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Production of glycine betaine and dimethylsulfoniopropionate in marine phytoplankton. I. Batch cultures

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Abstract The quantitative significance of the nitrogenous compound glycine betaine (GBT) and its sulfur analog dimethylsulfoniopropionate (DMSP) to intracellular pools in marine phytoplankton is not well known. In a series of experiments conducted in August 1993, we measured these compounds, as well as total organic sulfur, carbon, and nitrogen, over the growth cycle in six isolates of marine phytoplankton, *Amphidinium carterae* Hulburt, *Chrysochromulina* sp. Lackey, *Emiliania huxleyi* Hay et Mohler, *Prorocentrum minimum* (Pavillard) Schiller, *Skeletonema costatum* (Greville) Cleve, and *Tetraselmis* sp. At the same time, we measured cellular concentrations of protein, amino acids, chlorophyll, and inorganic nutrients. All six species produced DMSP, while three produced GBT at lesser levels. In the *Chrysochromulina* sp. isolate, levels of GBT were greater than DMSP during the exponential phase of growth, but declined sharply as the culture approached stationary phase. This change appeared to coincide with the onset of nitrogen limitation. Other nitrogenous osmolytes were produced in five of the six species but in much smaller quantities. DMSP contributed significantly to cellular sulfur throughout the growth cycle although, in some algae, the proportion of dissolved DMSP increased substantially during stationary growth. When present, GBT formed a sizeable fraction of the cellular nitrogen only during exponential growth. A significant percentage (ca. 50%) of the organic nitrogen could not be accounted for even when cellular pools of protein, amino acids, inorganic nitrogen, and nitrogenous osmolytes were combined. Based on these experiments, there does not appear to be a reciprocal relationship between DMSP and GBT production, although GBT production does appear to be correlated with nitrogen availability.

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Introduction

Plants and microorganisms that experience water stress, due either to salinity or desiccation, accumulate solutes (osmolytes) within their tissues in order to maintain favorable osmotic tensions and positive turgor (Yancey et al. 1982; Flowers 1985). Marine phytoplankton produce a variety of organic nitrogen- and sulfur-containing compounds which are present at osmotically significant concentrations. These osmolytes include the quaternary ammonium compound glycine betaine (GBT) and the tertiary sulfonium compound dimethylsulfoniopropionate (DMSP) (White 1982; Reed 1983; Roch et al. 1985; Blunden and Gordon 1986). Each compound appears to function as a true "compatible solute," not inhibiting enzyme functions and stabilizing macromolecules, even at intracellular concentrations in excess of 100 m*M* (Pollard and Wyn Jones 1979; Yancey et al. 1982; Gröne and Kirst 1992; Nishiguchi and Somero 1992).

DMSP has received considerable attention in the marine environment as the primary precursor of the important atmospheric trace gas dimethylsulfide (DMS). Oceanic flux of DMS is currently estimated at 0.6 to 1.7 Tmol S yr^{-1} (1 Tmol = 32×10^{12} g S) and accounts for a significant fraction (30 to 50%) of natural global sulfur emissions (Andreae 1990; Bates et al. 1992, and references within). Although marine phytoplankton are the source of DMSP in seawater, attempts to correlate DMS production to phytoplankton biomass (chlorophyll) have been only moderately successful (Barnard et al. 1982; Bates and Cline 1985). This lack of correlation can be attributed, at least in part, to the fact that not all phytoplankton produce DMSP (e.g. Keller

et al. 1989). In addition, the release of DMSP, and its conversion to DMS, is accomplished by a number of biologically mediated processes, including phytoplankton cell lysis, grazing by zooplankton, and bacterial degradation of DMSP (Dacey and Wakeham 1986; Nguyen et al. 1988; Belviso et al. 1990; Kiene and Bates 1990; Matrai and Keller 1993).

DMSP production generally follows taxonomic groupings in phytoplankton, with the classes Dinophyceae and Prymnesiophyceae being major sources of DMSP (Keller et al. 1989). Taxonomic trends in GBT production are evident in higher plants (Storey et al. 1977), but there is insufficient information to generalize about GBT production in marine phytoplankton. Some algae produce both GBT and DMSP (Blunden and Gordon 1986; Dickson and Kirst 1987a, b), and the relative proportions of GBT and DMSP are not constant, as was observed in cells of *Platymonas subcordiformis* grown at different salinities (Dickson and Kirst 1986) or different temperatures (Sheets and Rhodes 1996). There also appears to be an inverse relationship in phytoplankton between DMSP and available nitrogen. Turner et al. (1988) reported that in cultures of *Emiliania huxleyi* supplemented with 15 mg l^{-1} NO_3^-, the per-cell DMSP levels were less than in cultures with low nitrate. Similarly, Gröne and Kirst (1992) observed that DMSP levels in *Tetraselmis subcordiformis* were 75% higher in N-limited batch cultures. Recently, Keller and Bellows (1996) reported increases of 30 to 90% in intracellular DMSP concentrations in several species of phytoplankton grown in N-limited versus N-replete batch cultures. A reciprocal relationship between DMSP and GBT has been suggested in marine phytoplankton (Andreae 1986), with DMSP production being favored over that of nitrogen-containing osmolytes such as GBT in N-limited populations. The overwhelming abundance of sulfate in seawater (~28 m*M*) as compared to combined nitrogen (1 to 10 μ*M*), which is required in so many other physiological processes, may favor the production of sulfur-containing compounds.

Almost nothing is known about the role of GBT in the nitrogen economy of marine phytoplankton, or of marine systems in general (King 1988a). Although early considerations of sulfur partitioning in algal cells did not take DMSP into account, and sulfur was reported as being confined largely to structural protein (e.g. Andreae 1990 and references within), it is now known that DMSP accounts for >50% of the total sulfur in some phytoplankton (Matrai and Keller 1994). There are no data for phytoplankton on the contribution of GBT to cellular N, but GBT can make up a significant fraction (7 to 23%) of the organic N in certain halophytes (Stewart et al. 1979; Cavalieri and Huang 1981). It is possible that GBT could make up a similar percentage of cellular N in some phytoplankton. There is evidence that certain species of phytoplankton contain substantial pools of cellular N that are not accounted for when NO_3^-, NH_4^-, amino acids, protein, RNA, and DNA are summed (Conover 1975; Dortch et al. 1984; Doucette and Harrison 1991), but GBT and other nitrogenous osmolytes have not been considered in such budgets.

In the present study, we investigated the production of GBT and DMSP by selected species of marine phytoplankton, determining the quantitative importance of these compounds to cellular nitrogen and sulfur budgets. Since some phytoplankton produce both GBT and DMSP, the influence of nitrogen nutrition on the production of these compounds was also examined.

Materials and methods

Culture conditions

Six species of phytoplankton were used in these experiments, conducted in August 1993 (Table 1). The strains were obtained from the Provasoli-Guillard National Center for the Culture of Marine Phytoplankton (CCMP, Bigelow Laboratory, Maine USA) and tested with standard bacterial media for contamination. All of the cultures were axenic except the *Chrysochromulina* sp. (CCMP 288) strain. The cultures were grown in duplicate 2 liter batch cultures in *K*-medium (Keller et al. 1987), with nitrogen levels adjusted to *K*/10 levels (88.3 μ*M* NO_3^- and 5 μ*M* NH_4^+) to ensure that nitrogen limitation would occur after modest growth. The cultures were grown in continuous light (10^{16} quanta cm^{-2} s^{-1}) at 20 °C and were aerated through a 0.2 μm filter capsule. Cultures were inoculated with exponential phase cells grown in full-strength *K*, and their growth was monitored daily by measuring relative in vivo fluorescence with a Turner Designs 100 fluorometer (Brand et al. 1981). Samples were collected at five times in the growth cycle; twice during exponential growth, twice in stationary growth and once in senescence. For each sampling point, the following analyses were conducted.

Dimethyl sulfide (DMS) and dimethylsulfoniopropionate (DMSP)

Intra- and extracellular concentrations of DMSP and free DMS were analyzed on a Varian 3300 gas chromatograph with a flame

Table 1 Phytoplankton species used in this study. All cultures, with the exception of the *Chrysochromulina* sp. strain (CCMP 288), were axenic. Cultures were obtained from the Provasoli-Guillard National Center for the Culture of Marine Phytoplankton (CCMP; Bigelow Laboratory, Maine, USA). Cell volumes are in μm^{-3}

Species	Class	Strain	Cell volume
Chrysochromulina sp. Lackey	Prymnesiophyceae	CCMP 288	65.45
Emiliania huxleyi Hay et Mohler	Prymnesiophyceae	CCMP 378	65.45
Skeletonema costatum Cleve	Bacillariophyceae	CCMP 1332	196.00
Tetraselmis sp. Stein	Prasinophyceae	CCMP 896	551.42
Amphidinium carterae Hulburt	Dinophyceae	CCMP 1314	785.40
Prorocentrum minimum Schiller	Dinophyceae	CCMP 1329	1731.15

photometric detector and a Chromosil 330 teflon column. Whole water samples and the filtrates from samples gently filtered through glass fiber filters (GF/F) were collected in glass vials (15 ml; triplicate samples), base (5 *N* KOH) was added, and the vials were sealed. Samples were incubated for 24 h in the dark at room temperature and then sonicated before sparging. DMS was collected and analyzed via the cryogenic trap method, as described in Keller and Bellows (1996). DMS measured in these samples includes the free DMS, and the total and dissolved fractions of the DMSP pool, respectively. Particulate DMSP concentrations were determined by difference. DMS was also measured immediately upon sample collection, after sparging and cryotrapping. DMSP was obtained from Research Plus (Bayonne, New Jersey), and standards were prepared in DMSP-free filtered seawater. The concentration of S in these pools was calculated using the percent S of DMSP (18.8%).

Glycine betaine (GBT) and other nitrogenous osmolytes

GBT and other nitrogenous compounds were measured by the HPLC/ion chromatography method of Gorham (1984), also described by King (1988a). Cultures (50 ml; duplicate samples) were filtered gently onto a glass fiber filter (GF/F), which was subsequently extracted with a methanol:chloroform:water (12:5:1) mixture for 24 h. The extract was evaporated to dryness under a stream of air and reconstituted in 0.5 ml deionized water. The samples were then filtered through a 0.45 μm Teflon filter before injection. Mixtures were separated on a strong cation exchange column (Whatman Partisphere SCX), using 0.05 *M* KH_2PO_4 buffer and 2.5% methanol, a flow rate of 1 ml min^{-1}, and with detection by ultraviolet absorbance at 190 nm. Sensitivity was modest (minimum detectable amount of GBT ~0.5 nmol per 100 μl injection), but was more than adequate for analysis of phytoplankton cultures. GBT and trigonelline standards were prepared from reagents obtained from Sigma Chemicals. Homarine was not available at the time this study was carried out. Homarine was identified and quantified from the relative retention time and response factors of Gorham (1984). Identity was later confirmed with authentic homarine obtained from D. Rhodes (Purdue Univ.). Identification of homarine peaks was aided by its strong absorbance at 272 nm and by its separation from trigonelline. Peak identities were confirmed by standard addition to samples and co-chromatography. The concentrations of N in these pools was calculated using the %N of the standards, as supplied by the manufacturer.

Particulate organic sulfur (POS)

Samples were gently filtered (15 ml; duplicate samples) onto precombusted quartz fiber QM/A filters and rinsed with 3% isotonic ammonium formate to remove sulfate salts. Prior to analysis, the filters were dried at 60 °C. Analyses were carried out with an Antek 771 pyroreactor unit coupled with an Antek 701 C sulfur detector (Matrai 1989). Methionine was used as a standard. The limit of detection was 0.01 μg S and the precision of the method was within 5%.

Particulate organic carbon and nitrogen (POC and PON)

Samples (10 to 25 ml depending on cell density; duplicate samples) for POC and PON were gently filtered onto pre-combusted Whatman GF/F filters, stored at −20 °C in baked foil envelopes, and dried for 24 h at 50 °C, immediately prior to analysis. Analysis was on a Control Equipment Corp. CHN analyzer. Acetanilide was used as a standard.

Total protein and amino acids (AA)

Samples (50 ml; duplicate samples) were gently filtered onto glass fiber filters (GF/F), which were then ground in cold, 10% trichloroacetic acid (TCA). The sample was then centrifuged, and the pellet analyzed for protein by the Lowry method (Lowry et al. 1951), as modified by Dortch et al. (1984). Bovine serum albumin (BSA) was used as the standard for protein measurements. Amino acids were analyzed in the supernatant using the fluorescamine method (Packard and Dortch 1975). Glutamate was used as the standard for the amino acid analyses. Fluorescence was measured at 480 nm using a spectrophotometer with excitation at 390 nm. This method was determined to be the best in terms of recovery and sensitivity for marine phytoplankton samples (Clayton et al. 1988). The concentration of N in these pools was calculated using the %N of the standards, as supplied by the manufacturer (Sigma Chemical Co.).

Chlorophyll (chl), cell counts and volumes

Samples (2 to 5 ml depending on cell density; triplicate samples) for chlorophyll *a* measurement were gently filtered onto glass fiber filters (GF/F) and extracted in 90% acetone, following the methods of Strickland and Parsons (1972). Samples for cell counts were preserved in Lugol's fixative and counted either with a Speirs–Levy counting chamber or a 0.1 mm hemocytometer. Cell volumes were determined with live cells using light microscopy.

Nutrients in medium and intracellular inorganic nitrogen

Culture media samples (20 ml, unfiltered) were collected in nutrient vials, frozen and subsequently analyzed for inorganic nutrients using standard autoanalyzer methodology (T. Loder, UNH). Samples for intracellular nutrients (50 ml) were gently filtered through a glass fiber filter (GF/F), and the filter rinsed with 10 ml of low N seawater (Sargasso Sea water in this case). Fifty milliliters of boiling distilled deionized water were passed through the filter and collected for N analysis as described by Thoresen et al. (1982). Samples were frozen and subsequently analyzed for inorganic nutrients as above (T. Loder, UNH).

Results

Intracellular levels of DMSP and GBT, as well as dissolved DMSP and DMS, were measured over the growth cycle in six species of marine phytoplankton. At the same time, cellular concentrations of organic sulfur, carbon and nitrogen, protein, amino acids, chlorophyll and inorganic nutrients were measured. Cell numbers and volumes, in vivo fluorescence, and changes in extracellular nutrients were also monitored. Each alga exhibited typical growth patterns, with stationary growth coinciding with the depletion of extracellular nitrate (Figs. 1 to 3A for three algae; others not shown). Levels of other nutrients, phosphate and silicate, were not depleted (data not shown). Increases in C:N ratios (Figs. 1 to 3A for three algae; others not shown; Table 2 for C and N values in all strains) and changes in other indicators of cellular nitrogen limitation (increases in C:chl and decreases in AA:protein ratios) (Table 3) were observed for all of the algae except the *Tetraselmis* sp. culture (CCMP 896), which appeared to become more N-replete in stationary growth (Table 3). The C:N ratio did not change in the *Chrysochromulina* sp. culture (CCMP 288) (Fig. 1A), which is perhaps a reflection of its xenic state (i.e. contaminating bacteria have a very low C:N ratio), but the other indicators (C:chl and

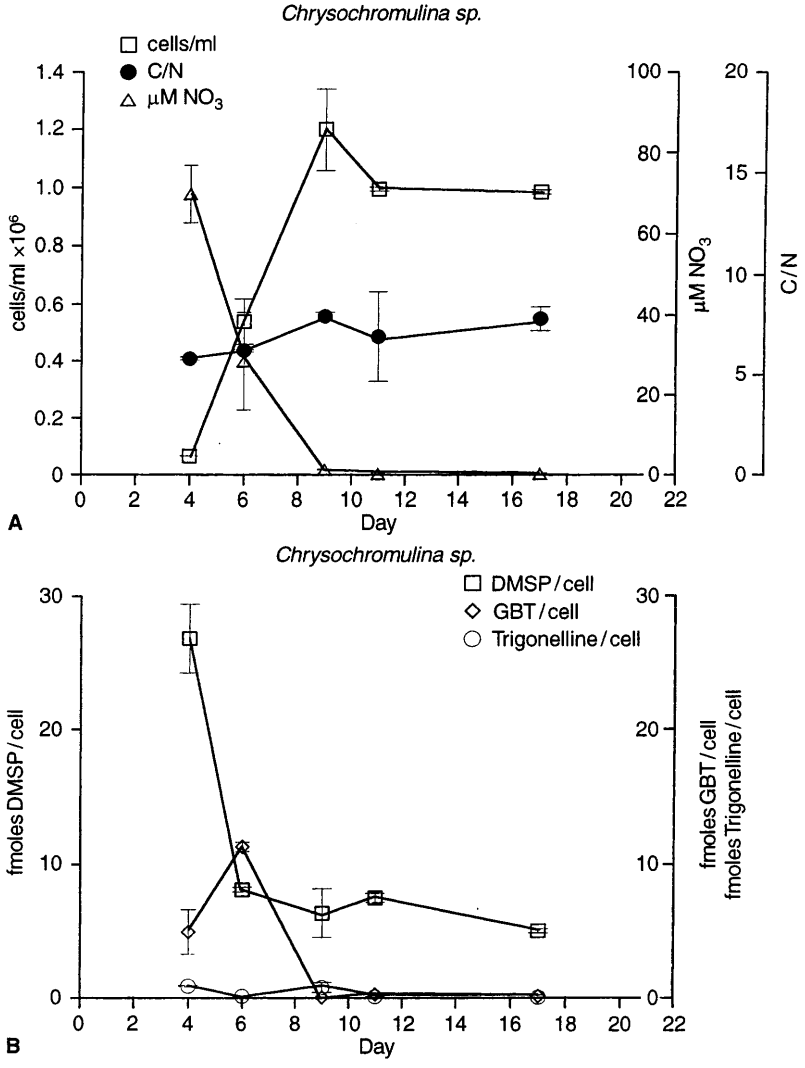

Fig. 1 *Chrysochromulina* sp. **A** Cell numbers, C:N ratio, and external concentrations of NO_3^- over growth cycle in batch culture. **B** Intracellular concentrations of DMSP, GBT, and trigonelline, another nitrogenous osmolyte, over growth cycle in batch culture. Means ± 1 SD are shown for each datum

AA:protein ratios) suggested N limitation (Table 3). Cell volumes did not change over the growth cycle in these algae.

All of the strains produced DMSP (Figs. 1 to 3B; Tables 2, 3). DMS production was insignificant during all stages of growth (data not shown), and DMSP dissolved in the medium [DMSP(D)] remained low throughout growth, except in two strains (*Prorocentrum minimum* and *Emiliania huxleyi*) where DMSP(D) increased significantly in late stationary phase (Fig. 4A, B). Intracellular levels of DMSP ranged from 18 to 219 μmol based on total cell volume (Table 3), with maximum production in the prymnesiophyte and dinoflagellate strains. Lower levels were observed in the prasinophyte and diatom cultures. Initial high concentrations of intracellular DMSP were seen in all of the algae (Figs. 1 to 3B for three clones; Table 2), a phenomenon observed in other parameters as well (POS, POC, PON, proteins, amino acids) (Table 2 for POS, POC, PON; other data not shown). Intracellular DMSP values per unit cell volume are reported for all species at two stages of growth (late exponential and mid-stationary) (Table 3). In all of the algae, the DMSP(P) (particulate) levels decreased over the growth cycle, with the decrease especially noteworthy in three species, the two dinoflagellates *P. minimum* (CCMP 1329) and *Amphidinium carterae* (CCMP 1314) and the prymnesiophyte *E. huxleyi* (CCMP 378). In two of these, the decrease may be attributable to the large increases in the fraction dissolved in the medium (Fig. 4A, B).

GBT was produced by three species, both prymnesiophytes, *Chrysochromulina* sp. (CCMP 288) and *Emiliania huxleyi* (CCMP 378), and one of the dinoflagellates, *Amphidinium carterae* (CCMP 1314) (Figs. 1 to 3B; Table 3). In *Chrysochromulina* sp. (CCMP 288), levels of GBT were greater than DMSP during exponential phase but declined sharply as the culture approached stationary phase (Fig. 1B). This change appeared to coincide with the onset of N limitation (Fig. 1A). In addition, a small amount of trigonelline appeared during exponential and early stationary growth. In the other prymnesiophyte, *E. huxleyi* (CCMP 378), there was also a maximum in GBT levels in exponential growth, but a small intracellular pool

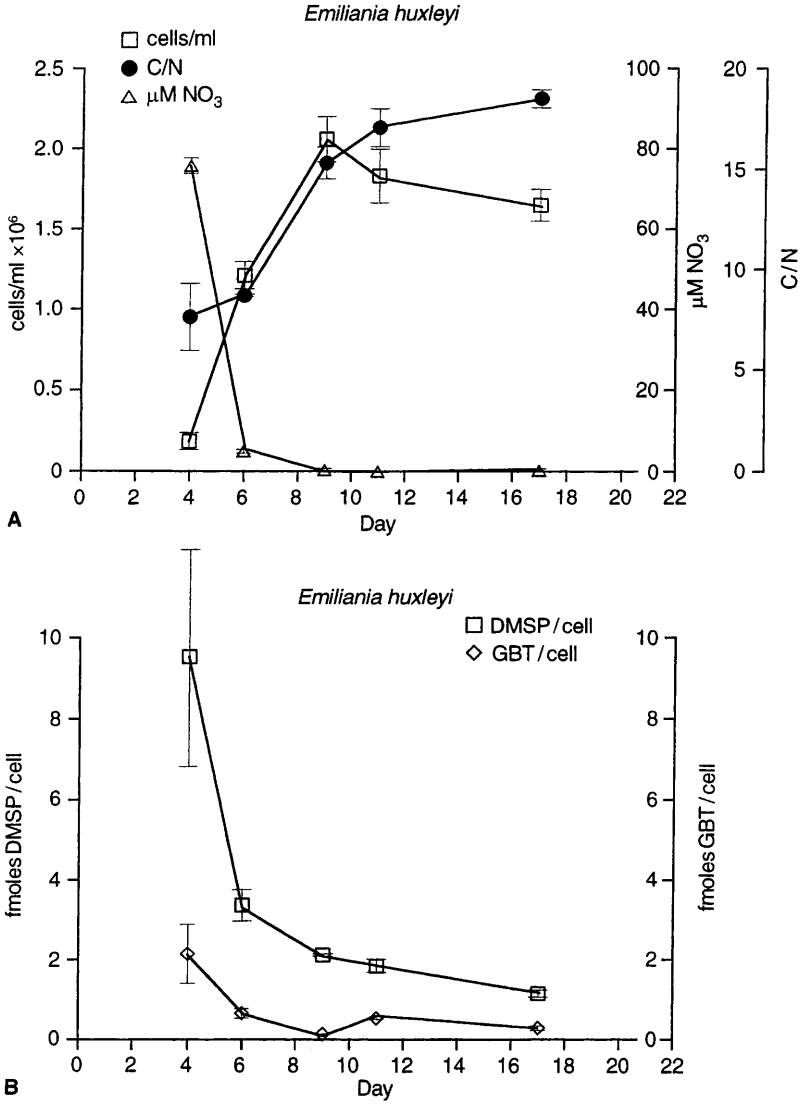

Fig. 2 *Emiliania huxleyi*. **A** Cell numbers, C:N ratio, and external concentrations of NO_3^- over growth cycle in batch culture. **B** Intracellular concentrations of DMSP and GBT over growth cycle in batch culture. Means ± 1 SD are shown for each datum

remained throughout the growth cycle (Fig. 2B). This culture did not appear to produce any other nitrogenous osmolytes detectable by our HPLC analysis. In the dinoflagellate *A. carterae* (CCMP 1314), GBT was present in low concentrations throughout the growth cycle but actually peaked at the onset of stationary growth (Fig. 3B). Another nitrogenous osmolyte, homarine, was maximal in exponential growth, but was absent in stationary phase. In the other three algae, no GBT was detectable and either very low levels or none of the other nitrogenous osmolytes were produced (Table 3).

DMSP contributed significantly to cellular sulfur throughout the growth cycle in all the algae, ranging from 27 to >100% of the measured POS (Tables 2, 3). GBT formed a significant fraction (7%) of the cellular nitrogen only during exponential or early stationary growth in only one species, *Chrysochromulina* sp. Its contribution was 2% or less for the other species (Tables 3, 4). A large percentage (ca. 50%) of the organic nitrogen could not be accounted for in these algae even when cellular pools of protein, amino acids, inorganic nitrogen, and nitrogenous osmolytes were combined (Table 4).

Discussion

The production of GBT and other nitrogenous osmolytes by marine phytoplankton appears to be highly variable, both in terms of differences among species and over the growth cycle. In this regard, GBT is similar to its sulfur analog, DMSP, the production of which is highly species-specific and related to physiological state. The previously observed relationship between DMSP and taxonomic groupings was reconfirmed in this study. The prymnesiophytes and dinoflagellates had greater concentrations of DMSP than either the prasinophyte or diatom on a per cell volume basis, although there were variations with growth stage (Table 3).

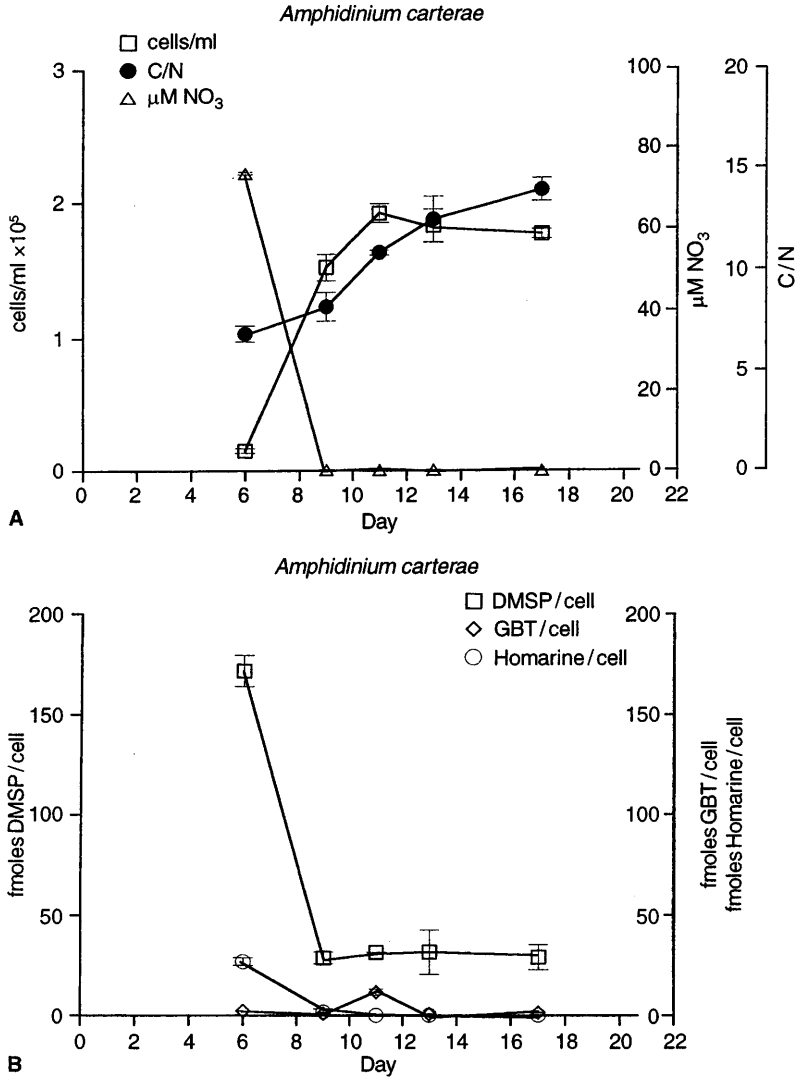

Fig. 3 *Amphidinium carterae*. **A** Cell numbers, C:N ratio, and external concentrations of NO_3^- over growth cycle in batch culture. **B** Intracellular concentrations of DMSP, GBT, and homarine, another nitrogenous osmolyte, over growth cycle in batch culture. Means ± 1 SD are shown for each datum

Levels of DMSP in the prymnesiophyte *Chrysochromulina* sp. (CCMP 288) in this study were comparable to previous observations with an average of 1.3 pg cell^{-1}. Keller et al. (1989) reported a value of 1.57 pg cell^{-1}, while Keller and Bellows (1996) observed levels of 1.1 pg cell^{-1} in N-replete cultures and 1.8 pg cell^{-1} in N-depleted cells of this alga. The diatom *Skeletonema costatum* (CCMP 1332) also had very similar levels to those previously observed, with an average of 0.9 pg cell^{-1} in the present study (Keller et al. 1989; Matrai and Keller 1994; Keller and Bellows 1996). Levels in the prasinophyte, *Tetraselmis* sp. (CCMP 896), were much greater than previously reported, at 4.5 pg cell^{-1} in this study, as opposed to 1.62 pg cell^{-1} (Keller et al. 1989) and 1.47 pg cell^{-1} (Keller and Bellows 1996). The culture conditions used in the present study were somewhat different from previous studies on batch cultures. In one, the N supply was almost an order of magnitude higher, resulting in cells that were presumably much more N-replete (Matrai and Keller 1994). In a second study, cultures were preadapted to either low or high nutrient levels (Keller and Bellows 1996). In addition, previous experiments were conducted with a 14 h light:10 h dark cycle and were not aerated, factors which may have altered DMSP concentrations in the present study.

Unlike previous studies, where intracellular DMSP concentration remained fairly constant over the growth cycle (Keller 1991; Matrai and Keller 1994; Keller and Bellows 1996), there was a general decrease in intracellular levels in all of the algal strains in this study, with especially notable decreases in the dinoflagellates *Prorocentrum minimum* (CCMP 1329) and *Amphidinium carterae* (CCMP 1314) and the prymnesiophyte *Emiliania huxleyi* (CCMP 378) (Tables 2, 3). *P. minimum* (CCMP 1329) had very low intracellular levels of DMSP, except in early exponential growth, in comparison to those seen previously. DMSP in these cultures decreased from 32.6 to 8 pg cell^{-1} in later stages of growth. This contrasts with the levels reported by Keller et al. (1989) for this alga (21.4 pg cell^{-1}) and Matrai and Keller (1994) (43 pg cell^{-1}). The organic sulfur (POS) concentrations in *P. minimum* were comparable between

Table 2 Intracellular concentrations of organic carbon (*C*), nitrogen (*N*), sulfur (*S*) and DMSP(S) (*DMSP*; 18.8% of DMSP) in each phytoplankton strain throughout the growth cycle. All concentrations are in pg cell^{-1}. Mean values, followed by standard deviation in first set of parentheses, and number of samples in second set (*Day* sampling date)

Strain (CCMP No.)	Day	pgC	pgN	pgS	pgDMSP
Chysochromulina sp. (288)	4	40.5 (2.3) (2)	6.9 (0.2) (2)	1.1 (0.6) (4)	0.9 (0.1) (4)
	6	14.1 (0.4) (4)	2.3 (0.1) (4)	0.4 (0.1) (4)	0.3 (0.0) (4)
	9	10.6 (1.5) (3)	1.3 (1.9) (3)	0.2 (0.0) (4)	0.2 (0.1) (4)
	11	14.2 (1.0) (3)	2.0 (0.7) (3)	0.2 (0.0) (4)	0.2 (0.0) (4)
	17	11.5 (0.5) (4)	1.5 (0.0) (4)	0.5 (0.1) (4)	0.2 (0.0) (4)
E. huxleyi (378)	4	20.8 (4.2) (3)	3.0 (1.2) (3)	0.4 (0.1) (4)	0.3 (0.1) (4)
	6	10.1 (0.5) (4)	1.2 (0.1) (4)	0.3 (0.1) (4)	0.1 (0.0) (4)
	9	11.6 (0.6) (4)	0.8 (0.1) (4)	0.2 (0.1) (4)	0.1 (0.0) (4)
	11	16.6 (1.8) (4)	1.0 (0.1) (4)	0.2 (0.0) (4)	0.1 (0.0) (4)
	17	20.7 (1.7) (4)	1.1 (0.1) (4)	0.3 (0.0) (4)	0.1 (0.0) (4)
S. costatum (1332)	2	32.1 (2.4) (4)	5.2 (0.9) (4)	1.1 (0.3) (4)	0.1 (0.0) (4)
	4	22.2 (0.9) (4)	2.4 (0.1) (4)	0.4 (0.0) (4)	0.2 (0.1) (4)
	6	22.8 (3.5) (4)	1.8 (0.1) (4)	0.3 (0.0) (4)	0.2 (0.1) (4)
	9	31.9 (1.5) (4)	7.9 (3.8) (3)	0.1 (0.0) (4)	0.2 (0.0) (4)
	11	27.6 (1.5) (4)	1.6 (0.1) (4)	0.2 (0.0) (4)	0.1 (0.0) (4)
	17	42.9 (4.4) (4)	2.3 (0.3) (4)	1.0 (0.5) (4)	0.2 (0.0) (4)
Tetraselmis sp. (896)	4	90.2 (12.2) (4)	6.5 (0.4) (4)	1.7 (0.4) (4)	1.1 (0.0) (4)
	6	91.5 (11.0) (4)	5.8 (0.9) (4)	1.5 (0.0) (4)	0.8 (0.1) (4)
	9	72.2 (19.6) (4)	4.4 (1.4) (4)	1.6 (0.6) (4)	0.7 (0.2) (4)
	11	70.0 (19.8) (4)	4.2 (1.0) (4)	1.0 (0.3) (4)	0.6 (0.3) (4)
	21	171.1 (37.5) (4)	7.5 (2.6) (4)	3.7 (0.9) (4)	1.3 (0.2) (4)
A. carterae (1314)	6	188.4 (19.3) (3)	27.1 (4.1) (3)	4.5 (0.4)(4)	5.5 (0.2) (4)
	9	75.7 (19.1) (4)	9.4 (3.0) (4)	1.4 (0.2) (4)	0.9 (0.1) (4)
	11	84.6 (1.3) (4)	7.8 (0.2) (4)	1.0 (0.0) (4)	1.0 (0.0) (4)
	13	89.2 (5.0) (4)	7.2 (0.9) (4)	1.3 (0.2) (4)	1.0 (0.4) (4)
	17	99.0 (7.8) (3)	7.0 (0.8) (3)	2.2 (0.6) (4)	0.9 (0.2) (4)
P. minimum (1329)	6	282.2 (48.8) (4)	27.8 (5.3) (4)	8.3 (2.9) (4)	5.3 (1.8) (4)
	9	237.1 (63.8) (4)	41.4 (3.0) (4)	4.7 (0.1) (4)	1.9 (0.1) (4)
	11	241.7 (48.4) (4)	14.8 (2.1) (4)	4.9 (0.3) (4)	1.6 (0.1) (4)
	13	344.4 (13.5) (4)	18.2 (1.7) (4)	7.5 (0.2) (4)	2.1 (0.6) (4)
	21	933.0 (61.7) (4)	26.0 (0.5) (3)	11.9 (2.1) (4)	5.9 (0.7) (4)

Table 3 Physiological indicators of N-nutrition, intracellular concentrations of measured osmolytes, and the relative contribution of DMSP(S) to organic sulfur pools in each phytoplankton strain in exponential (exp.) (N-sufficient) and stationary (stat.) (N-starved) phases of growth (*C* organic carbon; *chl* chlorophyll; *AA* amino acids; *prot*. protein; cm^{-3} unit cell volume). Intracellular concentrations based on live cell volume determinations for each strain. Values represent mean, $n = 4$ in each case, standard deviations in parentheses

Strain (CCMP No.)	Growth phase	C:chl	AA:prot.	µmol DMSP cm^{-3}	µmol GBT cm^{-3}	µmol Trigonelline cm^{-3}	µmol Homarine cm^{-3}	DMSP(S) as % of total sulfur
Chrysochromulina sp. (288)	exp.	87 (2)	0.30 (0.04)	124.0 (2.5)	172.3 (5.1)	1.9 (0.7)	0.0 (0)	68.0 (13.8)
	stat.	268 (66)	0.13 (0.04)	114.6 (5.3)	4.0 (1.3)	1.4 (0.0)	0.0 (0)	100.9[a] (2.6)
E. huxleyi (378)	exp.	105 (11)	0.19 (0.08)	145.6 (41.4)	32.6 (11.3)	0.0 (0)	0.5 (0.1)	68.7 (9.6)
	stat.	168 (14)	0.09 (0.01)	32.3 (0.4)	8.1 (0.26)	0.0 (0)	0.2 (0.1)	46.7 (15.8)
S. costatum (1332)	exp.	62 (2)	0.28 (0.03)	39.3 (12.6)	0.0 (0)	0.2 (0.0)	0.0 (0)	58.7 (19.9)
	stat.	224 (23)	0.15 (0.02)	18.1 (0.8)	0.0 (0)	0.4 (0.1)	0.0 (0)	59.1 (2.1)
Tetraselmis sp. (896)	exp.	394 (136)	0.12 (0.05)	61.7 (1.4)	0.0 (0)	0.1 (0.0)	0.0 (0)	66.9 (17.5)
	stat.	243 (66)	0.23 (0.01)	42.1 (8.9)	0.0 (0)	6.5 (1.9)	0.0 (0)	48.3 (7.8)
A. carterae (1314)	exp.	104 (10)	0.24 (0.00)	218.6 (9.8)	2.7 (0.2)	0.0 (0)	34.5 (2.5)	122.9[a] (5.5)
	stat.	193 (43)	0.07 (0.02)	40.2 (0.4)	15.0 (1.8)	0.0 (0)	0.2 (0.0)	100.7[a] (1.8)
P. minimum (1329)	exp.	121 (6)	0.28 (0.02)	94.8 (32.0)	0.0 (0)	0.0 (0)	0.0 (0)	63.8 (0.8)
	stat.	250 (30)	0.14 (0.01)	37.1 (11.3)	0.0 (0)	0.0 (0)	0.0 (0)	27.6 (9.0)

[a] ~20% of DMSP is volatilized and lost during POS analysis (Matrai and Keller 1994)

the present study and those of Matrai and Keller (1994), although the organic carbon (POC) levels were much greater in the present study (Table 2). This observation, in conjunction with the increasing levels of dissolved DMSP (Fig. 4A) observed in these cultures, suggests that the cells were under considerable physiological

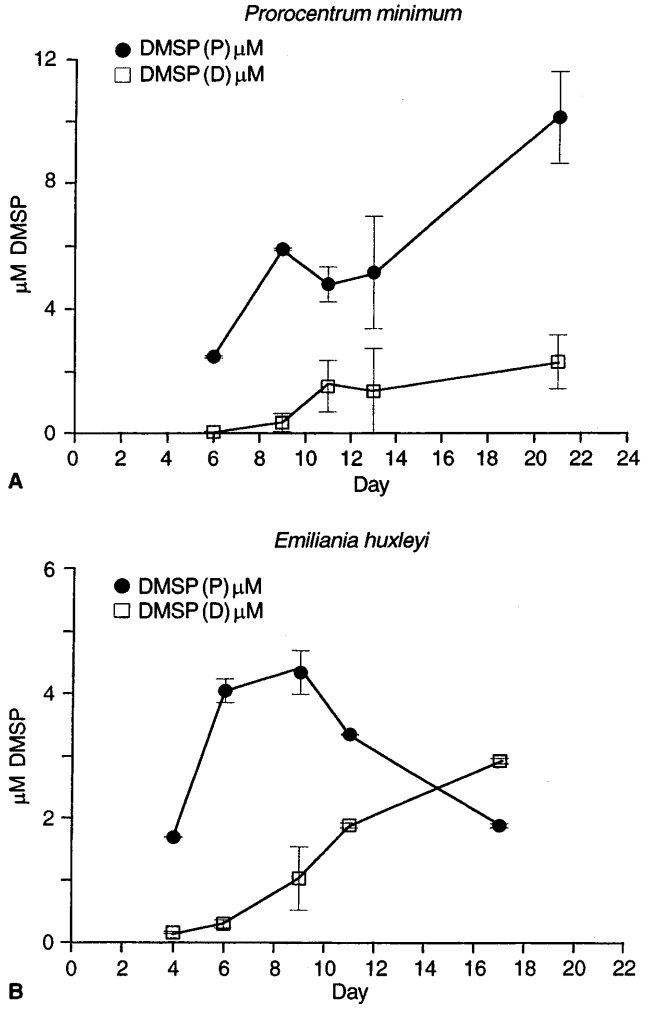

Fig. 4 *Prorocentrum minimum* (**A**), *Emiliania huxleyi* (**B**). Concentrations (μM l^{-1}) of particulate DMSP(P) and dissolved DMSP(D) over growth cycle in batch cultures. Means ± 1 SD are shown for each datum

stress. The prymnesiophyte *E. huxleyi* (CCMP 378) also exhibited quite variable and different production from that observed previously (range from 1.52 pg cell^{-1} initially to a low of 0.3 pg cell $^{-1}$ in senescence). This strain is not the same one used in two previous studies (CCMP 376), with levels of 1.1 pg cell^{-1} (Keller et al. 1989), 1.0 pg cell $^{-1}$ (Matrai and Keller 1994) and 0.8 pg cell^{-1} (Keller and Bellows 1996). The lesser intracellular levels were almost certainly due to the high levels of DMSP dissolved in the medium in these cultures. By senescence, the amount of DMSP in the medium exceeded the cellular DMSP levels (Fig. 4B). In healthy cultures of algae, DMSP remains largely intracellular, with increases in the extracellular fraction only in late stationary stages of growth (Keller 1991; Keller and Bellows 1996). The concentrations reported in the earlier studies were from cells harvested during exponential growth. The sixth alga, the dinoflagellate *A. carterae* (CCMP 1314), also exhibited levels of DMSP at odds with previous reports. Concentrations decreased from 24 to 5 pg cell^{-1} over the growth cycle, and levels of POC and POS (Table 2) were also substantially less than the levels previously reported (Matrai and Keller 1994). Keller et al. (1989) reported levels of 19.3 pg cell^{-1} in this alga, while Keller and Bellows (1996) reported 16.6 pg cell^{-1}. The present study differed in that continuous lighting was used; of the six algae used in this study, it was subsequently determined that only in *A. carterae* (CCMP 1314) did DMSP levels decrease when cultures were grown in continuous light (25% lower).

High initial intracellular values of DMSP were seen in these cultures. This phenomenon has been observed previously (Keller 1991; Matrai and Keller 1994; Keller and Bellows 1996), and similarly, cellular levels of POC, PON, POS, proteins, and amino acids were all elevated at this point (Table 2). Cell volumes did not change over the growth cycle. Originally thought to be an artifact of either carry-over of material during transfer, or an imprecise cell count, either of which could produce artificially high cellular values, the elevated cell concentrations appear to be accurate, if unexplained. DMSP may accumulate rapidly, as do many other small molecular weight precursors, during exponential growth and maximal biosynthesis.

We are reasonably certain that N was the limiting nutrient in these batch cultures. External nitrogen was exhausted when the cells entered stationary phase. Physiological indicators of nitrogen limitation, increasing C:N ratios, increasing C:chl ratios and decreasing AA:protein ratios, all point to cells experiencing increasing N starvation. The ratios we observed in these cultures are very comparable to other reports on N-limited phytoplankton (Sakshaug and Holm-Hansen 1977; Dortch et al. 1984). Dortch et al. (1984) determined that phytoplankton may remain N-replete into early stationary growth, even when external nitrogen is depleted days earlier in exponential growth. For this reason, data collected in late exponential to mid–late stationary growth stages were used to compare cells experiencing N-sufficient versus N-starved conditions (Tables 3, 4). These conditions appear to have been met for all of the strains with the exception of the *Tetraselmis* sp. cultures (CCMP 896). These cultures, based on C:chl and AA:protein ratios, appeared to be more N-replete in late stationary growth, suggesting some sort of nitrogen resupply. The internal inorganic pools of nitrogen in this alga did not increase during this period, confounding interpretation. In all the cultures, cellular pools of nitrate were depleted early but ammonia fluctuated substantially, with large quantities appearing at some points in stationary growth (Table 4). During unbalanced growth, as seen in batch cultures in stationary phase, the ammonia pool appears to be especially dynamic. The role of the nitrogen source on DMSP/GBT production was not examined, although it is known that NO_3^--grown cells require more energy to assimilate C or N than those grown on NH_4^+ (Levasseur et al. 1993 and references within). Symptoms of N limitation in algae may be induced by the type, as well as the supply rate, of

Table 4 Relative contributions of inorganic N pools, amino acids, proteins, GBT and other nitrogenous osmolytes to total particulate organic nitrogen pools in each phytoplankton strain in exponential (N-sufficient) and stationary (N-starved) phases of growth. Values represent means; standard deviation in parentheses

Strain (CCMP No.)	Growth phase	%NO_3	%NH_4	%GBT	%Homarine/ trigonelline	%Amino acids	%Protein	%Cumulative(N)
Chrysochromulina sp. (288)	exp.	0.0 (0.0)	0.9 (0.3)	7.4 (0.0)	0.1 (0.0)	8.5 (1.5)	44.5 (1.4)	61.0
	stat.	0.0 (0.0)	19.9 (20.3)	0.2 (0.0)	0.1 (0.0)	4.4 (0.9)	50.1 (4.3)	74.6
E. huxleyi (378)	exp.	0.0 (0.0)	3.5 (3.3)	1.1 (0.1)	0.0 (0.0)	4.0 (1.0)	34.1 (10.5)	42.8
	stat.	0.0 (0.0)	3.0 (0.7)	0.2 (0.0)	0.0 (0.0)	2.5 (0.0)	40.0 (3.5)	45.7
S. costatum (1332)	exp.	0.1 (0.1)	0.9 (0.1)	0.0 (0.0)	0.0 (0.0)	7.5 (0.2)	41.4 (3.6)	49.8
	stat.	0.0 (0.0)	26.8 (12.1)	0.0 (0.0)	0.1 (0.0)	3.9 (0.5)	41.5 (1.1)	72.2
Tetraselmis sp. (896)	exp.	0.5 (0.7)	0.5 (0.2)	0.0 (0.0)	0.0 (0.0)	3.0 (1.1)	39.4 (3.6)	43.5
	stat.	0.0 (0.0)	0.6 (0.1)	0.0 (0.0)	1.2 (0.8)	5.9 (1.7)	40.8 (1.1)	48.6
A. carterae (1314)	exp.	0.0 (0.0)	1.6 (0.4)	0.1 (0.1)	1.3 (0.1)	6.3 (0.0)	40.7 (0.2)	50.0
	stat.	0.0 (0.0)	10.8 (3.0)	2.1 (0.3)	0.0 (0.0)	2.4 (0.5)	54.1 (3.3)	69.5
P. minimum (1329)	exp.	0.0 (0.0)	2.6 (1.1)	0.0 (0.0)	0.0 (0.0)	7.6 (0.7)	42.1 (1.2)	52.3
	stat.	0.0 (0.0)	29.9 (2.0)	0.0 (0.0)	0.0 (0.0)	4.5 (0.3)	50.0 (0.4)	84.4

nitrogen, and may be exacerbated by the use of NO_3^- as the primary nitrogen source (Levasseur et al. 1993).

There is a direct relationship between N limitation and protein and amino acid production in phytoplankton, with the relative proportion of protein in the total nitrogen pool increasing as other pools, such as amino acids, are exhausted (Morris et al. 1974; Wynne and Rhee 1986; Flynn 1990; Flynn et al. 1993). This conservation of protein was observed in the cultures in the present study as well, with the relative contribution of the protein pool to total cellular nitrogen increasing, and the amino acid pool diminishing from exponential to stationary phase (Table 4). Again, it was only in *Tetraselmis* sp. (CCMP 896) cultures that the protein fraction did not increase proportionately. Instead, the amino acid pool increased in size during stationary growth, another indication that N may have been inadvertently resupplied.

Over the time course of these cultures, DMSP clearly did not increase with increasing N limitation. On the contrary, cellular concentrations of DMSP appeared to decrease with increasing senescence, an observation which is contrary to earlier reports of enhanced levels of DMSP in phytoplankton cultures that were N-depleted (Turner et al. 1988; Gröne and Kirst 1992; Keller and Bellows 1996). In each of these previous studies, the cells were preadapted to either N-replete or N-depleted conditions and were not monitored over a growth cycle with conditions from N sufficiency to N starvation. It was recently confirmed that DMSP is derived from methionine in marine algae (Gage et al. 1997). These authors reported that a transamination reaction initiates the DMSP synthetic pathway (followed by reduction and S-methylation) and suggested that this step may explain in part the observation that DMSP production is enhanced under conditions of N deficiency. The depletion of amino acids occurring with increasing N depletion would favor the transamination reaction, but if methionine was exhausted at some point of N starvation, DMSP production would cease. The depletion of DMSP precursors may explain the dramatic decrease in intracellular levels of DMSP observed in some cultures in the current study. The cells in these experiments were experiencing N starvation and many amino acids may have been exhausted.

DMSP formed a significant percentage of the organic sulfur in all the phytoplankton species examined in this study (Table 3). Relative contributions ranged from 27 to >100%, with the prymnesiophytes and dinoflagellates having much higher levels of POS than the prasinophyte or diatom (data not shown). This confirms the observations of Matrai and Keller (1994), who also determined that a significant percentage of the DMSP(S) could be lost during sampling and handling for POS analysis. This loss leads to overestimation of the contribution of DMSP to total cellular sulfur. This volatilization was especially problematic in those algae in which DMSP(S) is the major contributor to the organic sulfur pool; in these cases, the POS values can be considerably underestimated due to the breakdown of DMSP and the loss of DMS(S). Since the percentage of loss is not constant among algal strains, we did not correct the POS values, but simply acknowledge the discrepancy.

GBT and/or other nitrogenous osmolytes were present in five of the six algal strains examined in this study. The dinoflagellate *Prorocentrum minimum* (CCMP 1329) did not produce any of the identified nitrogenous compounds, GBT, homarine, or trigonelline. The diatom *Skeletonema costatum* (CCMP 1332) contained no GBT but did have small amounts of trigonelline in early stationary phase. In all species, the presence of significant amounts of these nitrogenous compounds appeared to be related to nitrogen availability. In the *Chrysochromulina* sp. (CCMP 288) cultures, the quantity of GBT present in exponential growth actually exceeded the molar quantities of DMSP (Fig. 1B; Table 3) but diminished quickly as the cells entered stationary growth. The other pry-

mnesiophyte alga, *Emiliania huxleyi* (CCMP 378), also produced GBT maximally when the cells were N-sufficient, and maintained a low level throughout stationary growth (Fig. 2B; Table 3). The dinoflagellate *Amphidinium carterae* (CCMP 1314) contained small amounts of GBT in exponential and early stationary growth and greater amounts of homarine when cells were N-sufficient (Fig. 3B; Table 3). Surprisingly, the prasinophyte *Tetraselmis* sp. (CCMP 896) did not contain GBT. This alga (although it was a different strain) was previously reported to contain large amounts of GBT (Dickson and Kirst 1986). The strain in the present study did produce trigonelline, especially in stationary growth, possibly another indication that N was introduced into these cultures at this point. Based on this study of six species, it is not possible to suggest whether there is a strong relationship between taxonomic groupings and GBT (or other nitrogenous osmolytes) production in marine phytoplankton, as has been found for DMSP. It is interesting that the representatives of the two groups producing the highest levels of these nitrogenous compounds, the dinoflagellates and prymnesiophytes, are also the major producers of DMSP.

Previous to this study, almost nothing was known about the roles of GBT or other nitrogenous osmolytes in the nitrogen economy of marine phytoplankton. Based on limited information in other taxonomic groups such as halophytes and cyanobacteria (Stewart et al. 1979; Cavalieri and Huang 1981; King 1988b), it seemed likely that GBT could make up a significant percentage (up to 20%) of cellular N in phytoplankton. Dickson and Kirst (1986) reported that *Tetraselmis subcordiformis* had a GBT concentration of 235 m*M* at a NaCl concentration of 500 m*M* (similar to that in seawater). In a separate study, Wheeler (1983) reported a range of N levels in *T. subcordiformis* from 1525 to 5520 mmol l^{-1} cell volume. In combination, these results suggest that GBT-N may contribute 4 to 15% of the total N in this species. In several species of marine phytoplankton, deAngelis and Lee (1994) measured methylamine production and found total concentrations of mono-, di- and trimethylamine ranging from 2.0 to 5.9 fmol $cell^{-1}$. Glycine betaine is thought to be the primary precursor of methylamines in the marine environment (King 1988a), and the concentrations measured by deAngelis and Lee are equivalent to the amounts of GBT we measured in exponential growth. We found that nitrogenous osmolytes typically contribute $< 2\%$ of the cellular organic N. The exception was the amount of GBT in the exponential phase cultures of the prymnesiophyte *Chrysochromulina* sp. (CCMP 288) (Table 3). The GBT in this alga was ca. 7% of the total nitrogen, a fraction of cellular nitrogen that equals or exceeds the amount typically observed in the total dissolved free amino acid pool of phytoplankton (Wheeler 1983; Dortch et al. 1984; present study). Based on our limited observations, the occurrence of GBT appears to be more ephemeral than that of the amino acid pool, and is highly dependent on N availability.

When N pools were summed in this study, it was clear that a large part of the cellular N was not identified (Table 3). This phenomenon has been observed previously in marine phytoplankton in which a substantial percentage of cellular N was not accounted for even when pools such as NO_3^-, NH_4^-, amino acids, protein, RNA, and DNA were considered (Conover 1975; Dortch et al. 1984; Doucette and Harrison 1991). We used the protein/amino acid sampling protocols recommended by Clayton et al. (1988), who tested several extraction and analysis methods to determine the best combination for marine phytoplankton, but our protein measurements were low in comparison to previous studies (e.g. Dortch et al. 1984). Based on our results, nitrogenous osmolytes, not measured in these previous studies, might make a small contribution to the nitrogen budget in cells, but would not explain the deficit. There is increasing recognition of the importance of dissolved organic N in marine systems (Bronk et al. 1994). These authors noted that in ^{15}N tracer experiments, the amount of added ^{15}N label could not be accounted for in the measured pools, the leftover ^{15}N, or the PON at the end of their experiments. Likewise, Flynn and Flynn (1992) identified a major unidentified peak in their amino acid extracts of marine phytoplankton, which eluted chromatographically near or with glutamine or histidine. This compound was present in dinoflagellates, prymnesiophytes, and diatoms, but was not present in the prasinophytes, chlorophytes, or rhodophytes examined. Clearly, work remains to be done in reconciling organic N distribution in marine phytoplankton and in marine systems in general.

The proposed reciprocal relationship between DMSP and GBT suggested by Andreae (1986) in marine phytoplankton, with DMSP production favored over that of N-containing osmolytes such as GBT in N-limited cells or populations, was not evident in this study. The species we examined continued to produce DMSP regardless of N availability, although variations were evident. The nitrogenous osmolytes, including GBT, did appear to be sensitive to N availability and formed a significant N pool only when cells were N-sufficient. Kadota and Ishida (1968) observed that cellular DMSP in the dinoflagellate *Gyrodinium cohnii* was inversely related to GBT concentration. When GBT was added to growing cultures, the cellular DMSP concentration decreased by half. Similarly, Kiene and Service (1993) demonstrated that the addition of 5 μ*M* GBT to estuarine water samples resulted in a decrease in DMSP(P) production, with coincident increases in DMS and DMSP(D) in the water. We did not amend our cultures directly with GBT additions, so no direct comparisons with these studies are possible, but it appears that the relationship between DMSP, GBT, and N availability is not directly inverse or straightforward.

Factors controlling the production of DMSP by marine phytoplankton are important to understanding the global sulfur cycle. Likewise, the potential importance of nitrogenous osmolytes, such as GBT, to intracellular N pools and N budgets deserves attention. Our

results suggest that both DMSP and GBT are highly dynamic compounds in marine phytoplankton, with their production being species- and perhaps strain-specific and dependent on physiological state. This variability confounds our ability to generalize about the likely responses in these pools to specific environmental parameters, such as N availability.

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