

Influence of CO₂, nitrate, phosphate, and silicate limitation on intracellular dimethylsulfoniopropionate in batch cultures of the coastal diatom *Thalassiosira pseudonana*

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Abstract

We measured intracellular dimethyl sulfoniopropionate (DMSP) concentrations in nutrient-limited batch cultures of the coastal diatom *Thalassiosira pseudonana*. Under N, P, CO₂, and Si growth limitation, intracellular DMSP concentrations decreased to low values during the exponential phase of growth and increased under nutrient limitation. The intracellular DMSP concentration increased exponentially with decreasing growth rate and cellular chlorophyll *a*, in response to the type and degree of nutrient limitation. For a given growth rate, N-limited cells showed the greatest increase in cellular DMSP concentrations, CO₂ and Si limitation had an intermediate effect, and P limitation caused the smallest increase. The results demonstrate the importance of nutrient limitation in enhancing DMSP concentrations in marine algae. This enhancement may be linked in part to the role of DMSP as a cellular antioxidant and to increased oxidative stress within cells during nutrient limitation. The replacement of DMSP, a sulfur-containing osmolyte, for N-containing osmolytes such as proline may also explain at least some of the DMSP increase under N limitation. Our results also point to the possible importance of diatom blooms in global or regional sulfur cycles.

In 1987, the CLAW hypothesis (after its authors, Charlson, Lovelock, Andreae, and Warren) postulated that planktonic marine emissions of the volatile organic sulfur compound dimethylsulfide (DMS) are involved in the biological regulation of climate (Charlson et al. 1987). The oxidation products of marine atmospheric DMS, mainly sulfuric acid, form aerosols that act as cloud condensation nuclei (CCN), thereby influencing the planetary albedo and climate (Bates et al. 1987; Charlson et al. 1987). This hypothesis is supported by the results of studies by Ayers et al. (1991) and Ayers and Gillett (2000), which showed direct correlations among DMS, aerosol non-sea salt sulphate, and CCN in the remote marine atmosphere.

Significant questions remain regarding the physiological roles of dimethyl sulfoniopropionate (DMSP) and DMS in marine algae and the environmental factors that regulate their production. DMSP is mainly produced by Dinophyceae (dinoflagellates) and Prymnesiophyceae (including the coccolithophorids) (Keller et al. 1989). Given its high intracellular concentration (up to 400 mmol L⁻¹), it serves in these species as an important osmolyte. It is cleaved into DMS and acrylate by DMSP lyase, an enzyme that is present in both marine phytoplankton (Stefels and van Boekel 1993) and bacteria (Kiene 1990). Through its cleavage to acrylate, a putative antigrazing compound, DMSP may act as a chemical defense against zooplankton (Wolfe et al. 1997). Also, a role in cry-

protection has been proposed for polar algae (Kirst et al. 1991). Finally, DMSP has been recently proposed to be the first link of an antioxidant system. DMSP and the products of its cleavage and oxidation (DMS, acrylate, and DMSO [dimethylsulfoxide]) all react avidly with hydroxyl radicals and, thus, can serve as effective scavengers of these highly toxic reactive oxygen species (Sunda et al. 2002).

Harmful reactive oxygen species, such as hydrogen peroxide and hydroxyl radicals, are produced as byproducts of photosynthesis (Jakob and Heber 1996; Niogi 1999). Limitation by nutrients leads to metabolic imbalances that can disrupt the photosynthetic mechanism. This, in turn, can lead to an increased production of reactive oxygen species and increased oxidative stress. Examples include CO₂ limitation (Vardi et al. 1999) and Fe limitation, which have been shown to increase the activity of antioxidant enzymes (e.g., ascorbate peroxidase) and cellular DMSP and DMS production in marine algae (Sunda et al. 2002). Although CO₂ is often not thought of as a nutrient, it is an essential growth factor, like N, P, and Si, and, thus, can act as a limiting nutrient. In the present study, we examined the effect of growth limitation by four different nutrients (nitrate, phosphate, silicate, and CO₂) on intracellular DMSP, chlorophyll *a*, and cell size in *Thalassiosira pseudonana*. This coastal diatom has an inducible DMSP system that is up-regulated under limitation by N, Fe, and CO₂ (Keller et al. 1999a; Sunda et al. 2002). The present experiments allowed us to compare the effect of growth limitation by different environmentally relevant nutrients and to examine interrelationships among nutrient limitation, Chl *a*, and intracellular DMSP concentrations. They also investigate the potential role of diatoms in the global sulfur cycle.

Materials and methods

Culture conditions—Axenic batch cultures of the diatom *T. pseudonana* (clone CCMP 1335) were grown in 1-liter

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polycarbonate bottles at 20°C under saturating fluorescent light (Vita Lite Plus, Durotest; irradiance of 560 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$, 12:12 h light:dark cycle). The complete medium consisted of filtered (0.4 μm) Gulf Stream seawater enriched with 64 $\mu\text{mol L}^{-1}$ nitrate, 4 $\mu\text{mol L}^{-1}$ phosphate, 80 $\mu\text{mol L}^{-1}$ silicate, 0.1 $\mu\text{g L}^{-1}$ vitamin B₁₂, 0.1 $\mu\text{g L}^{-1}$ biotin, 20 $\mu\text{g L}^{-1}$ thiamin, and 10 nmol L⁻¹ selenite. The medium also contained a trace-metal ion-buffer system that consisted of 0.5 mmol L⁻¹ nitrilotriacetic acid (NTA), 12 nmol L⁻¹ Cu, 32 nmol L⁻¹ Co, 645 nmol L⁻¹ Zn, 15 nmol L⁻¹ Mn, and 1 $\mu\text{mol L}^{-1}$ Fe. The buffer system generated free ion concentrations of Cu, Co, Zn, and Mn of 10^{-13.0}, 10^{-10.0}, 10^{-9.0}, and 10^{-8.0} mol L⁻¹, respectively, on the basis of equilibrium calculations at pH 8.2. Variation in pH within the experimental range (7.8–8.5) should have had little or no effect on these free ion values. The complete experimental medium had a Redfield N:P ratio of 16:1, so that cells would deplete both of these nutrients simultaneously. The culture media were sterilized by microwave treatment (Keller et al. 1988). The cultures were grown under axenic conditions, and axenicity was checked routinely by pipetting 1-ml culture aliquots into sterile peptone media. CO₂ limitation was generated by the addition of 1.5 mmol L⁻¹ H⁺, followed by degassing of CO₂. Nitrate, phosphate, and silicate limitations were generated by decreasing their respective concentrations by 7-, 20-, and 80-fold. Media were inoculated from dense, nutrient-limited cultures. The experimental cultures were sampled once or twice daily until the late stationary phase of growth.

Total cell volume, volume per cell, and specific growth rate—Total cell volume per liter of culture (CV, $\mu\text{L}_{\text{cell}} \text{ L}^{-1}$) and volume per cell (μm^3) were measured with a Coulter Multisizer II multichannel electronic particle counter with a 100- μm aperture size. The specific growth rate during the exponential phase of growth was determined by linear regression of ln CV versus time over several days (Sunda and Huntsman 1995). When the cells became limited and no longer exhibited a constant growth rate, the instantaneous specific growth rate was determined by the difference in ln CV over the 1-d period prior to sampling.

CO₂ availability—The pH was measured during the course of each experiment so that the concentration of CO₂ could be computed. This was done using the conditional constants for CO₂ and bicarbonate dissociation (Goyet and Poisson 1989), with $T = 20^\circ\text{C}$, $S = 36$, and CO₂ alkalinity = 2.5 mmol L⁻¹. In the CO₂ limitation experiment, the final CO₂ alkalinity was equal to 1.0 mmol L⁻¹ after the addition of 1.5 mmol L⁻¹ of H⁺.

DMSP and Chl a—Samples for intracellular DMSP were filtered onto glass-fiber filters (GF/F) under gentle vacuum (<5 mmHg). The loss of DMSP due to cell lysis did not occur because of the robustness of the diatoms' frustules (Sunda et al. 2002). Cellular DMSP samples were placed in 25-ml glass septum vials (duplicate samples) that contained 5 ml of 5 mol L⁻¹ NaOH. The vials were immediately sealed, and the samples were incubated for 24 h in the dark at room temperature. DMS was measured by gas chroma-

tography after the base hydrolysis of DMSP (Kiene and Gerard 1994). Samples for Chl *a* were filtered onto polycarbonate filters (0.45 μm) for the N-, P-, and Si-limitation experiments. Chl *a* was measured by fluorometry after extraction by DMSO/acetone (Sunda and Huntsman 1995).

Results

The growth of nutrient-limited cultures was compared with that of a control grown in fully enriched medium (64 $\mu\text{mol L}^{-1}$ nitrate, 4 $\mu\text{mol L}^{-1}$ phosphate, 80 $\mu\text{mol L}^{-1}$ silicate, and 2.5 mmol L⁻¹ CO₂ alkalinity) (Fig. 1). Each culture exhibited similar maximum specific growth rates at low to intermediate cell concentrations (Table 1) and then showed decreasing growth rate at higher cell densities as one or more nutrients became depleted. The exponential phase of growth was sometimes preceded by a lag phase because of the nutrient-limited condition of the inoculum (e.g., under CO₂ limitation; Fig. 1b). Experimental treatments exhibited growth limitation at lower cell densities than the controls because of growth limitation by specific nutrients: nitrate (Fig. 1a), CO₂ (Fig. 1b), phosphate (Fig. 1c), or silicate (Fig. 1d). During the P-, N-, and Si-limitation experiments, pH levels were 8.51, 8.34, and 8.45, respectively, at the cell volume maxima, yielding computed CO₂ concentrations of 3.9, 6.6, and 4.7 $\mu\text{mol L}^{-1}$. In previous experiments at the same CO₂ alkalinity, no increases in cellular DMSP or decreases in growth rate were observed in *T. pseudonana* at similar pH and CO₂ values (e.g., pH 8.55; [CO₂] = 3.4 $\mu\text{mol L}^{-1}$) (Sunda et al. 2002, supplementary materials). Thus, CO₂ limitation is unlikely to have been a factor in the N- and P-limitation experiments. It may, however, have occurred in the Si-limitation experiment because of the role of the silica frustule in cellular uptake of inorganic carbon (Milligan and Morel 2002; see "Discussion").

A significant increase of intracellular DMSP concentration occurred for each type of nutrient limitation (Figs. 2–5). The high value measured at the beginning of the experiment reflected the physiological condition of the inocula, which were taken from nutrient-stressed cultures at high cell concentrations. The growth curves of the inoculum cultures are shown in the gray shaded area in the figures. In the experimental cultures, intracellular DMSP decreased during the exponential growth phase because of a decrease in the specific rate of DMSP synthesis relative to the specific rate of biomass synthesis. However, cellular DMSP then increased again when the cell growth rate became limited by a specific nutrient—nitrate (Fig. 2, day 6), CO₂ (Fig. 3, day 17), silicate (Fig. 4, day 7.5), and phosphate (Fig. 5, day 11).

Cell Chl *a* concentrations in the nitrate-, phosphate-, and silicate-limitation experiments followed different trends. In the N-limitation experiment, Chl *a* was highest ($3.7 \pm 0.5 \text{ mmol L}_{\text{cell}}^{-1}$; $n = 6$) during the exponential phase of growth and decreased to 0.22 mmol L_{cell}⁻¹ 12 d after the onset of N limitation (Fig. 2). A lesser decrease was observed under phosphate limitation. Here, the cellular Chl *a* concentration was $3.4 \pm 0.4 \text{ mmol L}_{\text{cell}}^{-1}$ ($n = 4$) during the exponential growth period and decreased to 1.0 mmol L_{cell}⁻¹ 12 d after the onset of P limitation (Fig. 5). Chl *a* behaved differently

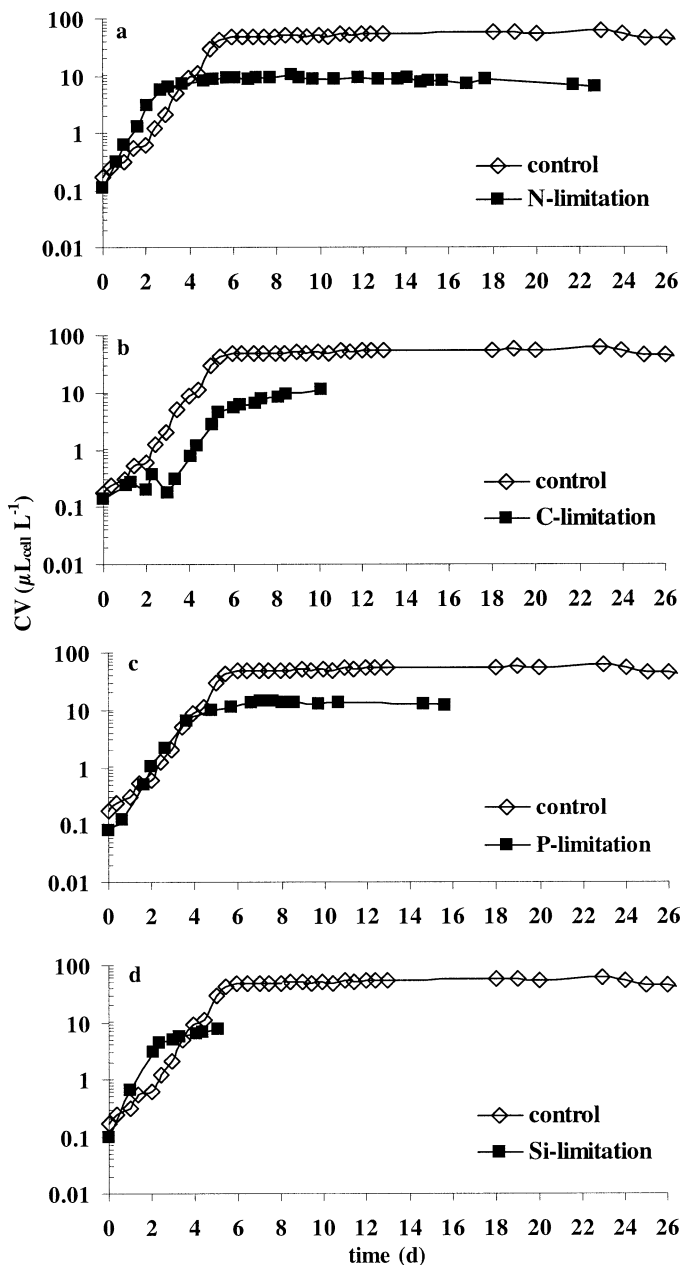


Fig. 1. Comparison of growth curves for cultures grown in nutrient-limited media with that of a control culture grown in fully enriched medium. Nutrient-depleted cultures exhibited growth limitation at lower cell densities than the control culture as a result of lowered concentrations of specific nutrients: (A) nitrate, (B) CO_2 , (C) phosphate, and (D) silicate.

Table 1. Specific growth rate μ (d^{-1}) during the exponential growth phase for the control experiment and the nitrate-, phosphate-, CO_2 -, and silicate-limitation experiments. The squared correlation coefficient for the regression of \ln cell volume versus time (r^2) and the number of data used for the regression (n) are also given.

	Control	N experiment	P experiment	CO_2 experiment	Si experiment
μ (d^{-1})	1.4	1.5	1.3	1.4	1.6
n	5	6	5	6	4
r^2	0.99	0.99	0.99	0.99	0.99

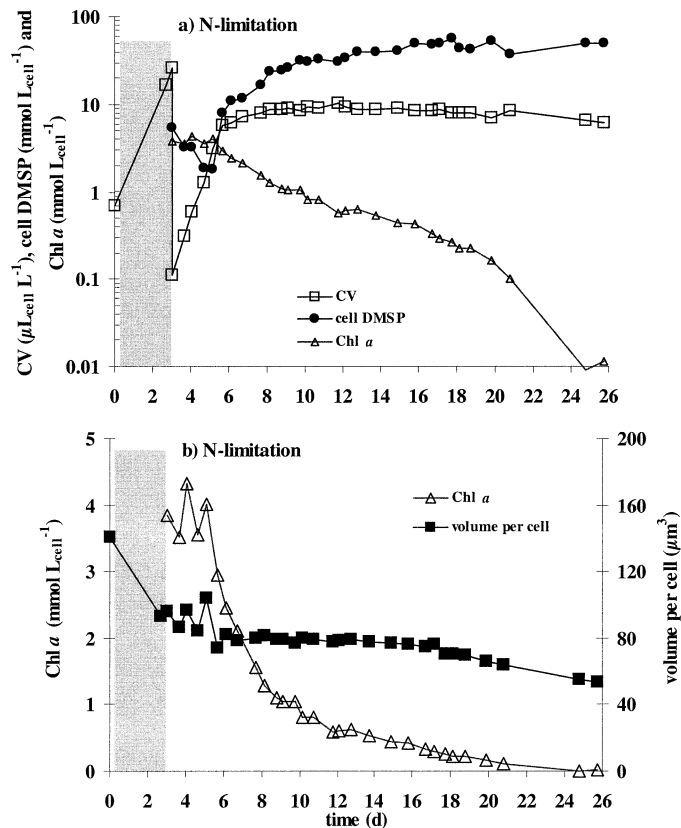


Fig. 2. Intracellular DMSP concentration (cell DMSP, $\text{mmol L}_{\text{cell}}^{-1}$), total cell volume (CV, $\mu\text{L}_{\text{cell}}^{-1}$), cell Chl *a* concentration ($\text{mmol L}_{\text{cell}}^{-1}$), and volume per cell (μm^3) vs. time (d) for the nitrate-limitation experiment. Values for the inoculum culture are shown in the gray-shaded area.

under silicate limitation (Fig. 4); it increased from 3.2 ± 0.3 $\text{mmol L}_{\text{cell}}^{-1}$ ($n = 3$) to 3.6 $\text{mmol L}_{\text{cell}}^{-1}$ at the beginning of the limitation and then slowly decreased thereafter to a relatively high value of 2.7 $\text{mmol L}_{\text{cell}}^{-1}$ at the end of the experiment. In a second experiment, Chl *a* content increased from 3.4 ± 0.2 $\text{mmol L}_{\text{cell}}^{-1}$ ($n = 4$) to 4.2 $\text{mmol L}_{\text{cell}}^{-1}$ at the onset of silicate limitation (data not shown).

The Chl *a* concentration is plotted versus specific growth rate in Fig. 6. Some data for CO_2 limitation measured by Sunda et al. (2002) and Sunda and Huntsman (unpubl. data) for the same species are also presented. Here the differing effects on Chl *a* of Si and CO_2 limitation versus those of N and P limitation were readily observed. Chl *a* showed remarkably similar behavior under both CO_2 and Si limitation of growth rate, with elevated values under moderate limi-

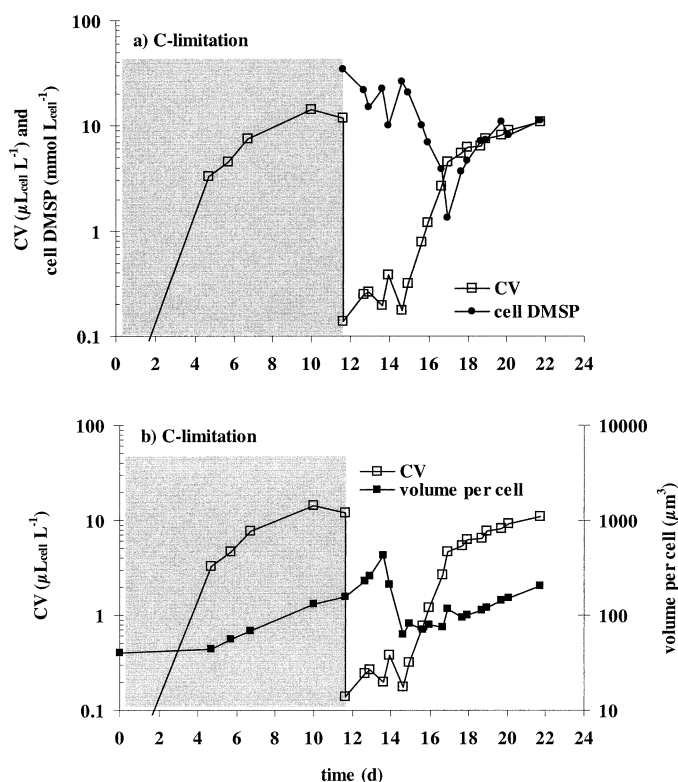


Fig. 3. Intracellular DMSP concentration (cell DMSP, mmol L_{cell}⁻¹), total cell volume (CV, μL_{cell} L⁻¹), and volume per cell (μm³) vs. time (d) for the CO₂-limitation experiment. Data for the inoculum culture are shown in the gray-shaded area.

tation ($\mu \sim 0.5\text{--}1.0 \text{ d}^{-1}$) and decreasing values under more severe limitation. Under N and P limitation, by contrast, Chl *a* exhibited lower values than those found under Si and CO₂ limitation for a given limited growth rate.

Intracellular concentrations of DMSP were plotted versus the instantaneous specific growth rate (μ) (Fig. 7) and cellular Chl *a* (Fig. 8) for each of the limitations studied. To minimize the bias caused by the nutrient stressed condition of the inoculum, data were considered from the day when the intracellular DMSP concentration was the lowest (during the late exponential growth phase) until the end of the experiment. Intracellular DMSP increased exponentially with decreasing growth rate (Fig. 7) for all limitations examined and with decreasing Chl *a* under N and P limitation but not under Si limitation. Linear regressions of log [cell DMSP] versus specific growth rate fit the equation

$$\log[\text{cell DMSP}] = A\mu + B \quad (1)$$

where *A* and *B* are the slope and *Y* intercept, respectively (Table 2). The *r*² values for the regression fits were 0.78, 0.93, 0.73 and 0.76 for N, P, CO₂, and Si limitation, respectively. Slopes of these regressions were always negative.

Likewise linear regressions of log [cell DMSP] versus cell chlorophyll *a* under N and P limitation fit a similar linear equation (Table 3):

$$\log[\text{cell DMSP}] = C\text{Chl } a + D \quad (2)$$

where *C* and *D*, again are the slope and *Y* intercept. The

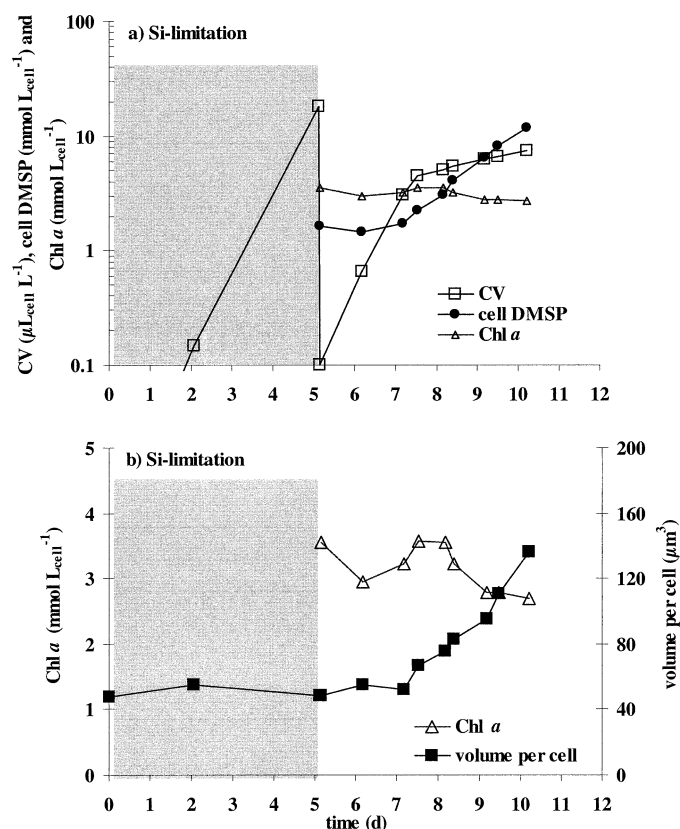


Fig. 4. Intracellular DMSP concentration (cell DMSP, mmol L_{cell}⁻¹), total cell volume (CV, μL_{cell} L⁻¹), cell Chl *a* concentration (mmol L_{cell}⁻¹), and volume per cell (μm³) vs. time (d) for the silicate-limitation experiment. Data for the inoculum culture are shown in the gray-shaded area.

regression *r*² values were 0.95 and 0.85 for N and P limitation, respectively. The *r*² value for the silica limited treatment was 0.46, indicating a much poorer fit to Eq. 2.

The plot in Fig. 7 readily allows us to compare the influence of the different nutrients at the same degree of growth-rate limitation. For a given reduced growth rate, nitrate limitation caused the greatest increase in cellular DMSP concentration, silica, and CO₂ limitations had a nearly identical intermediate effect, and phosphate had the least effect.

The volume per cell was also affected, and it behaved differently under limitation by the different nutrients. For the nitrate experiment, it decreased from 95 to 78 μm³ at the beginning of growth limitation and then remained stable from day 6 to day 17 before decreasing to 53 μm³ during the late stationary/senescence phase (days 17–26). Under CO₂ limitation, most of the inoculum cells were enlarged and misshapen, as observed by microscopic examination. The average volume per cell continued to increase during the first two days of a three day lag period (days 12–15), and then decreased substantially during the final day of the lag period. The volume per cell remained stable at 84 ± 17 μm³ during the exponential growth phase and then increased continuously from the beginning of the CO₂ limitation until the end of the experiment, reaching a final average value of 200 μm³. The same pattern was found under silicate limi-

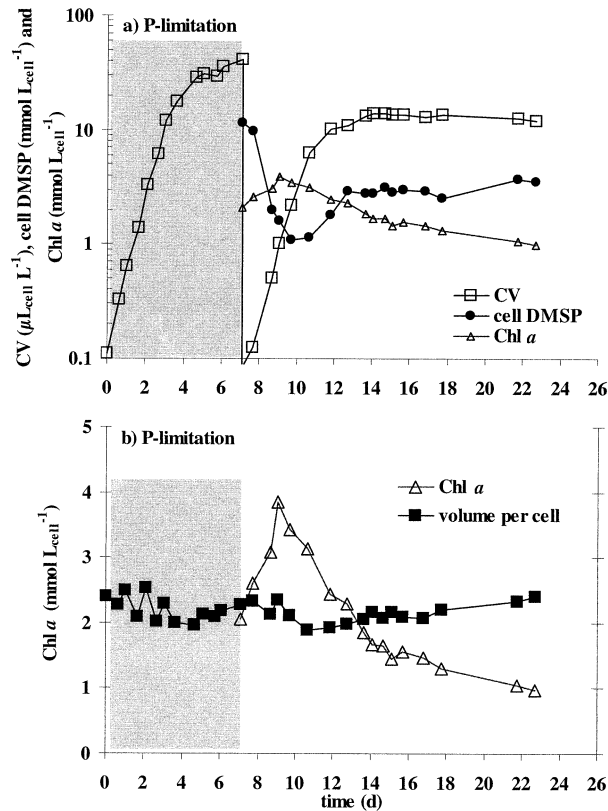


Fig. 5. Intracellular DMSP concentration (cell DMSP, $\text{mmol L}_{\text{cell}}^{-1}$), total cell volume (CV, $\mu\text{L}_{\text{cell}}^{-1}$), cell Chl *a* concentration ($\text{mmol L}_{\text{cell}}^{-1}$), and volume per cell (μm^3) vs. time (d) for the phosphate-limitation experiment. Data for the inoculum culture are shown in the gray-shaded area.

tation: volume per cell had a low constant value ($52 \pm 3 \mu\text{m}^3$) during the exponential phase of growth (days 5–7) and increased continuously under silica limitation, reaching a value of $137 \mu\text{m}^3$ at the end of the experiment. During the phosphate-limitation experiment, the volume per cell de-

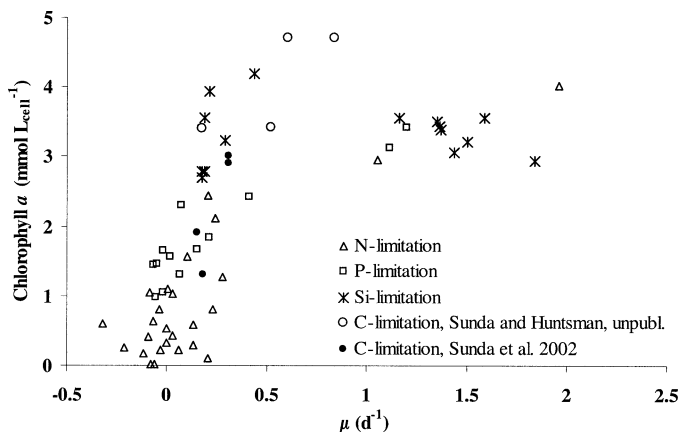


Fig. 6. Linear plot of Chl *a* concentration ($\text{mmol L}_{\text{cell}}^{-1}$) vs. the instantaneous specific growth rate μ (d^{-1}) for nitrate (this study), phosphate (this study), silicate (this study), and CO_2 (filled circles: Sunda et al. 2002, open circles: Sunda and Huntsman, unpubl. data) limitation experiments.

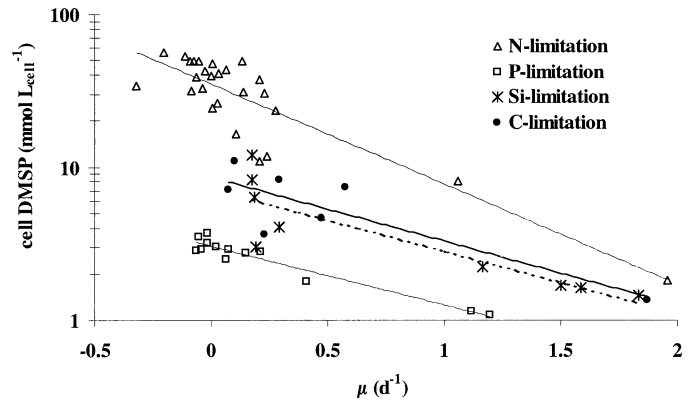


Fig. 7. Intracellular DMSP concentration (cell DMSP, $\text{mmol L}_{\text{cell}}^{-1}$) vs. the instantaneous specific growth rate μ (d^{-1}) for nitrate, phosphate, silicate, and CO_2 limitation.

creased from 93 to $75 \mu\text{m}^3$ during the exponential growth phase. It then increased again to $96 \mu\text{m}^3$ under phosphate limitation.

Discussion

Variations of the intracellular DMSP concentrations during the growth cycle—A high intracellular DMSP concentration at the beginning of batch culture experiments and decreasing concentrations during exponential growth are common features that have been reported in other culture studies (Matrai and Keller 1994; Keller and Korjef-Bellows 1996; Keller et al. 1999b). This pattern was clearly observed during the growth cycle in all of our experiments, with the exception of the Si-limitation experiment, where the experiment was initiated from an exponentially growing culture (Fig. 4a). The pattern supports the hypothesis of a direct link between intracellular DMSP concentration and the cell's physiological state that is related to the level and type of nutrient limitation. When cells were transferred from a limited culture to fresh medium, the cells decreased their intracellular DMSP concentrations as long as they were in the

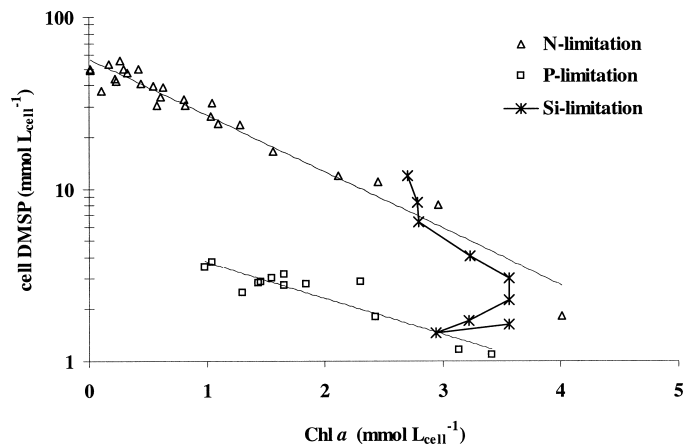


Fig. 8. Intracellular DMSP concentrations (cell DMSP, $\text{mmol L}_{\text{cell}}^{-1}$) vs. cell Chl *a* concentration ($\text{mmol L}_{\text{cell}}^{-1}$) for nitrate, phosphate, and silicate limitation experiments.

Table 2. Regression fit of intracellular DMSP ([cell DMSP]; $\text{mmol L}_{\text{cell}}^{-1}$) and instantaneous specific growth rate (μ) to the equation $\log[\text{cell DMSP}] = A \cdot \mu + B$. Values for A and B for each regression are presented, along with the number of data (n), correlation coefficient (r^2), and P value.

	N experiment	P experiment	CO ₂ experiment	Si experiment
A	-0.65 ± 0.07	-0.38 ± 0.03	-0.42 ± 0.11	-0.40 ± 0.09
B	1.54 ± 0.03	0.48 ± 0.01	0.93 ± 0.09	0.86 ± 0.09
n	26	13	7	9
r^2	0.78	0.93	0.73	0.76
P	<0.01	<0.01	<0.05	<0.01

exponential growth phase. When growth rate again became nutrient limited at high cell concentrations, intracellular DMSP concentration increased. It must be noted that, because of the nutrient-stressed condition of the inoculum and its associated initial high intracellular DMSP concentration, the lowest DMSP values measured during the exponential growth phase (Fig. 7) were often higher than those measured for fully acclimated, exponentially growing cultures ($1.1 \pm 0.2 \text{ mmol L}_{\text{cell}}^{-1}$; Bucciarelli and Sunda unpubl. data).

For the same species, Keller et al. (1999a) found that intracellular DMSP concentrations increased from $0.9 \text{ mmol L}_{\text{cell}}^{-1}$ at a high growth rate (0.8 d^{-1}) to $25.9 \text{ mmol L}_{\text{cell}}^{-1}$ for cells with a severely limited growth rate (0.3 d^{-1}) in N-limited chemostat cultures, which are observations comparable to ours.

DMSP, a multifunctional molecule—Because of its high intracellular concentration, which increased with increasing salinity, DMSP has been suggested to play a role in osmoregulation (Vairavamurthy et al. 1985). Its increase under nitrate limitation was originally explained by the replacement of N-containing osmolytes (e.g., glycine betaine [GBT]) by DMSP, a sulfur-containing osmolyte (Turner et al. 1988). In cultures of *Emiliania huxleyi* supplemented with extra nitrate, the DMSP level per cell was $\sim 20\%$ lower than that found in cultures grown with lower nitrate concentration (Turner et al. 1988). Similarly, Gröne and Kirst (1992) have shown that DMSP per cell in *Tetraselmis subcordiformis* was 75%–100% higher in N-limited batch cultures than that observed in a nonlimited control. Keller et al. (1999a) showed an inverse relationship between intracellular DMSP and GBT concentrations in N-limited chemostats of *Thalassiosira pseudonana*. But the intracellular GBT concentration ($2 \text{ mmol L}_{\text{cell}}^{-1}$) was not high enough for it to have served as a significant osmolyte, given the high osmolality

Table 3. Regression fit of intracellular DMSP ($\text{mmol L}_{\text{cell}}^{-1}$) and cellular Chl a ($\text{mmol L}_{\text{cell}}^{-1}$) concentration to the equation $\log[\text{cell DMSP}] = C \cdot \text{Chl } a + D$. Values for C and D for each limitation are presented, along with the number of data (n), correlation coefficient (r^2), and P value.

	N experiment	P experiment	Si experiment
C	-0.33 ± 0.01	-0.21 ± 0.03	-0.63 ± 0.26
D	1.75 ± 0.03	0.78 ± 0.05	2.52 ± 0.81
n	26	13	9
r^2	0.95	0.85	0.46
P	<0.01	<0.01	<0.05

($\sim 1 \text{ mol L}^{-1}$) of the seawater medium. Investigations of *Spartina anglica* Hubbard (van Diggelen et al. 1986) and *Spartina alterniflora* Loisel (Otte and Morris 1994) have also shown that intracellular DMSP concentrations did not correlate with salinity, which excludes an osmoregulatory role in these salt marsh grasses. Furthermore, although the use of DMSP as a substitute for N-containing osmolytes may contribute to the high DMSP under N limitation in our experiments, it cannot explain the increase in DMSP observed under CO₂, Si, and P limitations.

The production of DMSP under nitrogen limitation has also been explained as a way to eliminate excess reducing equivalents and to avoid an overproduction of carbohydrates, a so-called overflow mechanism (Stefels and van Leeuwe 1998). But if the production of DMSP indeed mainly served as an overflow mechanism for excess reducing equivalents, it should not increase under CO₂ limitation, as was observed in our study.

Grazing experiments have suggested a chemical defense role for DMSP, because its cleavage produces acrylate, a compound that appears to deter protozoan grazing (Wolfe et al. 1997). This role has to be considered, particularly under nitrogen limitation, which causes cells to become smaller. The resulting higher surface:volume ratio should increase uptake per unit of biovolume and, thus, may represent an adaptive mechanism (Hudson and Morel 1990). Smaller cells also are more easily grazed, which could promote anti-grazing adaptive strategies such as DMSP lysis to acrylate. The extent to which acrylate can reduce grazing is not yet clear, however, although grazing-mediated release remains a significant mechanism for DMSP conversion to DMS in seawater (Dacey and Wakeham 1986; Wolfe et al. 1997).

Another role has been recently proposed—that DMSP acts as the first link of a cellular antioxidant system. This system involves DMSP and the products of its cleavage and oxidation (DMS, acrylate, and DMSO) (Sunda et al. 2002). This high-capacity antioxidant system appears to be involved in the scavenging of hydroxyl radicals, highly toxic reactive oxygen species (Sunda et al. 2002). Oxidative stress occurs from the production of reactive oxygen species (superoxide radicals, hydrogen peroxide, and hydroxyl radicals) via the reaction of reduced components of the photosynthetic or respiratory electron transport chains with molecular oxygen. It also occurs when the excited states of pigments react with molecular oxygen to produce singlet oxygen (Foyer and Harbinson 1994). Any process that restricts metabolic efficiency and the smooth flow of excitation energy or electrons within the photosynthetic apparatus should increase the production

of reactive oxygen species and thereby increase oxidative stress within the cell. The formation of active oxygen species is accelerated under stress conditions, such as low temperature, high salinity, and CO₂ limitation (Butow et al. 1998; Noctor and Foyer 1998).

A number of enzymes and small molecules (e.g., ascorbic acid and glutathione) are involved in antioxidant protection, either in preventing the overexcitation or overreduction of the photosynthetic apparatus or in removing harmful reactive oxygen species. Among important antioxidant enzymes are superoxide dismutase and ascorbate peroxidase, which remove superoxide radicals and hydrogen peroxide within the photosynthetic water–water cycle (Asada 1999). Antioxidants and antioxidant systems are generally up-regulated under increased oxidative stress. For example, the cellular activities of catalase (Butow et al. 1998) and ascorbate peroxidase (Sunda et al. 2002) increase under CO₂ limitation, a known oxidative stressor. Similarly, ascorbic acid concentrations increase relative to chlorophyll under nitrogen limitation (Logan et al. 1999). An up-regulation of intracellular DMSP concentration or of enzymatic conversion of DMSP to DMS also occurs under increased oxidative stress linked to solar ultraviolet radiation exposure or to CO₂ and Fe limitation (Sunda et al. 2002). The increase in intracellular DMSP concentrations observed during our study under the different nutrient-limitations may also be linked to increased antioxidant protection.

The use of DMSP as an antioxidant under nutrient limitation—If, as we propose, the increase in the intracellular DMSP concentration is related to the level of oxidative stress, intracellular DMSP should be linked to the specific growth rate and cellular Chl *a* under nutrient limitation. These two parameters should be related to oxidative stress via metabolic imbalances brought on by nutrient limitation. Figs. 7 and 8 provide support to this hypothesis. Intracellular DMSP increases exponentially with decreasing growth rate (Fig. 7) and the associated decreases in Chl *a* (Fig. 8). The level of this effect depends on the type of nutrient limitation. Nitrate limitation has the greatest effect, silicate and CO₂ limitation cause a similar intermediate effect, and phosphate limitation has the least impact. CO₂, phosphate, nitrate, and silicate limitations all cause metabolic imbalances and, thus, should increase oxidative stress. CO₂ limitation directly affects the Calvin–Benson cycle and has been shown to stimulate the formation of reactive oxygen species in cultured phytoplankton to a level that induces programmed cell death (Vardi et al. 1999). Nitrate (Gröne and Kirst 1992; Berges and Falkowski 1998), phosphate (Sakshaug and Holm-Hansen 1977), and silicate limitation (present study, data not shown; Lippemeier et al. 2001) all decrease photosynthetic efficiency as indicated by a decrease in variable to maximum photosystem II fluorescence. This can be due to a reduced rate of CO₂ fixation in the Calvin–Benson cycle (Govindjee et al. 1973) and/or to inefficient electron transport within the photosynthetic electron transport chain.

The effect of nitrogen—At least some of the substantial increase in intracellular DMSP under nitrate limitation may be due to the replacement of N-containing osmolytes (e.g.,

proline) by DMSP, which contains no nitrogen. However, some portion of the effect may also be linked to increased oxidative stress under N limitation brought on by metabolic imbalances or by the restricted synthesis of N-containing antioxidants such as tripeptide glutathione and various antioxidant enzymes. Metabolic imbalances should occur under nitrate limitation. Because light harvesting and electron transport within the photosynthetic apparatus, CO₂ fixation within the Calvin–Benson cycle, and the enzymatic elimination of reactive oxygen species are all dependent on proteins that are rich in nitrogen. Under nitrogen limitation, the concentration of the photosynthetic carboxylation enzyme Rubisco (Berges and Falkowski 1998) and the ratio of Rubisco activity to electron transport activity (Evans and Terashima 1987) decline. Logan et al. (1999) noted that the resultant surplus electron flow could lead to enhanced oxygen photoreduction in the chloroplast and the production of elevated levels of reactive oxygen species. In the Berges and Falkowski (1998) study, nitrogen starvation induced programmed cell death, a process that is associated with oxidative stress in many organisms (Madeo et al. 1999; Vardi et al. 1999). Finally, nitrate limitation decreases the efficiency of enzymatic mechanisms that repair cell damage from oxidative stress (Litchman et al. 2002).

The effect of silica and CO₂—Silica and CO₂ limitations have an intermediate effect on intracellular DMSP and both exhibit strikingly similar features. Both cause an up-regulation of Chl *a* under mild growth limitation (Fig. 6), a similar increase in volume per cell (Figs. 3 and 4), and virtually indistinguishable exponential increases in intracellular DMSP with decreasing growth rate (Fig. 7). These similarities suggest common or linked causal mechanisms. In this regard, the silica frustule has recently been shown to play a role in CO₂ acquisition, which indicates that Si limitation can induce CO₂ limitation in diatoms (Milligan and Morel 2002). Specifically, the silica frustule facilitates the enzymatic conversion of bicarbonate to CO₂ at the cell surface by serving as a pH buffer (Milligan and Morel 2002). Silica limitation could thus lead to carbon limitation and thereby promote the production of reactive oxygen species.

The same pattern in cell Chl *a* content under CO₂ and silica limitation (Fig. 6), which differs from that under nitrate and phosphate limitation, lends support to this hypothesis. Under nitrate and phosphate limitation, cellular Chl *a* concentration decreased continuously with decreasing growth rate. The decrease in growth rate decreased the cellular requirement for adenosine triphosphate (ATP) and reducing equivalents, leading to a down-regulation in net Chl *a* synthesis, to maintain a balance between light acquisition and biosynthesis.

However, under CO₂ limitation, there is an increased ATP demand to fuel CO₂ acquisition. Evidence for active transport of inorganic carbon has been reported in *T. pseudonana* and other algae, and this so-called inorganic carbon pump requires energy, which is supplied from photosynthetically generated ATP (Falkowski and Raven 1997). The pump is also dependent on HCO₃⁻ conversion to CO₂ at the cell surface, which is catalyzed by carbonic anhydrase. Under CO₂ limitation, an intensification of the inorganic carbon pump

is needed to maintain adequate rates of CO₂ fixation (Raven and Johnston 1991). Up-regulation of the photosynthetic apparatus, and, hence, Chl *a*, is therefore needed to provide sufficient ATP to fuel the CO₂ pump. Because the silica frustule provides a pH buffer for the enzymatic conversion of HCO₃⁻ to CO₂ (Milligan and Morel 2002), silica limitation should reduce the efficiency of the inorganic carbon pump and lead to an additional requirement in photosynthetically derived ATP and, thus, an up-regulation of Chl *a* synthesis. The above scenario, therefore, provides an explanation for the increase in cell chlorophyll under moderate levels of both CO₂ and silica limitation (Fig. 6). Also, by inducing CO₂ limitation, Si limitation should increase the formation of reactive oxygen species within the chloroplast and thereby induce an up regulation of DMSP.

Both Si and CO₂ limitation also caused similar increases in volume per cell. Silicic acid uptake, silica frustule formation, and the cell division cycle are all tightly linked. Under silica limitation, the diatom cell cycle predominantly stops at the G2 phase, before the completion of cell division (Martin-Jézéquel et al. 2000). Thus, an inhibition of cell division linked to an inability to synthesize new cell wall material under silica limitation could readily explain the increase in the volume per cell observed in our study (Fig. 4). This increase could also be partly explained by the formation of auxospores with a larger cell diameter, as was observed under silicate limitation in the diatom *Skeletonema costatum* (Davis 1973, cited by Harrison et al. 1976). But a limitation of cell wall synthesis does not readily explain the similar increase in cell size observed under CO₂ limitation. Furthermore, microscopic observation revealed that the increase in cell size was largely due to an inhibition of cell division, as well as to some auxospore formation. Under increased oxidative stress, as occurs under CO₂ limitation, there is an increased production of reactive oxygen species (Vardi et al. 1999), and these can cause severe damage to DNA (Halliwell and Gutteridge 1984). This damage could inhibit DNA replication and cell division under both CO₂ and silica limitation, which would increase the volume per cell. Further experiments, however, are needed to establish firm links among oxidative damage to DNA, inhibition in cell division, and increased cell size under CO₂ or Si limitation.

The effect of phosphorus—Phosphorus occurs in DNA, RNA, phospholipids, and ATP. Sakshaug and Holm-Hansen (1977) noted a significant decrease in RNA and ATP per cell in P-deficient diatoms. Decreases in membrane phospholipids have also been observed under P limitation (Brussaard et al. 1997). The inability to produce nucleic acids under P limitation should inhibit cell division, which might explain the increase observed in the volume per cell (Fig. 5). By restricting the synthesis of RNA and ATP, P limitation will also lead to a decrease in the rate of protein synthesis. This includes proteins within the photosynthetic apparatus and the Calvin–Benson cycle, which should cause a decrease in rates of light utilization and carbon fixation (Falkowski and Raven 1997). The inhibition of protein synthesis under P limitation may thus have effects on cell metabolism and oxidative stress similar to those for inhibition of protein synthesis under N limitation, except that the effect is indirect and less

immediate. This less direct effect may explain why P limitation causes a smaller increase in cellular DMSP than does limitation by the other nutrients.

Significance of diatoms in the global sulfur cycle—Diatoms have been estimated to contribute to up to 40% of the global oceanic primary production (Nelson et al. 1995) and to play a major role in exporting organic carbon from upper layers to the deep ocean (Smetacek 1999). On the basis of this and other studies (see below), they might also play a role in the global sulfur cycle. They represent >75% of the total primary production in the Southern Ocean (Tréguer et al. 1995), an important region for atmospheric DMS emissions. Calculations have shown that the Antarctic Ocean, which represents only ~6% of the total area of the ocean, may contribute 10%–30% of global oceanic DMS emissions (Gibson 1990; Turner et al. 1995). This high DMS flux is generally attributed to high DMSP-containing *Phaeocystis* species (Stefels and van Leeuwe 1998). Although laboratory studies have concluded that diatoms are minor producers of DMSP (Keller et al. 1989), field studies have revealed significant cellular DMSP and DMS production by some bloom-forming Arctic diatom species (Levasseur et al. 1994; Matrai and Vernet 1997). A recent study in the Southern Ocean also showed significant DMSP production by lightly silicified diatoms in bottle-enrichment studies with natural phytoplanktonic communities (DiTullio et al. 2001). Our current study suggests that diatom DMSP production may be most intense at the end of blooms when nutrients (N, P, Si, and CO₂) become limiting. The production of DMS from the intracellular lysis of DMSP (Sunda et al. 2002) or from grazing, viral lysis, and bacterially mediated cleavage of DMSP might then become important.

Diatoms are generally considered to be low DMSP producers in the ocean. However, this and previous studies (Keller et al. 1999a; Sunda et al. 2002) have shown that limitation by environmentally relevant nutrients (phosphorus, nitrogen, silicon, CO₂, and iron) can substantially increase intracellular DMSP concentrations in some diatoms such as *T. pseudonana* and *S. costatum*. If other species, especially oceanic ones, were also found to express high intracellular DMSP concentrations under nutrient limitation, diatoms could be more important DMSP producers than has previously been thought, at least in some oceanic regions. Thus, DMS production by diatoms may have to be considered in global or regional sulfur budgets.

Concluding remarks—DMSP appears to be a multifunctional molecule—part osmolyte, cryoprotectant, and chemical defense against grazing. Its role as the first link of an antioxidant system now has to be considered, as does the linkage between nutrient limitation and oxidative stress. The relative importance of these different DMSP functions in phytoplankton cells, however, may vary among species and be highly dependent on environmental conditions such as differences in solar irradiance or nutrient limitation. Because of its wide spatial and temporal variations in the ocean, nutrient limitation may play an important role in regulating the dynamics of DMSP and DMS in marine surface waters and thereby influence DMS emissions to the atmosphere.

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