Grazing-activated production of dimethyl sulfide (DMS) by two clones of *Emiliania huxleyi*

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Abstract

Emiliania huxleyi clones CCMP 370 and CCMP 373 produced similar amounts of dimethylsulfoniopropionate (DMSP) during axenic exponential growth, averaging 109 mM internal DMSP. Both clones had detectable DMSP lyase activity, as measured by production of dimethyl sulfide (DMS) during in vitro assays of crude cell preparations, but activities and conditions differed considerably between clones. Clone 373 had high activity; clone 370 had low activity and required chloride. For both strains, enzyme activity per cell was constant during exponential growth, but little DMS was produced by healthy cells. Rather, DMS production was activated when cells were subjected to physical or chemical stresses that caused cell lysis. We propose that DMSP lyase and DMSP are segregated within these cells and reaction only under conditions that result in cell stress or damage. Such activation occurs during microzooplankton grazing. When these clones were grazed by the dinoflagellate *Oxyrrhis marina,* DMS was produced; ungrazed cells, as well as those exposed to grazer exudates and associated bacteria, generated no DMS. Grazing of clone 373 produced much more DMS than grazing of clone 370, consistent with their relative in vitro DMSP lyase activities. DMS was only generated when cells were actually being grazed, indicating that ingested cells were responsible for the DMS formation. We suggest that even low levels of grazing can greatly accelerate DMS production.

Many marine phytoplankton synthesize dimethylsulfoniopropionate (DMSP) (Keller et al. 1989), a sulfonium compound that seems to be the main biological precursor for dimethyl sulfide (DMS). DMSP is widespread among taxa but seems to be particularly abundant in specific groups, such as the dinophyceae and prymnesiophyceae (Keller et al. 1989). It may accumulate to high concentrations (mM–M) within cells and may be the dominant sulfur compound by mass in some species (Matrai and Keller 1994).

The biological function of DMSP, and especially of its cleavage to DMS, acrylate, and a proton, is still not clear.

High concentrations of DMSP contribute to the osmotic balance of cells, and DMSP belongs to a class of compounds known as "compatible solutes," which seem to be less damaging to cellular activities than are inorganic ions (Kirst 1990). However, studies have found little evidence for short-term modulation of DMSP in response to osmotic stress (Dickson and Kirst 1986; Edwards et al. 1988), and DMSP is only one of a number of such solutes, all of which contribute toward overall osmoregulation. Specifically, it is not yet established that the cleavage of DMSP to DMS and acrylate has a primary role in osmotic adjustment or maintenance. Another function of very high solute concentrations, cryoprotection, has also been suggested for ice-algae that contain DMSP (Kirst et al. 1991), but this function does not seem to be general for the many nonpolar species that contain large amounts of this compound. It is likely that marine phytoplankton may utilize DMSP for other biochemical reactions, such as methyl transfer, as was suggested for the heterotrophic flagellate *Crypthecodinium* (*Gyrodinium*) *cohnii* (Ishida and Kadota 1968).

Studies of phytoplankton DMS production have largely

been motivated by its potential climatic impact (Charlson et al. 1987). Culture studies have focused mainly on environmental cues that may result in increased DMS emissions (Baumann et al. 1994; Vairavamurthy et al. 1985; Vetter and Sharp 1993), and field studies have focused on large blooms of high-DMSP titer species (Holligan et al. 1993; Matrai and Keller 1993; Stefels et al. 1995). In culture, production of DMS by healthy, axenic phytoplankton during exponential growth, such as by *Hymenomonas carterae* (Vairavamurthy et al. 1985) and *Phaeocystis pouchetii* (Stefels and van Boekel 1993), seems to be relatively rare. It is not clear that all algae that synthesize DMSP are able to cleave it to DMS (Steinke et al. 1996). The observations of DMS production with nonaxenic clones are complicated by evidence that many bacteria utilize DMSP and produce DMS (Kiene 1992; Kiene and Service 1991). Other microbial processes, such as mesozooplankton grazing of high-DMSP species, have been shown to generate DMS through zooplankter or bacterial enzymatic action (Dacey and Wakeham 1986). Studies of microzooplankton grazing have shown contradictory results: Wolfe et al. (1994) found that little DMS was produced during grazing by the dinoflagellate *Oxyrrhis marina* on *Emiliania huxleyi* (strain CCMP 370), but a similar study with *E. huxleyi* strain CCMP 379 showed increased production of DMS during grazing (Malin et al. 1994). Without an understanding of the function and mechanism of DMS production from DMSP, it has been difficult to predict when and where DMS is produced.

In this study we compare two axenic clones of *E. huxleyi*, CCMP 370 and CCMP 373, which both synthesize DMSP but differ in their abilities to convert it to DMS. We measured DMS and DMSP as well as in vivo and in vitro DMSP lyase activity during batch growth, following cell stress and injury, and also when cells were grazed by the dinoflagellate *O. marina* in order to gain insights into the mechanism and function of DMS formation by phytoplankton in the marine environment.

Methods

Culture growth conditions—Axenic *E. huxleyi* cultures were obtained from the Provasoli-Guillard National Center for the Cultivation of Marine Phytoplankton (CCMP, West Boothbay Harbor, Maine). Cultures were inoculated into 1-liter volumes of filtered, autoclaved seawater enriched with nutrients (f/2, Guillard and Ryther 1962) in polycarbonate bottles and incubated at 80–100 μmol m^{-2} s^{-1} under a 16:8 L/D cycle at 15°C. Cells were checked for bacterial contamination throughout experiments by epifluorescence microscopy following staining with acridine orange and by plating on 1% peptone agar plates. No bacteria were detected by either method, except in treatments where bacteria were introduced intentionally or with grazers. Bottles were capped and maintained with minimal headspace to avoid degassing of DMS during sampling. Bottles were rotated gently before sampling to distribute cells but were otherwise unshaken, and DMS samples were not taken until at least 5 min after rotation

to allow gas equilibration between water and headspace. Typical cell densities during grazing experiments were 5–30×10^3 ml^{-1}. During growth studies, cell densities reached 5–8×10^5 ml^{-1} in stationary phase.

Sulfur determinations—Sulfur analyses were made by gas chromatography using a Shimadzu GC-14 chromatograph equipped with a flame photometric detector. The column packing was Chromosil 330 (Supelco), operated isothermally at 60°C. Helium was the carrier gas and was also used for sample sparging. DMSP was analyzed as DMS by alkaline hydrolysis. DMS was introduced via headspace samples (0.1–100 μM samples) or following cryotrapping (0.1–1,000 nM samples). Detection limit was ~1 pmol sulfur. Other analytical details were the same as those reported by Wolfe et al. (1994), except that samples for DMSP (2 ml) were filtered under low vacuum (<5 mm of Hg) rather than by syringe to minimize cell breakage.

DMSP lyase assays—Phytoplankton cells were concentrated by centrifugation (in vivo tests: 4,000 × g for 20 min; in vitro tests: 20,000 × g for 10 min) at 15°C. The supernatent was removed by pipette, and the pellet was resuspended by gentle pipetting into 0.3–1 ml f/2 or buffer based on 50 mM 2-[N-morpholino] ethanesulfonic acid (MES) with 13–20 mM CaCl$_2 \cdot$2H$_2$O. For clone CCMP 370, this buffer was amended with 600 mM NaCl and 2 mM DL-dithiothreitol (DTT or Cleland's Reagent) and adjusted to pH 6.5. For clone CCMP 373, the buffer was amended with 0.1–0.5% (v/v) of the nonionic detergent polyoxyethylenesorbitan monooleate (Tween 80) and adjusted to pH 6.2. For storing frozen extracts, 10% (v/v) glycerol was also added; tests showed extracts were stable under such storage. For in vitro assays, cells resuspended in buffer were sonicated by brief (2×10 s) bursts while on ice.

DMSP lyase was assayed by adding DMSP-Cl [synthesized by the method of Larher et al. (1977) or obtained from Research Plus] to a sample of live cells or cell extract in buffer and incubating 295 μl in 1.8-ml glass screwcap vials with Teflon-coated septa. Whole-cell (in vivo) assays were incubated in the light at in situ temperatures (15°C). In vitro assays were incubated in a water bath at 30°C. DMS production was measured by headspace analysis (50 μl). Before adding DMSP, samples were monitored for endogenous DMS production for 10–20 min, then the vials were uncapped, 5 μl of a 60 mM stock DMSP-Cl solution were added (1 mM final concn), and the samples were immediately recapped with fresh, unpunctured septa and monitored again for 30–60 min. Typically, only 1–5% of the DMSP was converted during this time, so rates were nearly first-order even though substrate concentrations were not saturating. When necessary, the pH of the final solution was checked to verify that the reaction products did not acidify the solution. DMSP standards were prepared in NaOH for headspace calibration.

Cellular chlorophyll and fluorescence—Chlorophyll was extracted from GF/F-filtered cells (5–10 ml) with 90%

Table 1. Comparison of initial prey and predator densities for four grazing experiments. Numbers are means (or ranges, in the case of *Oxyrrhis marina*) of duplicate bottles.

Initial density (ml^{-1})	Exp. 1*	Exp. 2†	Exp. 3‡	Exp. 4§
E. huxleyi CCMP 373	13,810		9,310	10,390–10,400
E. huxleyi CCMP 370		14,920	7,930	
D. tertiolecta				22,160
O. marina	1,380	930	240–450	380–390

* *O. marina* feeding on *Emiliania huxleyi* CCMP 373.
† *O. marina* feeding on *E. huxleyi* CCMP 370.
‡ *O. marina* feeding on either *E. huxleyi* 373 or 370.
§ *O. marina* feeding on *E. huxleyi* 373 with or without *Dunaliella tertiolecta*.

acetone for 24 h, then measured by a Turner Designs 10-AU fluorometer (Strickland and Parsons 1972). In vivo fluorescence was measured by fluorometer.

Cell enumerations—Phytoplankton cells were enumerated by epifluorescence microscopy after staining with acridine orange, as by Wolfe et al. (1994). Whole-cell (in vivo) fluorescence was also used to monitor growth in some experiments. *O. marina* cells were enumerated live with a dissecting microscope (Wild M3Z) in 1–10 μl drops.

Grazing experiments—A culture of *O. marina* was maintained on *Dunaliella tertiolecta*. This prey produces minimal DMSP, can sustain high *O. marina* numbers (up to 40,000 ml^{-1}), and can be removed from culture by placing the prey and grazers in the dark at 15°C for several days, allowing *O. marina* to completely clear the prey from the bottles and reach a starved state.

E. huxleyi cultures were inoculated into f/2 and allowed to grow for several days, until densities were ~1–2×10^4 ml^{-1} as determined by epifluorescence microscopy or calculated from in vivo fluorescence. Concentrated grazer cultures were added to prey bottles; typical grazer densities were 200–1,000 ml^{-1}. Table 1 summarizes initial prey and predator densities for four feeding experiments utilizing clones 370, 373, and *D. tertiolecta* as prey. Because *O. marina* and *D. tertiolecta* cultures contained bacteria that might affect DMSP and DMS pools, filtrates of the concentrated *O. marina* and *D. tertiolecta* cultures were prepared by gravity filtration through 3-μm (*D. tertiolecta*) or 5-μm (*O. marina*) Nuclepore filters and added to controls in order to keep bacterial populations similar in all treatments. A few *D. tertiolecta* passed through the 3-μm filter, but no *O. marina* cells were observed to pass through 5-μm filters.

Grazing experiments were conducted at 80–100 μmol m^{-2} s^{-1} under a 16 : 8 L/D cycle at 15°C. Prey and predator cell numbers, DMS, and DMSP concentrations were measured every 6–12 h for 24–48 h. Exponential growth rates were calculated from log-transformed cell densities in prey-only control bottles. Net growth rates (production
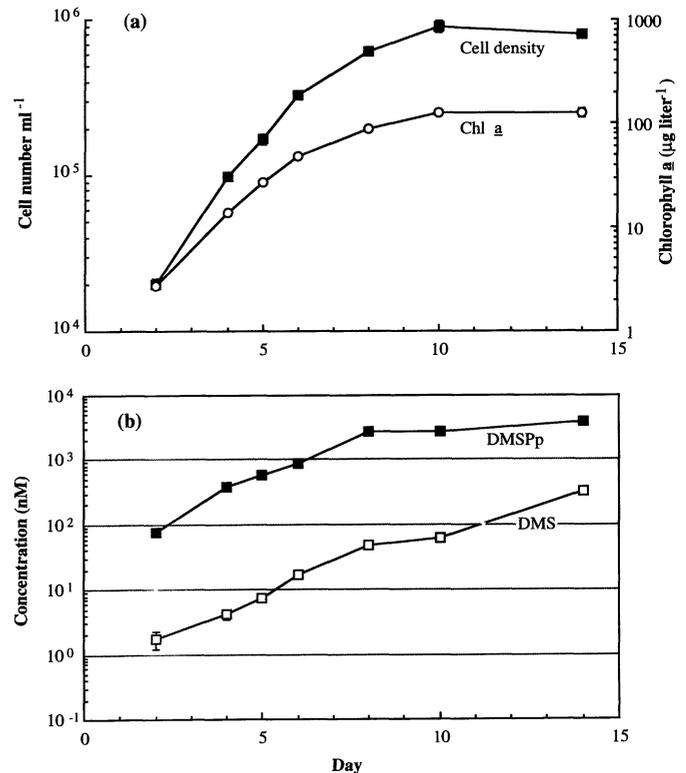

Fig. 1. *Emiliania huxleyi* clone 370 during batch growth. [a.] Cell density and chlorophyll *a* vs. time. [b.] DMS and particulate DMSP vs. time. Numbers are means of duplicates, with ranges shown by error bars.

− grazing) were calculated similarly from predator+prey bottles, and grazing rates were deduced by difference.

Results

Production of DMSP and DMS during batch growth— During exponential growth, clones 370 and 373 grew at rates of 0.70 and 0.47 d^{-1} to final concentrations of 8.5×10^5 and 5.8×10^5 ml^{-1}, respectively (Figs. 1a, 2a, Table 2). Clone 370 reached stationary phase at day 6, but clone 373 continued exponential growth until day 10 (Figs. 1, 2). Under our growth conditions, neither culture produced coccoliths. Clone 373 was larger than clone 370 (5.1 μm diameter vs. 3.9 μm based on observations of live cells) and had a correspondingly larger DMSP titer per cell (7.6 vs. 3.6 fmol). These titers were constant during exponential growth (Fig. 3a), similar to results shown by Matrai and Keller (1994), who found ~6 fmol DMSP cell^{-1} for clone 8613C. Because of the different cell volumes, both clones produced similar concentrations of internal DMSP during growth, averaging 109 μmol cm^{-3} cell volume. Dissolved DMSP, defined operationally by passage through a GF/F filter during gentle filtration, was consistently ~6–7% of internal DMSP during all stages of growth for both clones (data not shown). Dissolved DMSP seemed to rise during stationary phase for both clones, but this may have been an artifact of filtration of easily broken or leaky cells, because both
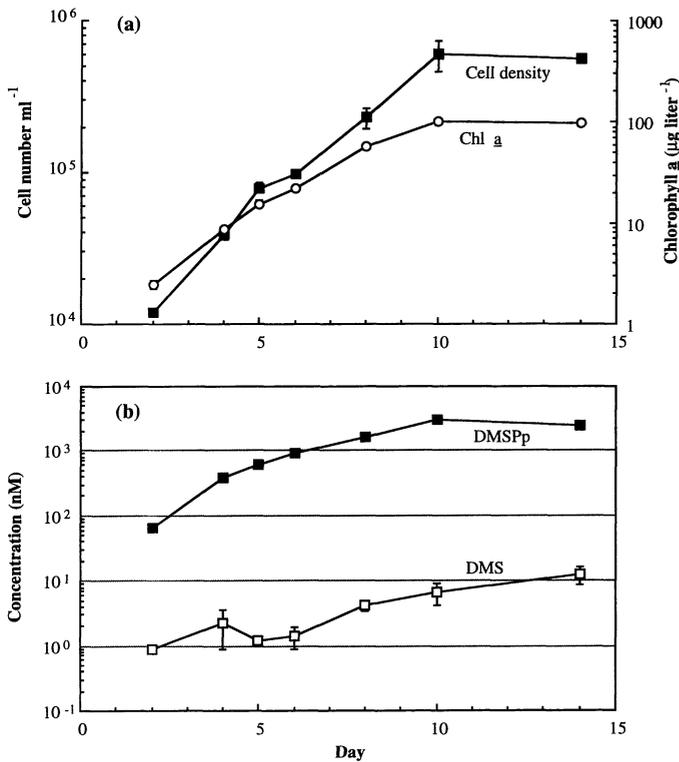
Fig. 2. As Fig. 1, but for clone 373.

Table 2. Comparison of growth and DMS(P) characteristics for *Emiliania huxleyi* clones CCMP 370 and 373 during exponential growth.

Parameter	Clone 370	Clone 373
Growth rate (μ, d^{-1})	0.70	0.47
Final cell density (ml^{-1})	8.5×10^5	5.8×10^5
Cell diam, μm ($n = 20$)	3.93 ± 0.29	5.13 ± 0.53
Cell vol., $\times 10^{-12}$ cm^3	31.8	70.7
Chl a $cell^{-1}$ (ng)	0.15	0.22
DMSP $cell^{-1}$ (fmol)	3.58	7.59
Internal DMSP concn (mM)	113	107
Dissolved DMSP $cell^{-1}$ (fmol)	0.29	0.51
DMS $cell^{-1}$ (fmol)	0.07	0.03
In vitro DMSP lyase activity (fmol $cell^{-1}$ min^{-1})	0.05	1.03

particulate DMSP per cell and chlorophyll a per cell decreased during stationary phase.

In contrast to the high concentrations of internal DMSP, very little DMS was produced by exponentially growing cells (Figs. 1b, 2b). Clone 370 consistently produced more
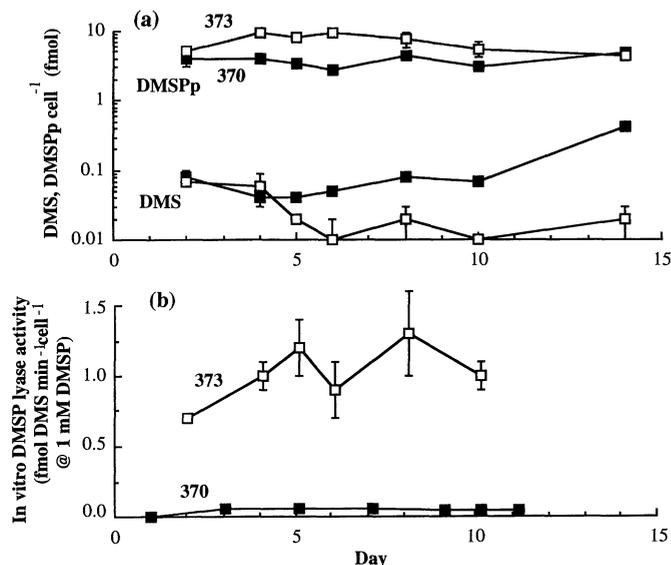

Fig. 3. Comparison of *Emiliania huxleyi* clones 370 (■) and 373 (□) during batch growth. [a.] Particulate DMSP and DMS per cell vs. time. [b.] In vitro DMSP lyase activity per cell vs. time. Numbers are means of duplicates, with ranges shown by error bars.

DMS than clone 373 did on a per-cell basis during exponential growth (0.07 vs. 0.03 fmol $cell^{-1}$; Table 2, Fig. 3a). During growth, DMS levels were a small fraction of dissolved DMSP ($\sim$7%) for clone 373, but a significant fraction (51%) for clone 370. When cells reached stationary phase and stopped dividing, DMS production continued, so that DMS per cell increased (clone 370, Fig. 3a). This increase was also seen for clone 373 in other experiments (data not shown). However, DMS production rates per cell during stationary phase were no higher than during exponential phase.

In vitro production of DMS in cell extracts—Intriguingly, we found in vitro DMSP lyase activity in both clones despite their limited DMS production during growth. Clone 370, which produced more DMS during growth, had low but detectable DMSP lyase activity (0.02–0.05 fmol DMS min^{-1} $cell^{-1}$ at 1 mM DMSP). Clone 373 showed 20-fold higher in vitro DMSP lyase activities, averaging 1.03 fmol DMS min^{-1} $cell^{-1}$ at 1 mM DMSP; however, this strain produced almost no DMS during exponential growth. Furthermore, biochemical characterization of the crude cell extracts showed distinct differences between the two clones (Table 3; Steinke et al. in prep.). In particular, clone 370 showed an absolute salt requirement, and enzyme preparations were stabilized by addition of a reducing agent (DTT or β-mercaptoethanol). In contrast, clone 373 lyase activity was unaffected by NaCl concentration or reductant, but improved slightly by addition of detergent. Solubilities of the two enzymes were also quite different.

Despite these contrasts, in vitro enzyme activity per cell did not change for either clone during exponential growth or when cells reached stationary phase (Fig. 3b), suggesting that total enzyme titer was constitutive. Clone 373 cells grown in high-nitrate (883 μM) and low-nitrate (50 μM) f/2 showed similar in vitro rates (data not shown). Neither enzyme exhibited any lytic ability with the closely related sulfonium compound, dimethylsulfonioacetate (DMSA), similar to a DMSP lyase isolated from a marine bacterium (de Souza and Yoch 1995). However, cell-free extracts of either enzyme failed to cross-react by western blot or ELISA with a polyclonal antibody prepared against

Table 3. Comparison of DMSP lyase characteristics in crude cell extracts of *Emiliania huxleyi* clones CCMP 370 and 373.

DMSP-lyase characteristic	Clone 370	Clone 373
Salt requirement	yes*	no
Sulfhydryl group requirement	yes†	no
pH optimum	>8	6
Half-saturation (mM DMSP)	5.5	1.4
Rate @ 1 mM DMSP (fmol DMS cell^{-1} min^{-1})	0.05	1.2
Soluble activity (fraction of crude cell)‡	36–58%	3–10%

* No activity below 0.2 M NaCl; maximum activity at >1 M NaCl.

† Enzyme showed greater stability and activity with reductants such as DTT.

‡ Range of values includes results with no detergent, 3% non-ionic detergent (Tween 80), or 3% ionic detergent (Zwittergent Z-312, CalBiochem).

the bacterial DMSP-lyase (Mark de Souza pers. comm.). This antibody has been shown to react against extracts from some DMS-producing macroalgae and one microalga (de Souza et al. in prep.). Although these results are preliminary and interferences from buffers or detergents cannot be ruled out, they suggest that the DMSP lyases in both *E. huxleyi* clones may be antigenically quite different from other DMS-producing bacteria and algae.

Production of DMS from dissolved DMSP by whole and lysed cells—When exponentially growing cells were concentrated by centrifugation and gently resuspended into fresh f/2 medium, no DMS production from endogenous DMSP was observed in short-term incubations, as shown for clone 373 (Fig. 4, inset). Microscopic examination showed healthy, intact cells, and little DMS was leaked from cells during concentration steps. However, if the same culture was resuspended into MES buffer (with or without NaCl, as appropriate), DMS production rates became measurable, although low (Fig. 4, inset), and examination showed bloated, fragmented cells. DMSP quickly leaked from cells resuspended into MES buffer, producing ~12–48 μM DMSP in the concentrated cell solutions (Fig. 4, pie graphs). If these cells were then sonicated, DMS production rates increased only slightly. Cells heated for 5 min and cell-free filtrates showed no production (data not shown).

The fact that DMS production occurred only when cells were ruptured during handling suggested that the DMSP lyase enzyme and its substrate are segregated within the cell, reacting only upon lysis. When 1 mM exogenous DMSP was added to cells in f/2 or buffer (Fig. 4, time zero), unlysed cells in f/2 did produce a small amount of DMS, but rates were far below those for lysed cells in buffer, indicating that the lyase enzyme was much more accessible in broken cells. This pattern of DMS production was seen in both clones, although DMS production rates were much higher in clone 373. These results suggest that DMSP lyase is located inside the cells rather than on the cell surface.
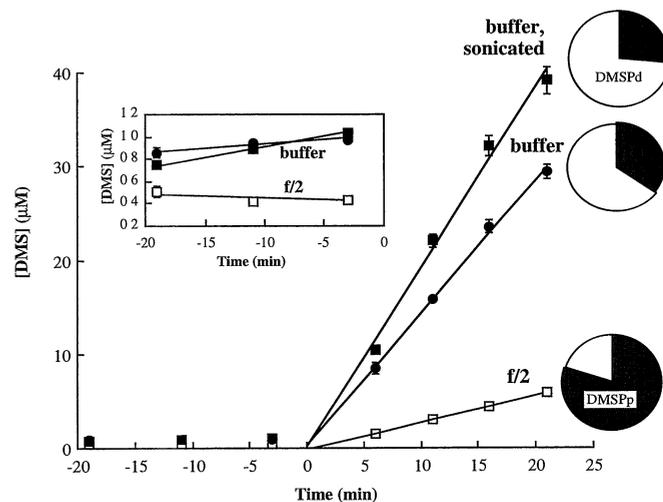

Fig. 4. Comparison of production of DMS from DMSP by *Emiliania huxleyi* clone 373 during resuspension into f/2 or MES buffer, with and without sonication; 1 mM DMSP was added at 0 min. Inset shows production of DMS from endogenous DMSP by cells resuspended in MES buffer before addition of DMSP. Pie graphs show relative fractions of particulate DMSP (■, DMSPp) and dissolved DMSP (□, DMSPd) found following resuspension in each case. Numbers are means of duplicates, with ranges shown by error bars.

Production of DMS during microzooplankton grazing—When the dinoflagellate *O. marina* was added to cultures of *E. huxleyi* 373 (Table 1, Exp. 1), DMS production began immediately and continued for 24–48 h as cells were grazed (Fig. 5a). The production of DMS was not seen in ungrazed 373 controls or in prey incubated with a filtrate of the *O. marina* culture, which contained bacteria associated with this predator (Fig. 5a). Total in vitro DMSP lyase activity decreased in grazed bottles as prey were removed, so that activity per cell was constant across treatments and over time (Fig. 5b). Dissolved DMSP did not show any trend during the incubation, remaining at ~10–20 nM in all treatments (data not shown). Grazing removed both cell production (Fig. 6a) and prey DMSP (Fig. 6b). Similar results were seen in several experiments with different initial densities of predator and prey cells (Tables 1 and 4). Ungrazed growth rates were somewhat variable (Table 4) in these short-term incubations, probably due to unequal cell division rates during daylight and dark periods, as has been shown for other strains of *E. huxleyi* (Van Bleijswijk et al. 1994).

When the same experiment was performed with clone 370 as prey (Table 1, Exp. 2), DMS concentrations also increased, but production rates were much lower (Table 4) and not as consistent across experiments (data not shown). *O. marina* grazing rates were higher on 370 than on 373 (Table 4). To ensure that different preconditioning of the grazer culture did not affect the results, we performed a comparison experiment with the same *O. marina* culture feeding on either prey clone (Table 1, Exp. 3). Grazed prey numbers decreased similarly for both clones, but ungrazed 370 had higher growth rates, so that
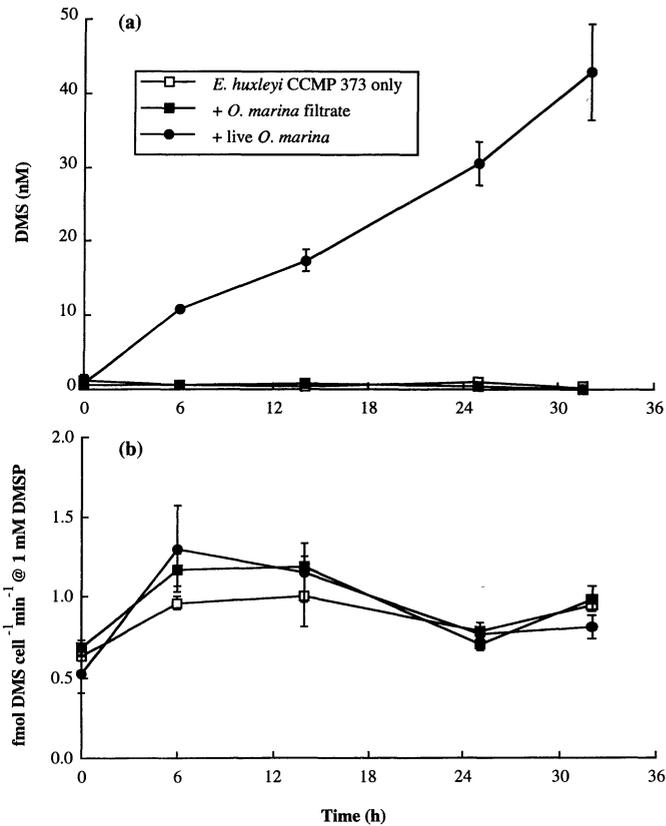
Fig. 5. Production of DMS (a) and in vitro DMSP lyase activity per cell (b) during grazing of *Oxyrrhis marina* on *Emiliania huxleyi* clone 373. Numbers are means of duplicates, with ranges shown by error bars.
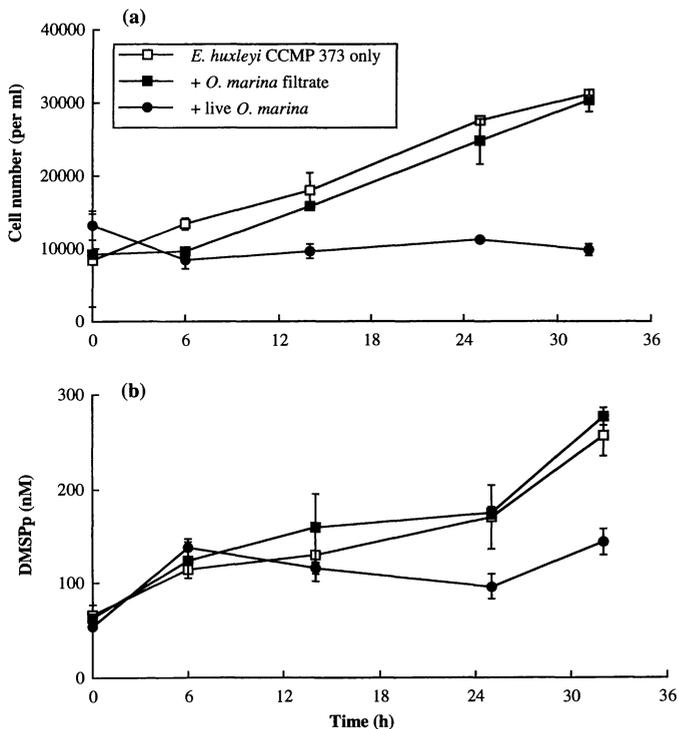

Table 4. Comparison of growth, grazing, and DMS production rates from *Oxyrrhis marina* grazing on *Emiliania huxleyi* and *Dualiella tertiolecta*. Numbers are averages with ranges in parentheses.

Parameter	*E. huxleyi* clone		*D. tertiolecta*‡
	373*	370†	
Ungrazed growth rate (μ, d^{-1})	0.53(0.05)	0.61(0.13)	0.78
Grazing rate (g, d^{-1})	0.59(0.16)	1.40(0.48)	2.10
Grazer clearance rate (μl predator^{-1} d^{-1})	1.05(0.35)	2.31(0.41)	4.34
Predator-specific grazing rate (prey predator^{-1} d^{-1})	8.6(3.9)	16.2(2.2)	53.2
DMS production rate (nM d^{-1})	23.0(7.0)	3.5(1.5)	1.2
DMS produced per grazed prey (fmol cell^{-1})	4.6(2.4)	0.3(0.2)	0.0

* Range of values from three experiments; rates are averaged over experimental period.

† Range of values from two experiments; rates are averaged over experimental period.

‡ Values from one experiment.

grazing removed more "new production" for clone 370 than for clone 373. Because predator numbers increased similarly in both treatments, this implied that *O. marina* cleared clone 370 at higher rates (Table 4).

Because it was not clear whether the DMS observed in the grazed cultures was produced by the grazed or ungrazed prey, we performed an experiment in which clone 373 cells were present during grazing and exposed to chemical or physical cues due to grazing (grazing exudates, shear stresses) but were not actually grazed (Table 1, Exp. 4). To do this, we took advantage of the preference of *O. marina* for the prey *D. tertiolecta,* a non-DMSP-producing chlorophyte. We incubated clone 373 (10,000 cells ml^{-1}) with a 2-fold higher concentration of *D. tertiolecta* (22,000 cells ml^{-1}). When *O. marina* cells were added (500 cells ml^{-1}), *D. tertiolecta* cells were rapidly removed by grazing (Fig. 7a, 0–30 h). Clone 373 numbers increased until *D. tertiolecta* cells had been grazed to ~5,000 ml^{-1} at 25 h, at which time *O. marina* began grazing on clone 373 and its numbers decreased (Fig. 7a). DMS was not produced during the grazing of *D. tertiolecta* (Fig. 7b, 0–25 h), but production began as soon as clone 373 began to be eaten (Fig. 7b, 25–55 h). In treatments that contained only clone 373 as prey, DMS was produced throughout the experiment (data not shown), as clone 373 cells were grazed. In the clone 373-only treatment, both grazing rates and DMS production rates were highest initially and decreased over the experiment as prey became scarce. In contrast, when *D. tertiolecta* prey were also

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Fig. 6. Removal of prey cells (a) and prey DMSP (b) during grazing *Oxyrrhis marina* on *Emiliania huxleyi* clone 373. Numbers are means of duplicates, with ranges shown by error bars.

present, grazing rates and DMS production rates both increased sharply at 25 h, when predators switched their grazing to clone 373. Once again, control bottles of clone 373 incubated with *D. tertiolecta* and with *O. marina* culture filtrate showed no DMS production. These observations confirmed that production of DMS originates from grazed clone 373 cells.

Discussion

The two *E. huxleyi* strains synthesized similar concentrations of internal DMSP and also produced constitutive DMSP-lyase enzymes. However, production of DMS during growth was a trivial fraction of potential production given the measured rates of in vitro DMSP lyase. For example, clone 370, which produced ~0.05 fmol DMS $cell^{-1}$ min^{-1} in vitro (1 mM DMSP), generated only 6.5×10^{-6} fmol DMS $cell^{-1}$ min^{-1} during growth—about 0.04% of potential production. For clone 373, the discrepancy was even greater. Instead, DMS production was clearly associated with damaged cells, as demonstrated by increased DMS production when cells were lysed by chemical or physical means (Fig. 4) or when cells were grazed (Figs. 5, 7). The very low cleavage of DMSP by growing *E. huxleyi* cells contrasts strongly with another important DMS-producing phytoplankter, *P. pouchetii*, which produces large quantities of DMS during growth. That species averaged 3.05 fmol DMS $cell^{-1}$ min^{-1} during exponential growth in axenic culture (Stefels and van Boekel 1993) and cleaved exogenous DMSP at rates several thousand-fold greater than we found for healthy, undamaged *E. huxleyi*. It seems likely that the DMSP lyase enzyme, and possibly its physiological role, is quite different for these species.

One explanation for the behavior we observed is that DMSP and the DMSP lyase enzyme are physically segregated within the cell and only react under conditions that rupture the compartments and allow mixing. Cell manipulations clearly showed increased rates of DMS production from endogenous or exogenous DMSP when cells were ruptured. One potential model for such a segregated enzyme-substrate system is a cell-surface enzyme with the active site outside the cell or imbedded in the plasma or cell membrane. Although we found that whole, uninjured cells exposed to mM exogenous DMSP could form DMS (Fig. 4), rates were much lower than for lysed cell extracts. Application of proteinase K, shown to degrade other cell-surface proteins in clones of *E. huxleyi* under similar growth conditions (Palenik and Morel 1990), did not decrease DMS production in our whole-cell tests (data not shown). Therefore, we believe that the enzyme is internal to the cell. We were not able to detect significant uptake of the exogenous DMSP into cells (data not shown), so cleavage of exogenous DMSP by whole cells is still somewhat mysterious. It is possible that external DMSP initiates conversion of internal DMSP pools through some signal mechanism, and it is conceivable that DMSP-lyase may play some role in detecting external stress or environmental cues.
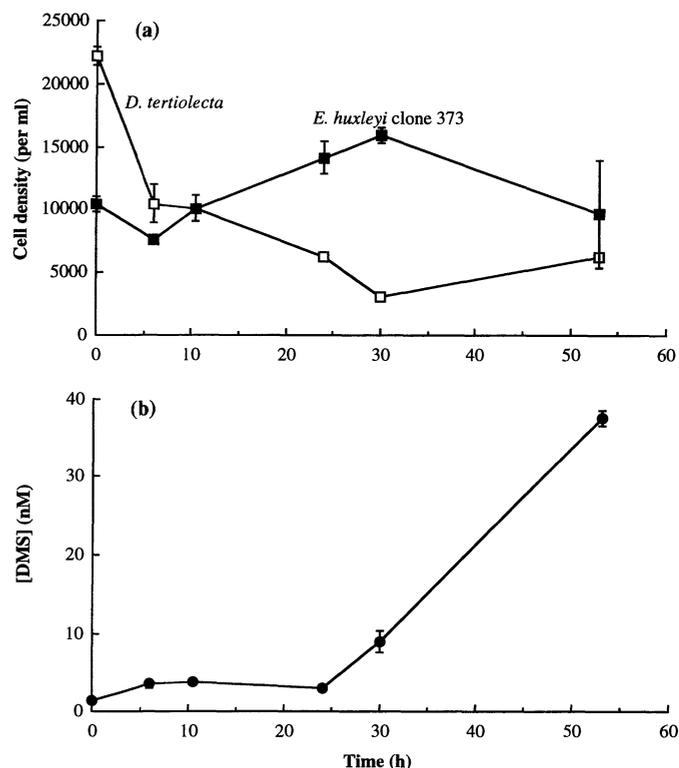

Fig. 7. Experiment with *Oxyrrhis marina* grazing both *Dunaliella tertiolecta* and *Emiliania huxleyi* clone 373. [a.] Prey cell densities. [b.] DMS concentrations. Numbers are means of duplicates, with ranges shown by error bars.

The dramatic contrast in lyase activity and function between two clones of the same species is surprising, but there is precedent for other biochemical and genetic diversity among *E. huxleyi*. Van Bleijswijk et al. (1991) found two distinct morphotypes of *E. huxleyi* based on an antibody test to a coccolith polysaccharide, and Conte et al. (1995) found different biomarker compounds and different amounts of fucoxanthin in oceanic and neritic strains. These studies suggested that *E. huxleyi* may in fact be multispecific. Although genetic testing using DNA sequence variation information (Medlin et al. 1994) showed little difference among widely distributed isolates, preliminary evidence from amplified polymorphic DNA (RAPD) analysis (Barker et al. 1994) in mesocosm and bloom studies suggests that there may be genetic variations at the subspecies level that are not detected by DNA methods. There is also biochemical evidence for phenotypic diversity among *E. huxleyi* clones. Palenik and Koke (1995) found that a cell-surface enzyme expressed under nitrogen limitation was present in some but not all of five axenic *E. huxleyi* clones, suggesting that closely related clones may have significantly different enzyme systems.

Wood and Leatham (1992) pointed out that many studies on diverse marine phytoplankton have shown intraspecies phenotypic variation and suggested that strain designation should be considered essential information when experimental results are reported. We therefore pre-

dict that other phytoplankton species will also show diversity among strains with respect to DMSP lyase behavior. There is already evidence for DMSP lyase diversity among related macroalgae. Steinke et al. (1996) found that the DMSP lyase enzyme seems to be widespread, but activity can vary greatly between species. Three species of *Enteromorpha* (*E. clathrata, E. intestinalis,* and *E. compressa*) had high specific lyase activities, but another species (*E. bulbosa*) had very low activity. Although the assay of Steinke et al. was developed and optimized for *E. clathrata* and may not have detected other enzymes that operate under different conditions, it is likely that DMSP lyase activity is often species- or strain-specific. It is even possible that the genetic ability to cleave DMSP to form DMS may not always be related to the ability to synthesize DMSP. Thus, these results reinforce the notion that DMSP may serve other biochemical functions inside cells aside from DMS-acrylate production.

Production of DMS during microzooplankton grazing—DMS can be formed during grazing when lyase enzymes are present in either the prey or predator. Previous work with predators such as copepods (Dacey and Wakeham 1986) and fish (Dacey et al. 1994) has suggested that either the grazer or bacteria associated with grazer digestive tracts or fecal material could be responsible for DMSP cleavage during grazing. Our work shows that algal DMSP lyases may also be activated during grazing.

We were not able to perform grazing treatments without bacteria, but we believe their contribution to DMS production was minimal. Although bacteria were likely present in the *O. marina* culture that cleaved or demethylated DMSP, activities were probably low because treatments without grazers but with grazer exudates and bacteria produced little or no DMS (Fig. 5a) even when substantial pools of dissolved DMSP (10–20 nM) were present. Furthermore, in grazed treatments with the same grazer-bacteria populations and different prey, DMS production varied greatly but was always correlated with prey DMSP lyase in vitro activity (Table 4). We also believe bacterial DMS consumption was minimal. Once grazing had removed *E. huxleyi* cells and DMS production stopped, DMS levels usually remained steady over many hours (not shown).

It is clear that our lyase assay measured DMSP lyase activity in live, ungrazed cells. In vitro DMS production rates were proportional to live cell numbers, decreasing as cells were grazed and as grazer populations increased, so that rates per live cell were constant, as they were for ungrazed cultures (Fig. 5b). However, we believe that the DMS produced during grazing came not from live *E. huxleyi* cells but only from those which had been ingested by *O. marina,* as was seen clearly in the experiment in which clone 373 was exposed to grazers but was not grazed due to the presence of an alternate prey, *D. tertiolecta.* Until *D. tertiolecta* cells were grazed to low numbers, no DMS was formed, but as soon as consumption of clone 373 began, DMS levels rose sharply (Fig. 7). During grazing, degradation of the prey cells inside *O. marina* digestive vacuoles must briefly allow the enzyme-substrate

reaction to proceed before prey enzymes are destroyed by predator digestion. For example, with clone 373 we found that DMS production rates in three grazing experiments averaged 4.6 fmol DMS per grazed cell (Table 4). If we assume that production rates by grazed cells were similar to in vitro rates (~ 1.0 fmol DMS cell^{-1} min^{-1}), then the lyase need only have been active for 3–5 min following ingestion. Clone 373 had a titer of ~ 7.6 fmol DMSP cell^{-1} (Table 2), so roughly 60% of prey DMSP was converted to DMS following grazing. Because *O. marina* grazed clone 373 at low rates (~ 0.4 prey predator^{-1} h^{-1}, Table 4), it seems reasonable that digestion would have taken longer than a few minutes, allowing slightly digested or broken prey cells to produce DMS for a short period following ingestion. Similar calculations for clone 370 yield similar time estimates for DMS production following ingestion, but because enzyme activities were lower, a much smaller fraction of cellular DMSP was converted to DMS during grazing.

Such a lysis-activated reaction has analogs among marcophytic defense reactions, such as the hydrolysis of glucosinolates (Chew 1988) and the rapid conversion upon injury of halimedatetraacetate to the feeding deterrent halimedatrial in the marine macroalga *Halimeda* (Paul and van Alstyne 1992). We hypothesize that this reaction may also serve as a chemical deterrent against protozoan herbivory. DMS is merely a byproduct, and the acrylate produced acts as a toxin, as has long been suggested (Sieburth 1960). Obviously, since *E. huxleyi* cells were readily grazed by *O. marina,* the reaction is not grossly toxic. However, for clone 373, cleavage of 60% of the prey DMSP following ingestion would leave the grazer food vacuole with 65 mM acrylic acid (neglecting dilution), and we often observed multiple prey inside protozoan food vacuoles. *O. marina* repeatedly cleared clone 373 at lower rates than clone 370, which produced the same amount of DMSP but much less DMS and, presumably, acrylate. Furthermore, both *E. huxleyi* clones were grazed at lower rates than was the non-DMSP-producing *D. tertiolecta* prey (Table 4). Whether this reaction might function for defense in natural situations is unknown. There is no indication that *E. huxleyi* is particularly resistant to grazing pressure, and one study found evidence for preferential grazing of this species compared to all phytoplankton (Holligan et al. 1993). We are currently testing this hypothesis with other *E. huxleyi* strains and with grazers more representative of surface marine waters (Wolfe et al. in prep.).

The differing production of DMS during microzooplankton grazing on these two clones helps explain some of the diversity seen in previous experiments. When *O. marina* grazed *E. huxleyi* clone 370 (Wolfe et al. 1994) some DMS was formed, but only a small fraction of the prey DMSP that was metabolized during grazing. That study suggested that the DMS production was bacterial, but it now seems that at least some of the DMS produced was due to a low-activity prey DMSP lyase, activated during grazing. However, another study using the same grazer species with a different *E. huxleyi* clone (strain PLY 379) found significant DMS production (Malin et al. 1994),

quite similar to our results with clone 373. These results suggest that production of DMS by grazed *E. huxleyi* will be strain-specific, and we believe it is critical to specify the clones used in experiments.

Implications for DMS production in natural waters— Our results yield some insight into the patterns of DMSP and DMS seen in the field. First, if the results we observed in our two *E. huxleyi* clones are representative of other strains, there is significant intraspecies phenotypic variability, and we will need to know not just which species are present but which strains. Two *E. huxleyi* blooms might show very different temporal patterns of DMS production. Second, our work reinforces the diverse nature of DMS formation, because grazing-activated prey production of DMS must now be added to other known DMS production mechanisms, including production by growth-active phytoplankton DMSP lyases as in *P. pouchetii* (Stefels and van Boekel 1993), inducible bacterial DMSP lyases (de Souza and Yoch 1995), and heterotroph (Ishida 1968) or predator-associated (Dacey et al. 1994) DMSP lyases.

Our experiments reinforce the importance of grazing processes to the production of DMS. During growth, DMS production rates for both clones were very low. Over the life cycle of an individual cell (1.0–1.5 d), only ~0.01 fmol of DMS was produced by either clone. However, in the few minutes following ingestion, 0.3–4.6 fmol DMS was produced from clones 370 or 373. Thus, production per cell increased 30-fold to 400-fold during grazing. Although herbivory rates in our experiments were high, these results suggest that even low rates of herbivory will result in greatly increased DMS production. Furthermore, cells that have the segregated enzyme-substrate lyase system do not have to be grazed to become ruptured, and senescent cells might produce DMS in the absence of grazing. DMS has been observed to be highest in the older parts of *E. huxleyi* blooms (Matrai and Keller 1993). This pattern is consistent with the mechanism we have observed, but could also be explained by bacterial or mesozooplankton actions. Our results clearly need to be extended to other grazers and prey, including other *E. huxleyi* strains and other DMSP-producing phytoplankton.

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