values. Symbols as in Fig. 3.

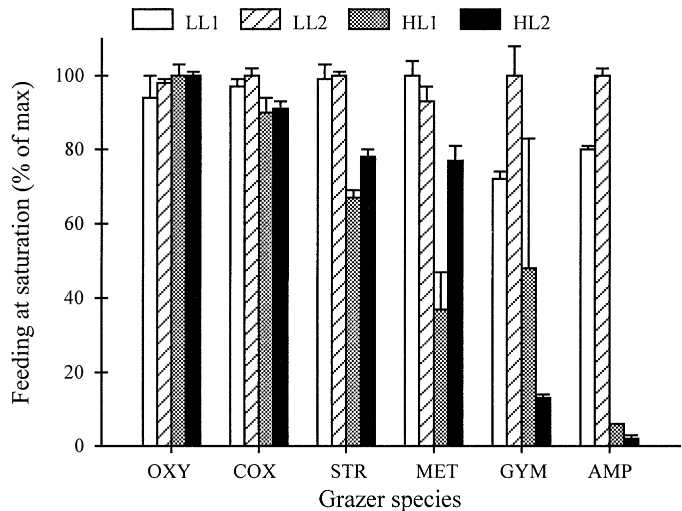

Fig. 5. Feeding levels at saturation (mean ± SD, $n = 2$ or 3), normalized to the maximum value observed for each of six protist grazer species fed *Emiliania huxleyi* strains with low (LL1, LL2) and high (HL1, HL2) DMSP lyase activity. See Table 3 for a description of feeding rate measurement methods and units. OXY, *Oxyrrhis marina*; COX, *Coxliella* sp.; STR, *Strombidinopsis* sp.; MET, *Metacylis* sp.; GYM, *Gymnodinium* sp.; AMP, *Amphidinium longum.*

were observed in single-prey experiments (i.e., no alternate prey was available to the grazers).

Some grazer species showed larger strain-dependent feeding effects than others, with *A. longum,* as the most extreme case, feeding almost not at all on HL strains (Fig. 4a). *Gymnodinium* sp. was also strongly deterred by HL strains (Fig. 5), although the deterrence was variable depending on the stage of the experiment (Table 3). Rates of feeding in these two dinoflagellates also differed between the two LL strains, with LL-2 ingested considerably more readily than LL-1 (Fig. 5). (Note that feeding levels for *A. longum* on LL-2 are minimum estimates because of the sampling time frame of the experiment; *see* Fig. 4a.) The three ciliate species all showed reduced grazing on HL strains (Table 3), but to a lesser extent than *A. longum* and *Gymnodinium* sp. (Fig. 5). The dinoflagellate *O. marina* showed the smallest prey strain–dependent differences in feeding rate; the data in fact suggest slightly higher rates of feeding on HL strains (Table 3).

Growth on E. huxleyi *alone*—Growth of protists on the four *E. huxleyi* strains was consistent with feeding rates (Table 4). The grazer species least deterred by HL strains, *Coxliella* sp. and *O. marina,* grew on all four strains. *Coxliella* showed no lyase-related growth response, whereas *O. marina* grew at very low rates (<0.1 d^{-1}) on LL strains, and more rapidly (0.18–0.37 d^{-1}) on HL strains. *Strombidinopsis,* which exhibited an intermediate level of feeding deterrence, grew only on LL strains, whereas *Gymnodinium* sp. did not appear capable of significant growth on a diet of *E. huxleyi* alone regardless of strain chemistry. For both of these grazers, mortality was greater in the unfed control treatments than in any of the four *E. huxleyi* treatments (Table 4), indicating that some modest nutritional benefit was derived from all strains. *A. longum,* the grazer most strongly deterred by HL strains, grew at rates proportional to ingestion (Fig. 4b): fastest on LL-2, more slowly on LL-1, and not at all on HL strains.

Dual-prey experiment—The results of the dual-prey experiment (Fig. 6) demonstrate that exposing *A. longum* to strain HL-2 did not result in acute toxicity or even the reduction of feeding potential. Rather, we observed strong selectivity. *A. longum* fed exclusively on *R. salina* even at the highest *E. huxleyi* : *R. salina* ratio tested (Fig. 6). The rate

Table 4. Population growth rates for protists fed four strains of *Emiliania huxleyi* and control (no food) diets. Values are averages, with 1 SD in parentheses. nd, not determined.

Grazer	Date	*n*	Growth rate (d^{-1})				
			Control	LL-1	LL-2	HL-1	HL-2
Ciliates							
Coxliella sp.	Sep 98	1	nd	0.33	0.56	0.32	0.42
	Jul 99	2	−0.19(0.06)	0.43(0.16)	0.35(0.09)	0.61(0.13)	0.28(0.02)
Strombidinopsis sp.	Mar 99	2	−0.19(0.14)	0.26(0.10)	0.10(0.07)	−0.06(0.06)	0.02(0.02)
Dinoflagellates							
Amphidinium longum	Oct 98	2	nd	0.13(0.05)	0.51(0.005)	−0.13(0.13)	−0.05(0.04)
	Nov 98	2	nd	0.07(0.03)	0.16(0.02)	−0.05(0.01)	−0.02(0.002)
Oxyrrhis marina	Jul 99	2	−0.04(0.06)	0.07(0.05)	0.03(0.05)	0.18(0.10)	0.37(0.03)
Gymnodinium sp.	Aug 99	2	−0.09(0.02)	−0.03(0.02)	−0.07(0.05)	−0.05(0.03)	0.01(0.03)

of feeding induction was the same for all treatments containing *R. salina*; apparently, the presence of high proportions of *E. huxleyi* HL-2 did not impair feeding on the preferred species at these high prey abundances. Consistent with previous experiments, there was virtually no ingestion of HL-2 even when it was the sole available prey.

Amphidinium longum *growth inhibition experiment*—There was no negative effect of exposure to HL strains on subsequent *A. longum* growth (Fig. 7). Postacclimation (interval iii) growth rate on the high-quality *I. galbana*/*R. salina* mixture was approximately 0.25 d^{-1} (range 0.16–0.37 d^{-1}) whether *A. longum* had previously been offered LL-2, HL-1, HL-2, or the same high-quality mixture (Fig. 7e). Curiously, growth on the mixture was reduced to 0.05 d^{-1} when *A. longum* had previously been fed LL-1, the strain supporting moderate ingestion and growth rates. This suggests a cost to dietary switching under some circumstances. There was also a high *A. longum* mortality rate (−0.9 d^{-1}) during the 2-d acclimation phase following transition from HL-1 to the mixed diet (interval ii, Fig. 7d).

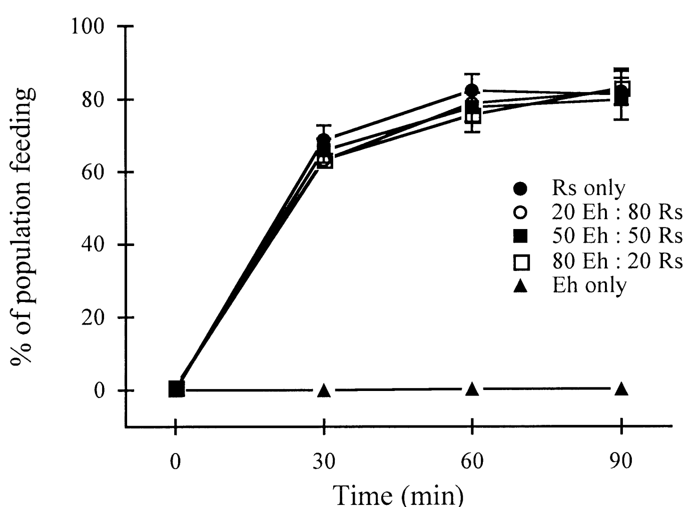

Fig. 6. Feeding response (as percentage of grazer population with ingested prey) over time for *Amphidinium longum* fed mixtures of the high-quality prey *Rhodomonas salina* and *Emiliania huxleyi* strain HL-2. Legend shows ratios of *E. huxleyi* : *R. salina* by cell volume (*see text*).

Discussion

Overview—We observed consistently lower protist feeding rates on HL *E. huxleyi* strains (Fig. 5). This reduction in feeding was observed for both heterotrophic dinoflagellates and ciliates and included species that feed by both phagocytosis and myzocytosis. Reductions in feeding on HL strains were observed during both feeding induction (as accumulation rates of prey in food vacuoles of a previously starved grazer population) and feeding saturation (as food vacuole content once food vacuoles were filled), and results were consistent among experiments conducted over a period of several years. For most studied protist species, population growth rates were low (Table 4), probably because of the monospecific diet and the dark incubation conditions. Observed growth rate reductions in the presence of HL strains were, however, consistent with feeding responses, demonstrating that feeding deterrence has the potential to be realized as reduced population growth for some protist species. Together, these findings demonstrate that the negative response to HL strains is widespread among protist taxa, persistent in time, and characteristic of all portions of the feeding response.

Our results are consistent with feeding deterrence caused by or related to high DMSP lyase activity. Data presented in a companion paper (Strom et al. 2003) show that DMSP itself inhibits protist feeding, further supporting the hypothesis that sulfur chemistry is involved in phytoplankton chemical defense. In further support of this hypothesis, Olson and Strom (2002) measured reduced microzooplankton grazing rates on the <8-μm phytoplankton size fraction during a Bering Sea *E. huxleyi* bloom. Many other nuisance bloom-forming taxa (e.g., *Alexandrium, Prorocentrum, Phaeocystis, Aureococcus, Chrysochromulina*) have high intracellular DMSP concentrations, and some exhibit high levels of DMSP lyase as well (Keller et al. 1989*a,b*; Wolfe et al. 2002). Although *E. huxleyi* contains high levels of DMSP, all four strains had similar intracellular concentrations, and the relationships among DMSP lyase activity, DMSP release

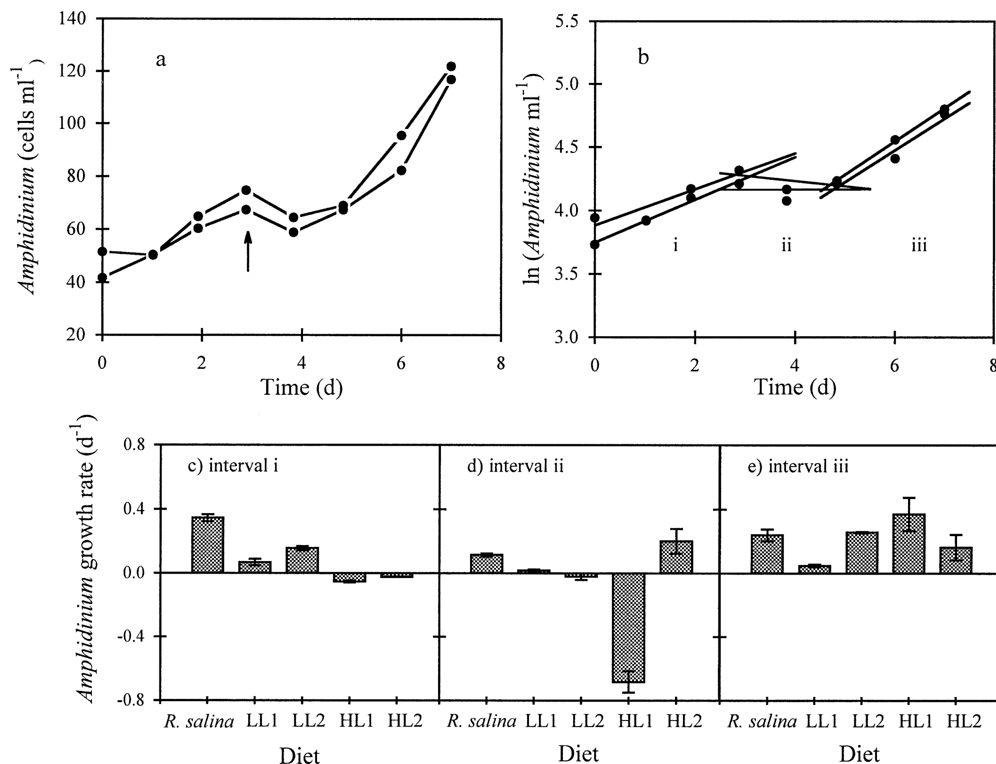

Fig. 7. Population growth rates of *Amphidinium longum* exposed to four strains of *Emiliania huxleyi* for 3 d, then switched to a diet of the high-quality prey mixture *Rhodomonas salina* + *Isochrysis galbana.* Change in (a) population density over time and (b) natural log-transformed population density showing experiment intervals (i: 3-d exposure to "treatment" diet; ii: 2-d lag phase following switch to high-quality diet; iii: 2-d realized growth on high-quality diet). Data for duplicate flasks (treatment diet: *E. huxleyi* LL-2) are shown. Arrow in (a) shows time of diet switch; slopes of regression lines in (b) used as estimates of population growth rate (d^{-1}) during the three intervals. (c–e) Population growth rates of *A. longum* (mean and range, $n = 2$) for the three experiment intervals are shown. Diet indicated is treatment diet offered during interval i.

by algal cells, and other possible chemical signals are unclear at this time.

Differences in cellular protein and carbohydrate content also correlated with feeding reductions in our study, so that reduced feeding on HL relative to LL strains could be interpreted as a response to prey nutritional quality. However, higher growth rates of the heterotrophic dinoflagellate *O. marina* on HL strains indicate that these were actually nutritionally superior to the LL strains for grazers willing and able to eat them. *O. marina* was the only grazer that did not show a negative response to high-lyase *E. huxleyi.* This dinoflagellate is known to be catholic in its feeding tastes, ingesting bacteria, inert particles, and a wide range of phytoplankton species and even feeding cannibalistically when densities are high (Droop 1966; Schumann et al. 1994). This suggests that *O. marina* is less responsive than other protists to cues associated with food quality. Enhanced chemical defense of prey types that are otherwise more nutritious is a feature of both terrestrial plants and seaweeds. Furthermore, most chemical defenses studied to date show a similar range in effectiveness, from nearly complete deterrence of some grazers to no effect on others (Stachowicz 2001).

The feeding deterrence we observed appears to be due to grazer behavioral responses rather than to HL strain toxicity. There are several lines of evidence in support of this. Grazer species showing little or no deterrence grew equally well on all four *E. huxleyi* strains or even, in the case of *O. marina,* fastest on HL strains. These growth responses show that, at least for some protist grazers, HL cells are not toxic in the short term. For more strongly deterred grazer species (*Strombidinopsis* sp., *Gymnodinium* sp., *A. longum*), exposure to HL strains did not cause mortality exceeding that in starved controls (Table 4), nor did such exposure reduce growth potential, as evidenced when *A. longum* was subsequently offered a high-quality prey mixture (Fig. 7). Similarly, *A. longum* was able to ingest *R. salina* at high rates even in the presence of a 4:1 mixture of HL-2 and *R. salina* (Fig. 6). Finally, neither *A. longum* nor *Coxliella* sp. exhibited gross changes in swimming behavior upon addition of HL cells to healthy grazer cultures (not shown). Indeed, for *A. longum,* the most strongly deterred grazer species studied, we found no evidence that grazers even need to ingest HL cells in order to avoid them (Fig. 4a).

How do grazer and E. huxleyi *cells interact?*—If grazers need not ingest an *E. huxleyi* cell to determine its palatability, then how is information exchanged between grazer and prey? One possibility is that grazers are responding to a dis-

solved cue associated with prey cells (Wolfe 2000). A generalized negative cue associated with high-lyase *E. huxleyi* could not be uniformly dispersed throughout the seawater medium because *A. longum* fed actively on high-quality prey (*R. salina*) in the presence of HL cells (Fig. 6). Dissolved cues could be closely associated with the area around individual prey cells, and there has been considerable speculation that phytoplankton cells in general exist within a ''phycosphere'' of emitted, dissolved substances (e.g., Azam and Ammerman 1984). In additional research, we have shown that added DMSP causes feeding reductions in protists at low levels (μmol L^{-1}, Strom et al. 2003). Although bulk DMSP levels in seawater are typically less than this (e.g., Cantin et al. 1996; Dacey et al. 1998), higher local concentrations could easily be achieved by a pulse-like release from a prey cell (Wolfe 2000).

A second possible means of information exchange between grazers and prey is via prey surface characteristics. Once contacted by a grazer cell, prey surface compounds might signal ''don't eat me'' (Wolfe 2000); high-speed video photography and behavioral assays have shown that planktonic protists can indeed respond to cell surface-associated cues (Taniguchi and Takeda 1988; Snyder 1991; Sakaguchi et al. 2001). Although previous work with lectins has demonstrated that closely related phytoplankton species or even strains can have widely differing cell surface sugars (Costas and Rodas 1994; Hori et al. 1996), our lectin binding results show no major differences among the four *E. huxleyi* strains in this regard (Table 2). Distinguishing cell surface cues, if any, must lie in other compound classes for these *E. huxleyi* strains.

Consideration of protist feeding mode supports the idea that protist grazers are responding to chemical cues on or near prey cell surfaces. *A. longum,* the most strongly deterred species, feeds by myzocytosis, piercing and ingesting the contents of prey cells using a feeding tube (Calado et al. 1998). In contrast, the three species least deterred by HL strains all consume large numbers of *E. huxleyi* cells per hour, suggesting less time for or a lower investment in sensing and rejecting individual prey cells. The extent of feeding deterrence by HL *E. huxleyi* and other unpalatable prey species might interact with grazer feeding mode.

Why would protist grazers reduce feeding?—In our experiments, feeding reductions translated into reduced population growth rates for at least two grazer species. On one hand, this suggests that ingestion of high-lyase prey must confer a substantial cost, to make avoidance of those prey worthwhile in terms of individual fitness. On the other hand, our growth rate experiments offered no prey choices, whereas in nature, protists have a variety of prey available to them. Thus, the severe reductions in population growth exhibited in our experiments might rarely be realized in the sea, and avoidance of suboptimal prey might actually increase the probability that grazers could ingest and utilize high-quality prey when encountered in a mixture. In a mixed-prey environment, the costs and benefits of avoidance are likely to depend on relative time and energy required for seeking, ingesting, and digesting prey, as well as the abundance and composition of the prey community (Stephens and Krebs 1986).

Assuming that feeding inhibition in our experiments was in response to a chemical defense, how might such a defense function? For a chemical defense to retain its effectiveness in the evolutionary sense, there must be a cost associated with ingestion of the defense chemical (Berenbaum 1995). There is no evidence that either DMS or acrylate are acutely toxic to protists or metazoans. Both are substrates for bacterial growth (Kiene and Bates 1990; Noordkamp et al. 1998). It has been hypothesized that high concentrations of acrylic acid (protonated acrylate, the form assumed by the compound at low pH levels representative of some gut and food vacuole states) can destroy gut bacteria (Sieburth 1979 and references therein). Wolfe (2000) estimated that complete conversion of *E. huxleyi*–contained DMSP in protist food vacuoles could generate ~70 mmol L^{-1} acrylic acid, a concentration known to be toxic to some microorganisms (Thijsse 1964). Following this reasoning, it is possible that feeding reductions are a response to the potential negative consequences of vacuoles full of acrylic acid. Grazers that showed little or no feeding deterrence either might be resistant to acrylic acid effects or might have experienced negative consequences that we did not measure, such as decreases in gross growth efficiency (e.g., because of reduced assimilation efficiency). Alternatively, there might be other, as yet unidentified deleterious compounds associated with HL *E. huxleyi* cells.

Summary—We have demonstrated large strain-dependent differences in protist grazing on the coccolithophorid *E. huxleyi.* Reductions in grazing rate in single-prey experiments were variable in extent but nearly always associated with high DMSP lyase activity in *E. huxleyi* strains; large reductions in grazing usually led to large reductions in protist population growth rates. Numerous lines of evidence indicate that grazers were responding to preingestion cues, such as dissolved chemicals or prey cell surface properties, rather than to postingestion physiological consequences of prey composition. Related work (Strom et al. 2003) demonstrates that dissolved DMSP is itself a feeding inhibitor, supporting the idea that high DMSP lyase activity is associated with a chemical defense against grazers, although seemingly not through the direct mechanism of DMSP cleavage and product release. Prymnesiophytes, along with other small marine phytoplankters, are largely removed from marine waters by microzooplankton grazing. This suggests a strong selective pressure for the evolution of grazing resistance strategies such as chemical defense among marine phytoplankton. Grazing resistance could be an important determinant of the ability to form persistent phytoplankton blooms, and it is noteworthy that many bloom-forming dinoflagellate and prymnesiophyte species contain high levels of DMSP. The current challenges are to determine the mechanisms by which chemical signals influence protist–phytoplankton interactions and the extent to which these are important in natural planktonic communities.

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