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## Increased intracellular concentrations of DMSP and DMSO in iron-limited oceanic phytoplankton *Thalassiosira oceanica* and *Trichodesmium erythraeum*

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### Abstract

We investigated the link between iron (Fe) limitation and intracellular dimethylsulfoniopropionate (DMSP) concentration in two oceanic phytoplankton species, the diatom *Thalassiosira oceanica* and the diazotrophic cyanobacterium *Trichodesmium erythraeum*. Dimethylsulfoxide (DMSO) concentrations were also measured in Fe-replete and Fe-limited *T. oceanica*. Fe limitation decreased the growth rates of *T. oceanica* and *T. erythraeum* by 33-fold and 3.5-fold, respectively and increased intracellular DMSP (DMSP<sub>p</sub>) concentrations by 12-fold (from 2.8 to 33.7 mmol L<sub>cell</sub><sup>-1</sup>) and by 45-fold (from 0.05 to 2.27 mmol L<sub>trichome</sub><sup>-1</sup>), respectively. Intracellular dimethylsulfoxide (DMSO<sub>p</sub>) concentrations in *T. oceanica* increased by 5-fold under severe Fe limitation, from 0.78 mmol L<sub>cell</sub><sup>-1</sup> in Fe-replete cells to 3.86 mmol L<sub>cell</sub><sup>-1</sup>. The increase in DMSP<sub>p</sub> and DMSO<sub>p</sub> under Fe limitation provides support for the role of these sulfur compounds as antioxidants. Under severe Fe limitation, the large increase in DMSP<sub>p</sub>:C and DMSP:chlorophyll *a* (Chl *a*) ratios for both *T. oceanica* (by 16- and 40-fold, respectively) and *T. erythraeum* (by 18- and 145-fold, respectively) places these species above the range of values generally attributed to diatoms and cyanophytes. Comparison of these values with in situ results, such as those from Fe fertilization experiments, suggests that the decrease in DMSP<sub>p</sub>:Chl *a* and DMSO<sub>p</sub>:Chl *a* that is generally observed with alleviation of Fe limitation may be partly related to decreases in DMSP<sub>p</sub> and DMSO<sub>p</sub> in individual species. The role of diatoms and diazotrophic cyanobacteria in the biogeochemical cycle of dimethylsulfide and associated sulfur compounds in Fe-limited oceanic environments should not be overlooked.

The CLAW hypothesis, named after the initials of the authors (Charlson, Lovelock, Andreae, and Warren), postulates that atmospheric oxidation products of dimethylsulfide (DMS), a volatile sulfur compound produced by phytoplankton, are cloud condensation nuclei (CCN) that can increase cloud droplet formation and the planetary albedo (Charlson et al. 1987). It also proposes that increased albedo and resulting lower temperature may lower phytoplankton growth and decrease DMS production (Charlson et al. 1987). Such hypotheses, postulating biogeochemical feedbacks at the global scale, are very difficult to prove or even to test because of the complexity of the Earth system. Recently, the existence and significance of the CLAW hypothesis has been challenged by Quinn and Bates (2011), who provided evidence that sea salt and organics are better candidates for CCN production in the remote marine boundary layer than DMS. However, using 9 yr of global satellite data and ocean climatologies to derive parameterizations of the seasonal variability of sea

salt particles, sulfur aerosols derived from DMS, and primary and secondary organic aerosols, Lana et al. (2012) concluded that both sulfur and organic aerosols are important in CCN production. This issue is still strongly debated.

There are also many uncertainties in the various processes that control the biogeochemical cycle of DMS. Oceanic emissions of DMS to the atmosphere result from complex physical, biological, and chemical interactions that are not well constrained (Stefels et al. 2007). For example, the mechanisms that regulate phytoplankton production of dimethylsulfoniopropionate (DMSP, the DMS precursor) and its enzymatic cleavage into DMS and acrylate are still debated. The contribution of dimethylsulfoxide (DMSO), an oxidation product of DMSP and DMS, to the dimethylated sulfur pool and biogeochemical cycle of DMS is also poorly understood (Simo 2004).

Intracellular DMSP concentrations and DMS-producing enzyme activity vary with many different physical and chemical parameters. For example, they appear to be up-regulated by increased salinity (Vairavamurthy et al. 1985)

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and growth rate limitation by major nutrients (N, P, and CO<sub>2</sub>; Keller et al. 1999; Bucciarelli and Sunda 2003; Sunda et al. 2007) and the micronutrient iron (Fe; Stefels and van Leeuwe 1998; Sunda et al. 2002). DMSP has a chemical structure similar to the osmolyte glycine betaine, and it serves as a sulfur-containing osmolyte in marine phytoplankton (Vairavamurthy et al. 1985). This could explain why DMSP concentrations increase under nitrogen limitation, where it can be used to replace N-containing osmolytes like glycine betaine or proline (Keller et al. 1999). Stefels and van Leeuwe (1998) also proposed that DMSP production can act as a photosynthetic overflow mechanism to eliminate excess reducing equivalents and ATP and thereby avoid carbohydrate overproduction under nitrogen-limiting conditions. Because of their ability to react with hydroxyl radicals, Sunda et al. (2002) postulated that DMSP and its breakdown products (acrylate, DMS, and the oxidation products DMSO and methane sulfonic acid) serve as antioxidants. Antioxidants react with reactive oxygen species (ROS, like hydroxyl radicals, superoxide radicals, hydrogen peroxide) and thereby protect the cell against their damaging and potentially lethal effects (Lesser 2006). Any process that disrupts the transfer of electrons within the photosynthetic apparatus will increase the formation of ROS and thereby increase oxidative stress. For example, nutrient limitation (e.g., by Fe) increases the production of ROS and can induce programmed cell death, which is associated with oxidative stress (Berman-Frank et al. 2004). In addition, Fe limitation decreases the concentration and activity of known Fe-containing antioxidant enzymes like superoxide dismutase and ascorbate peroxidase (Sunda et al. 2002; Peers and Price 2004).

Due to its low, subnanomolar concentrations in surface waters of the open ocean, Fe is estimated to limit phytoplankton growth in ~ 50% of the world ocean, including high-nutrient, low-chlorophyll (HNLC) regions and nitrogen-poor low-latitude waters where Fe often limits nitrogen fixation by diazotrophs (Boyd and Ellwood 2010). During the last two decades, mesoscale Fe fertilization experiments in oceanic HNLC regions have all shown that Fe addition increases phytoplankton growth and biomass (Boyd and Ellwood 2010) and generally affects DMSP and DMS production (Turner et al. 2004; Liss 2007). While there is no obvious trend in the variations of DMS concentrations following Fe fertilization (Turner et al. 2004; Levasseur et al. 2006), DMSP concentrations generally increase with bloom development (Turner et al. 2004; Merzouk et al. 2006). However, the ratio of DMSP:chlorophyll *a* (Chl *a*) generally decreases. This decrease has been attributed partly to a shift from high-DMSP producers, such as nanophytoplankton, to low-DMSP producers, such as diatoms (Liss 2007). High DMSP production is indeed generally linked to dinoflagellates and prymnesiophytes, while diatoms and cyanobacteria are considered to be low-DMSP producers (Keller et al. 1989; Stefels et al. 2007). Diatoms, however, can considerably increase their DMSP content under nutrient limitation (Sunda et al. 2002; Bucciarelli and Sunda 2003; Sunda et al. 2007) and are the phytoplankton group whose

growth is stimulated most dramatically by Fe addition in the HNLC regions: the Subarctic Pacific, Equatorial Pacific, and Southern Ocean (Boyd and Ellwood 2010).

In low-nutrient, low-chlorophyll regions, Fe-rich mineral dust deposition stimulates blooms of *Trichodesmium*, a colonial nitrogen-fixing cyanobacterium genus (Lenes et al. 2001). In tropical and subtropical nutrient-poor ocean waters, *Trichodesmium* forms extensive blooms and may contribute a larger fraction of total marine nitrogen fixation than any other organisms (Capone et al. 2005). Due to the high iron requirement for N<sub>2</sub> fixation, diazotrophic growth by *Trichodesmium* and other N<sub>2</sub>-fixing cyanobacteria requires much more cellular iron than equivalent photosynthetic growth on fixed nitrogen chemical species such as ammonium or nitrate (Kustka et al. 2003). As a consequence, N<sub>2</sub> fixation and resultant growth of *Trichodesmium* can be limited by iron availability (La Roche and Breitbart 2005).

Despite its potential importance, the influence of Fe on intracellular DMSP levels has been investigated only in the prymnesiophyte *Phaeocystis* sp. (Stefels and van Leeuwe 1998) and the coastal diatoms *Thalassiosira pseudonana* and *Skeletonema costatum* (Sunda et al. 2002). If Fe limitation significantly increases DMSP production by oceanic diatoms and diazotrophic cyanobacteria, these groups may have to be considered in global or regional DMSP and DMS biogeochemical cycles. Iron limitation may also affect intracellular concentrations of DMSO, which influences the DMS cycle. DMSO can be produced intracellularly by marine phytoplankton (Simo et al. 1998; Hatton and Wilson 2007) from the oxidation of DMSP or DMS (Sunda et al. 2002). It can be enzymatically reduced back to DMS, possibly fueling a coupled DMS and DMSO antioxidant cycle (Spiese et al. 2009). Under increased oxidative stress, phytoplankton may thus increase DMSO and DMS production (Spiese et al. 2009). In the Peruvian upwelling region, Riseman and DiTullio (2004) found that DMSP and DMSO correlated well with the antioxidant  $\beta$ -carotene. They measured higher DMSP and DMSO concentrations in low-iron open ocean waters off Peru than in the adjacent high-iron waters of the continental shelf, which they suggested may be caused by increased oxidative stress due to iron deficiency. Asher et al. (2011) recently showed that biological DMSO reduction is the main source of DMS (rather than DMSP lysis) in Antarctic sea ice, indicating that such reduction may be an important overlooked process in DMS production.

In this study we investigated the influence of Fe limitation on intracellular DMSP in two oceanic species, the diatom *Thalassiosira oceanica* and the diazotrophic cyanobacterium *Trichodesmium erythraeum*. We also measured intracellular DMSO in *T. oceanica* to help us assess the role of these sulfur compounds as antioxidants.

## Methods

The oceanic diatom *T. oceanica* (CCMP 1005) and the oceanic diazotrophic cyanobacterium *T. erythraeum* (IMS 101) were grown at different iron concentrations, ranging from growth sufficient to growth limiting. All bottles and

labware were thoroughly cleaned with suprapure hydrochloric acid and ultrapure water (MilliQ; > 18.2 M $\Omega$ ), and all manipulations were conducted in a clean laboratory within a class 100 laminar flow hood using sterile and trace metal clean techniques.

**Culture conditions**—Cultures of *T. oceanica* were grown in polycarbonate bottles at 18°C under fluorescent light (Vita Lite Plus, Durotest) at a light intensity of 75  $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$  and a 14:10 light:dark (LD) cycle. Cultures of *T. erythraeum* were grown at 27°C at a light intensity of 150  $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$  of fluorescent light (MLR350T) and a 12:12 LD cycle. Cultures were gently mixed each day by hand (*T. oceanica*) or using orbital shakers (*T. erythraeum*) to minimize cell sedimentation. Cells were grown semicontinuously in Fe-ethylenediaminetetraacetic acid (EDTA) buffered batch cultures (Sunda and Huntsman 1995; Sunda et al. 2005). Prior to cellular measurements, they were cultured at the different iron concentrations until their growth rate remained constant for at least 10 generations. Cultures of *T. oceanica* were sampled every 1–2 d over a 2–4 d period, and the volume sampled was replaced by fresh medium to refill the bottles and leave no headspace. For *T. erythraeum*, four replicate cultures were grown at each iron concentration and were sampled on the same day. Samples were collected at the same time of the day to avoid diel cycle variations among treatments (Bucciarelli et al. 2007). Depending on specific growth rate and initial cell concentration, the cultures were diluted with fresh medium every 3–25 d for *T. oceanica* and every 7–10 d for *T. erythraeum* to maintain low total cell biovolumes. The low biovolumes prevented significant depletion of CO<sub>2</sub> (and associated rise in pH) and depletion of nutrients such as N, P, or Si, which might otherwise cause a decrease in growth rate. Culture pH varied between 8.01 and 8.20, which indicated little or no CO<sub>2</sub> depletion. Cultures were not axenic as evidenced by bactopectone tests.

**Culture media**—The complete media consisted of artificial Aquil seawater for *T. oceanica* (Price et al. 1988–1989) and modified Yi-Bu Cheng II (YBC II) medium for *T. erythraeum* (after Chen et al. 1996). These media were enriched with macronutrients, vitamins, and trace metals and were buffered by EDTA (Table 1). *T. erythraeum* was cultured without added fixed nitrogen and thus was grown diazotrophically on dinitrogen (N<sub>2</sub>). An EDTA concentration of 100  $\mu\text{mol L}^{-1}$  was used for the *T. oceanica* cultures (Sunda and Huntsman 1995) while a lower concentration (2  $\mu\text{mol L}^{-1}$ ) was used in the *T. erythraeum* cultures because of their known sensitivity to higher EDTA levels (Kustka et al. 2003). By forming stable iron chelates, the added EDTA reduces concentrations of biologically available dissolved inorganic iron hydrolysis species (Fe<sup>3+</sup>; Fe(OH)<sub>2</sub><sup>+</sup>, Fe(OH)<sub>3</sub>, and Fe(OH)<sub>4</sub><sup>2-</sup>) to 1:26th–1:1000th of the total Fe concentrations, depending on the EDTA concentration, temperature, culture pH, and light conditions (Sunda and Huntsman 2003). It thus acts as an Fe<sup>3+</sup> buffer and ensures that the Fe<sup>3+</sup> concentration ([Fe<sup>3+</sup>]) remains constant with time during the growth of the cells in the batch cultures (Sunda et al. 2005). Added iron

Table 1. Chemical composition of the culture media used for *Thalassiosira oceanica* (Aquil; Price et al. 1988–1989) and for *Trichodesmium erythraeum* (modified YBC II; after Chen et al. 1996).

Chemical	Aquil	Modified YBC II
Nitrate ( $\mu\text{mol L}^{-1}$ )	300	—
Phosphate ( $\mu\text{mol L}^{-1}$ )	10	50
Silicate ( $\mu\text{mol L}^{-1}$ )	100	—
B12 (nmol L <sup>-1</sup> )	0.35	0.37
Biotin (nmol L <sup>-1</sup> )	2.05	2.05
Thiamin (nmol L <sup>-1</sup> )	332	296
Se (nmol L <sup>-1</sup> )	10	—
Mo (nmol L <sup>-1</sup> )	100	11
Cu (nmol L <sup>-1</sup> )	20	1
Co (nmol L <sup>-1</sup> )	50	2.5
Zn (nmol L <sup>-1</sup> )	80	4
Mn (nmol L <sup>-1</sup> )	121	20
Fe (nmol L <sup>-1</sup> )	0, 10, 30, and 500	8 and 400
EDTA ( $\mu\text{mol L}^{-1}$ )	100	2

concentrations to the Aquil medium for *T. oceanica* were 0 (no addition), 10, 30, and 500 nmol L<sup>-1</sup>. The media for *T. erythraeum* contained either 8 or 400 nmol L<sup>-1</sup> Fe. The mean culture [Fe<sup>3+</sup>] were computed from the complexation data in Sunda and Huntsman (2003). In the *T. oceanica* cultures containing 10, 30, and 500 nmol L<sup>-1</sup> Fe, the computed [Fe<sup>3+</sup>] were 10, 30, and 500 pmol L<sup>-1</sup>, respectively. In the *T. erythraeum* cultures containing 8 and 400 nmol L<sup>-1</sup> Fe, the computed [Fe<sup>3+</sup>] were 303 and 15,000 pmol L<sup>-1</sup>. The latter value is invalid, as it exceeds the solubility limit for iron with respect to ferric hydroxide precipitation ( $\sim 700 \text{ pmol L}^{-1}$ ; Sunda and Huntsman 1995). The media were sterilized by microwave treatment (Keller et al. 1988).

**Cell concentration, volume per cell, and specific growth rate**—Cell numbers per liter of culture (N) and mean volume per cell ( $V_{\text{cell}}$ ;  $\mu\text{m}^3$ ) of *T. oceanica* were measured with a Coulter Multisizer II multichannel electronic particle counter with a 100  $\mu\text{m}$  aperture size. Total cell volume per liter of culture (CV;  $\mu\text{L}_{\text{cell}} \text{ L}^{-1}$ ) was calculated from N and  $V_{\text{cell}}$ . For *T. erythraeum*, 300–500  $\mu\text{L}$  of culture were filtered onto a 25 mm diameter, 0.2  $\mu\text{m}$  pore polycarbonate filter. The number of trichomes ( $n_{\text{T}}$ ) was counted using an epifluorescence microscope (Nikon 50i) equipped with Nikon imaging software. On the day of sampling, the width and length of 200 trichomes from each culture bottle (i.e., 800 trichomes for each Fe treatment) were also measured using an analogic camera. The mean volume of the trichomes was calculated using the geometric formula for a cylinder. Specific growth rate ( $\mu$ ) during the exponential phase of growth was determined by linear regression of  $\ln(\text{CV})$  and  $\ln(n_{\text{T}})$  vs. time for *T. oceanica* and *T. erythraeum*, respectively, after correcting for culture dilution by added fresh medium.

**Cellular C, N, and Chl a**—Cells from culture samples were filtered onto glass fiber filters (GF/F) and rinsed with artificial seawater that had been previously sterilized by microwave treatment. All the glassware used for cell C and



N analysis (filter holders, filtration funnels, and vials) was washed with 10% HCl, rinsed with Milli-Q water, and dried. The cleaned glassware and the GF/F filters were precombusted at 450°C for 4.5 h before use. After filtration, samples were immediately frozen at -60°C (for Chl *a* determination) or dried at 60°C (for C and N determination) until analysis. Chl *a* was measured by fluorometry after extraction into 90% acetone (Lorenzen 1966). Samples for C and N measurement were analyzed with a Carlo-Erba NA-1500 elemental analyzer. Samples for Chl *a* and for C and N were collected in duplicate each day for *T. oceanica* over 2–4 d and once in each of the four replicate cultures for *T. erythraeum* on the day of sampling.

**DMSP and DMSO**—To measure intracellular DMSP (DMSP<sub>p</sub>), low-volume samples (0.5–5 mL for *T. oceanica* and 10–20 mL for *T. erythraeum*) were filtered by gravity onto GF/F filters. The filters were placed in 25 mL glass septum vials containing 5 mL of 5 mol L<sup>-1</sup> NaOH. Aliquots (< 4.5 mL) of the filtrates and the unfiltered culture were sampled to obtain measurements of dissolved DMSP (DMSP<sub>d</sub>) and total DMSP (DMSP<sub>t</sub>), respectively. NaOH (5 mol L<sup>-1</sup>) was added to adjust the total volume of liquid in the vial to 5 mL. The vials were immediately sealed and the samples were incubated for 24 h in the dark at room temperature. The DMS released from base hydrolysis (and any that was originally present) was measured with a Varian 3800 gas chromatograph equipped with a Poraplot Q capillary column and a pulsed flame photometric detector. A cryotrap was used to preconcentrate the DMS samples. DMS was calibrated using DMSP standards (Centre for Analysis, Spectroscopy and Synthesis [CASS], University of Groningen).

For *T. oceanica*, we observed no difference between DMSP<sub>p</sub> measured directly in filtered cells and DMSP<sub>p</sub> calculated as the difference between DMSP<sub>t</sub> and DMSP<sub>d</sub>. But for *T. erythraeum* and 20 mL filtration volumes, directly measured DMSP<sub>p</sub> (i.e., that collected on the GF/F filters) was higher than that calculated from the total culture DMSP (DMSP<sub>t</sub>) minus that measured in the culture filtrate (DMSP<sub>d</sub>) ([measured DMSP<sub>p</sub>] = 1.77 ± 0.15 × [calculated DMSP<sub>p</sub>], *n* = 16, *r*<sup>2</sup> = 0.68). In some cases, measured DMSP<sub>p</sub> was even higher than DMSP<sub>t</sub>, which points to a possible analytical artifact when filtering relatively large (20 mL) culture volumes. By contrast, measured and calculated DMSP<sub>p</sub> agreed well for 10 mL filtration volumes ([measured DMSP<sub>p</sub>] = 1.09 ± 0.05 × [calculated DMSP<sub>p</sub>], *n* = 10, *r*<sup>2</sup> = 0.96). Consequently, for this species, DMSP<sub>p</sub> was always calculated from the difference between DMSP<sub>t</sub> and DMSP<sub>d</sub>, whatever the volume filtered, which may provide a lower estimate of DMSP<sub>p</sub> in some cases.

Samples of *T. oceanica* were also measured for intracellular DMSO (DMSO<sub>p</sub>). These samples were filtered by gravity onto precombusted GF/F filters using precombusted filter holders and filtration funnels (1 h at 450°C). The filters were placed in precombusted 25 mL glass septum vials containing 1 mL of culture medium. One milliliter of culture was also sampled for total DMSO

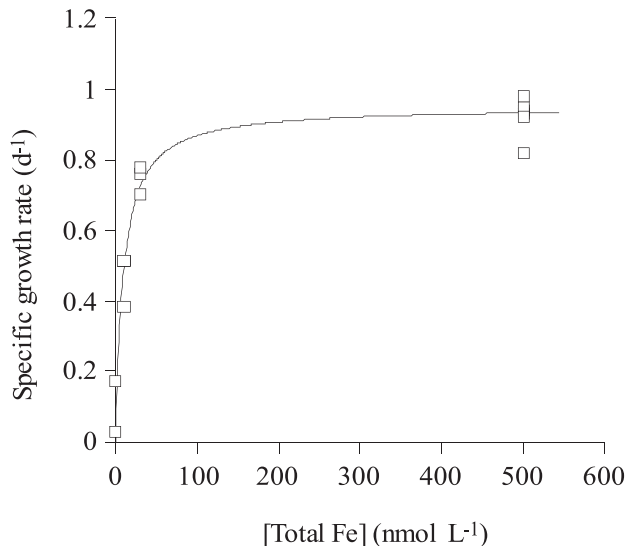
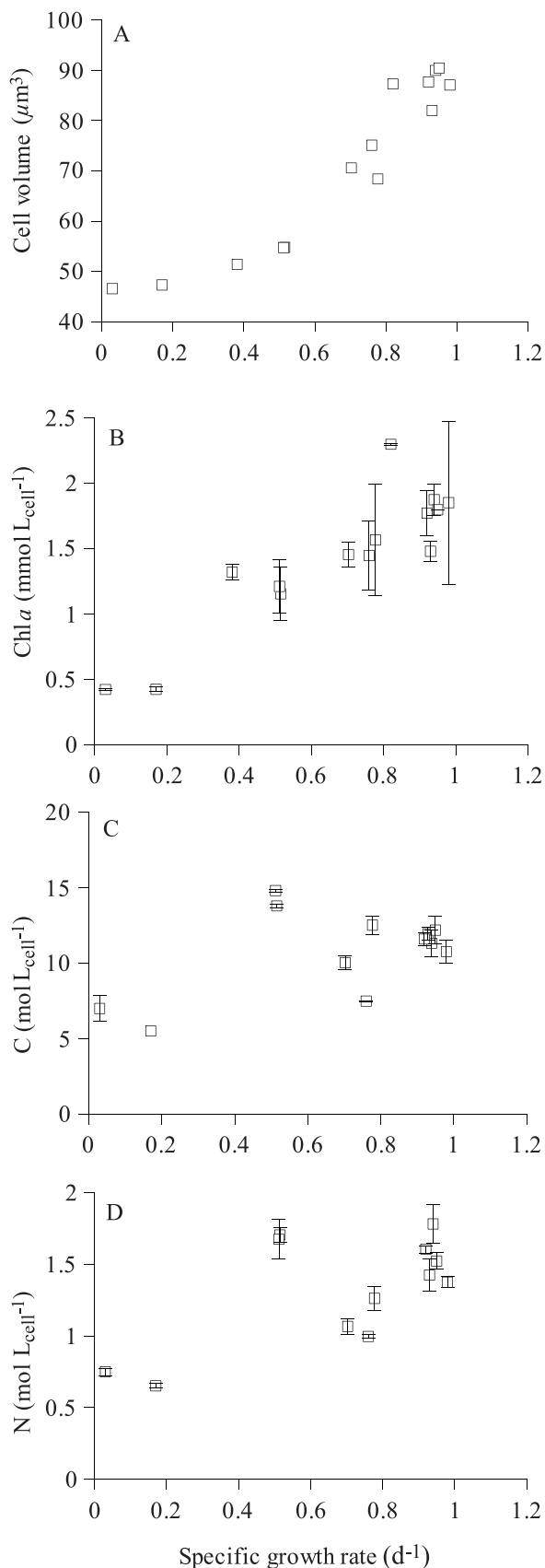


Fig. 1. Specific growth rate of *Thalassiosira oceanica* vs. total iron concentration in the medium. The Monod saturation function is represented by the solid curve.

(DMSO<sub>t</sub>). Thirty milliliters of 5 mol L<sup>-1</sup> NaOH were added to these samples and incubated for 6 h in the dark at room temperature to transform DMSP into DMS. The samples were then sparged with He to remove DMS. They were subsequently amended with 200 μL of 20% TiCl<sub>3</sub> and heated at 50°C for 1 h to reduce DMSO to DMS (Kiene and Gerard 1994). Blanks for DMSO<sub>p</sub> and DMSO<sub>t</sub>, respectively, were measured by analyzing a precombusted GF/F filter in 1 mL of Aquil medium and by analyzing 1 mL of Aquil medium, following the same protocol as used for the cultures. Blank values ranged from 0 to 7 pmol for DMSO<sub>p</sub> and from 0 to 6 pmol for DMSO<sub>t</sub>. They represented less than 6% of the sample values, except for one DMSO<sub>t</sub> value in a severely Fe-limited culture containing no added Fe where the blank value reached 5.4 pmol, 27% of the sample value. All culture values presented below were corrected for blanks.

The different fractions of DMSP and DMSO were measured in duplicate or triplicate. DMSP<sub>p</sub> and DMSO<sub>p</sub> were assumed to be present within algal cells and were expressed on an algal biovolume basis (i.e., mmol L<sub>cell</sub><sup>-1</sup> for *T. oceanica* and mmol L<sub>trichome</sub><sup>-1</sup> for *T. erythraeum*). However, we cannot rule out the possibility that at least some of the DMSP<sub>p</sub> and DMSO<sub>p</sub> was present in co-occurring bacteria. To allow comparisons with intracellular DMSP and DMSO, total and dissolved DMSP and DMSO were also normalized to algal biovolume.

DMS was measured in *T. oceanica* by sparging 5 mL of cultures immediately after collection of the sample. Measured DMS values varied between 0.32 ± 0.06 mmol L<sub>cell</sub><sup>-1</sup> (Fe-replete conditions, *n* = 2, 95% confidence interval) and 1.13 ± 0.64 mmol L<sub>cell</sub><sup>-1</sup> (0 nmol L<sup>-1</sup> added Fe, *n* = 4, 95% confidence interval). Because the cultures were not axenic, these values are not discussed further. Statistical analyses were performed using Statgraphics Plus®.



## Results

*Effect of Fe limitation on algal growth, cell size, and cell composition*—Decreasing concentrations of iron decreased the growth rate of *T. oceanica* (Fig. 1). This decrease was accompanied by a decrease in volume per cell and cellular Chl *a*, cell C, and cell N normalized to cell volume (Fig. 2). The relationship of iron in the medium fits a Monod saturation function ( $n = 14$ ,  $R^2 = 0.95$ ) with a maximum specific growth rate ( $\mu_{\max}$ ) of  $0.95 \pm 0.03 \text{ d}^{-1}$  and a half-saturation constant for growth with respect to total iron ( $K_{\mu\text{Fe}}$ ) of  $9.4 \pm 1.7 \text{ nmol L}^{-1}$  (Fig. 1). Over the range of Fe limitation, specific growth rate, cell volume, and Chl *a* decreased by 33-, 1.8-, and 4.4-fold, respectively (Figs. 1, 2A,B).

The *T. oceanica* cultures were classified as severely Fe-limited for growth rates of 3–18% of  $\mu_{\max}$ , moderately limited for growth rates of  $\sim 55\%$  of  $\mu_{\max}$ , or mildly Fe-limited for growth rates of 76–85% of  $\mu_{\max}$  (Table 2). We observed an approximately 2-fold decrease in cellular C and N concentrations in severely Fe-limited cultures (Fig. 2C,D). Although there was some scatter in the data at mild to intermediate iron stress, the decreases were significant (analysis of variance, carbon:  $p < 0.00001$ ,  $F_{20,21} = 45.30$ , nitrogen:  $p < 0.00001$ ,  $F_{21,21} = 50.66$ ). Cellular C:N increased significantly with increasing Fe stress (i.e., decreasing  $\mu$ ; analysis of variance,  $p = 0.002$ ,  $F_{20,21} = 7.23$ ; Table 2).

Decreasing iron concentration decreased the specific growth rate of *T. erythraeum* from 0.46 to 0.13  $\text{d}^{-1}$  (28% of  $\mu_{\max}$ , indicating severe iron limitation) and decreased the mean volume of trichomes by 3-fold, from  $14,855 \pm 317 \text{ } \mu\text{m}^3$  ( $n = 800$ , 95% confidence interval) to  $5020 \pm 241 \text{ } \mu\text{m}^3$  ( $n = 800$ , 95% confidence interval). The C:N ratios of iron-limited cells increased by  $\sim 1.6$ -fold (from  $5.1 \pm 0.1 \text{ mol mol}^{-1}$ ,  $n = 4$ , 95% confidence interval, to  $8.1 \pm 1.1 \text{ mol mol}^{-1}$ ,  $n = 4$ , 95% confidence interval; Table 3). Cellular Chl *a*:C decreased by 7-fold under severe Fe limitation (from  $0.20 \pm 0.03 \text{ mmol mol}^{-1}$ ,  $n = 3$ , 95% confidence interval, to  $0.03 \pm 0.01 \text{ mmol mol}^{-1}$ ,  $n = 4$ , 95% confidence interval; Table 3), consistent with iron's direct negative effect on photosynthesis and N<sub>2</sub> fixation (Kustka et al. 2003).

*Intracellular and dissolved DMSP and DMSO*—For *T. oceanica*, total and intracellular DMSP concentrations increased with increasing iron limitation (i.e., decreasing growth rate; analysis of variance,  $\text{DMSP}_t$ :  $p < 0.00001$ ,  $F_{33,34} = 68.60$ ,  $\text{DMSP}_p$ :  $p < 0.00001$ ,  $F_{33,34} = 372.67$ ; Fig. 3A). Total and intracellular DMSO also increased

←

Fig. 2. (A) Mean volume per cell, (B) cellular Chl *a* normalized to cell volume, (C) cellular carbon normalized to cell volume, and (D) cellular nitrogen normalized to cell volume vs. specific growth rate in *Thalassiosira oceanica* under varying degrees of iron limitation.

Table 2. Specific growth rate ( $\mu$ ) and cellular ratios of C:N, DMSP<sub>p</sub>:Chl *a*, DMSP<sub>p</sub>:C, DMSP<sub>p</sub>:N, and DMSO<sub>p</sub>:Chl *a* for *Thalassiosira oceanica* growing under different degrees of Fe limitation.

Degree of Fe limitation	Total Fe (nmol L <sup>-1</sup> )	$\mu$ (d <sup>-1</sup> )	C:N (mol mol <sup>-1</sup> )	DMSP <sub>p</sub> :Chl <i>a</i> (mol mol <sup>-1</sup> ) [mmol g <sup>-1</sup> ]	DMSP <sub>p</sub> :C (mmol mol <sup>-1</sup> )	DMSP <sub>p</sub> :N (mmol mol <sup>-1</sup> )	DMSO <sub>p</sub> :Chl <i>a</i> (mol mol <sup>-1</sup> ) [mmol g <sup>-1</sup> ]
Severe	0	0.03	9.34±1.48	79.9±2.3 [89.4±2.6]	4.8±0.6	45.1±2.0	9.1±2.3 [10.2±2.5]
	0	0.17	8.48±0.21	70.2±7.2 [78.6±8.1]	5.4±0.3	45.7±3.9	6.3±0.6 [7.0±0.7]
Moderate	10	0.51	8.84±0.78	13.2±3.7 [14.8±4.2]	1.1±0.1	9.6±1.9	2.1±0.4 [2.4±0.5]
	10	0.52	8.08±0.30	13.5±3.4 [15.1±3.8]	1.1±0.1	9.1±1.0	2.5±0.5 [2.8±0.5]
Mild	30	0.70	9.40±0.91	3.7±0.4 [4.2±0.4]	0.5±0.0	5.1±0.4	1.9±0.3 [2.1±0.3]
	30	0.76	7.51±0.14	3.7±0.9 [4.1±1.0]	0.7±0.1	5.3±0.4	1.0±0.3 [1.2±0.3]
	30	0.78	9.91±1.14	4.4±1.3 [4.9±1.4]	0.6±0.0	5.5±0.4	n.d.
Replete	500	0.82	n.d. <sup>a</sup>	1.3±0.1 [1.5±0.1]	n.d.	n.d.	0.36±0.04 [0.40±0.04]
	500	0.92	7.22±0.39	2.1±1.0 [2.4±1.1]	0.3±0.1	2.4±0.9	n.d.
	500	0.93	8.39±0.97	2.5±0.1 [2.8±0.2]	0.3±0.0	2.6±0.2	n.d.
	500	0.94	6.35±0.98	1.5±0.2 [1.7±0.2]	0.2±0.0	1.6±0.2	0.41±0.03 [0.46±0.03]
	500	0.98	7.82±0.76	2.0±0.9 [2.3±1.0]	0.4±0.1	2.8±0.4	n.d.

<sup>a</sup> nd, not determined.

Table 3. Specific growth rate ( $\mu$ ), cellular Chl *a*:C, cellular C:N, (DMSP<sub>p</sub>, DMSP<sub>p</sub>:Chl *a*, total and intracellular DMSP normalized to cell carbon (DMSP<sub>t</sub>:C and DMSP<sub>p</sub>:C, respectively), and DMSP<sub>p</sub>:N in iron-replete and iron-limited cultures of *Trichodesmium erythraeum*.

Degree of Fe limitation	Total Fe (nmol L <sup>-1</sup> )	$\mu$ (d <sup>-1</sup> )	Chl <i>a</i> :C (mmol mol <sup>-1</sup> )	C:N (mol mol <sup>-1</sup> )	DMSP <sub>p</sub> (mmol L <sub>trichome</sub> <sup>-1</sup> )	DMSP <sub>p</sub> :Chl <i>a</i> (mol mol <sup>-1</sup> ) [mmol g <sup>-1</sup> ]	DMSP <sub>t</sub> :C (mmol mol <sup>-1</sup> )	DMSP <sub>p</sub> :C (mmol mol <sup>-1</sup> )	DMSP <sub>p</sub> :N (mmol mol <sup>-1</sup> )
Severe	8	0.13±0.06	0.03±0.01	8.1±1.1	2.27±0.82	2.32±1.11 [2.60±1.24]	0.088±0.011	0.054±0.010	0.430±0.075
Replete	400	0.46±0.08	0.20±0.03	5.1±0.1	0.05±0.02	0.016±0.005 [0.017±0.005]	0.006±0.001	0.003±0.001	0.016±0.005

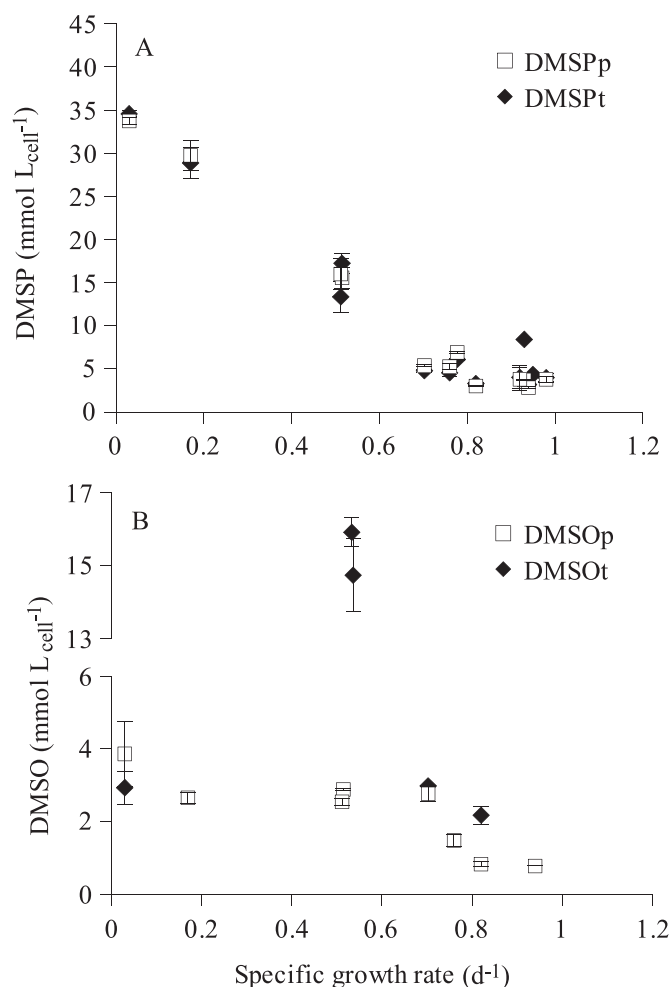


Fig. 3. (A) Intracellular DMSP concentration (DMSP<sub>p</sub>) and total DMSP (DMSP<sub>t</sub>) normalized to cell volume and (B) intracellular DMSO concentration [DMSO<sub>p</sub>] and total DMSO (DMSO<sub>t</sub>) normalized to cell volume vs. specific growth rate for *Thalassiosira oceanica* growing under varying degrees of iron limitation. (B) Scale on the y-axis has been modified to allow a better definition at the lowest DMSO values.

with increasing Fe limitation of growth rate in *T. oceanica* (analysis of variance, DMSO<sub>t</sub>:  $p < 0.00001$ ,  $F_{10,34} = 247.02$ , DMSO<sub>p</sub>:  $p = 0.0001$ ,  $F_{18,34} = 15.61$ ; Fig. 3B) but to a lesser extent than the increase in DMSP<sub>p</sub>. DMSP<sub>p</sub> and DMSO<sub>p</sub> per unit of cell volume increased by  $\sim 12$ -fold and  $\sim 5$ -fold, respectively, in the severely Fe-limited *T. oceanica* cells compared to iron-sufficient values (Fig. 3A,B). Because of the larger increase in DMSP<sub>p</sub>, DMSP<sub>p</sub>:DMSO<sub>p</sub> ratios in *T. oceanica* increased with iron limitation, from  $4.2 \pm 1.2 \text{ mol mol}^{-1}$  in the Fe-sufficient cells to  $9.8 \pm 3.4 \text{ mol mol}^{-1}$  at severely Fe-limited growth rates (analysis of variance,  $p < 0.00001$ ,  $F_{18,34} = 24.21$ ; Fig. 4). In *T. oceanica*, there was no measurable DMSP in the dissolved phase over the range of Fe limitation (DMSP<sub>d</sub> =  $104 \pm 11\%$  DMSP<sub>t</sub>,  $n = 13$ ), except for one replete culture (Fig. 3A). For that culture, the contribution of DMS to the DMSP<sub>t</sub> pool was negligible ( $0.32 \pm 0.06 \text{ mmol L}_{\text{cell}}^{-1}$  of DMS [ $n = 4$ , 95% confidence interval] vs.  $8.4 \pm$

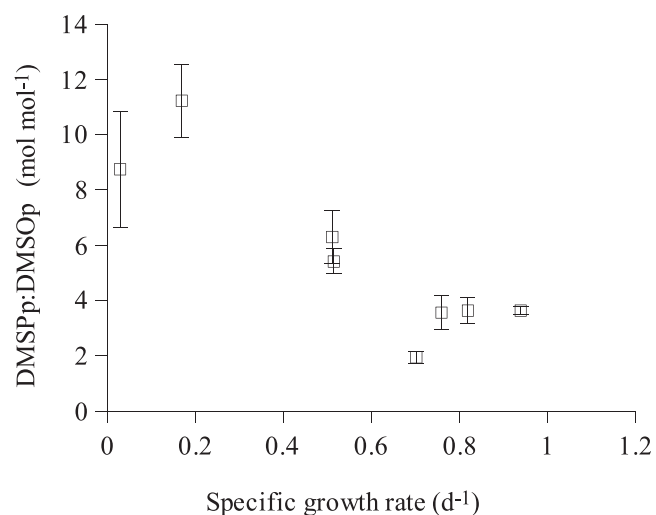


Fig. 4. Intracellular DMSP:DMSO ratios (DMSP<sub>p</sub>:DMSO<sub>p</sub>) for *Thalassiosira oceanica* growing at varying iron-limited specific growth rates.

$1.1 \text{ mmol L}_{\text{cell}}^{-1}$  of DMSP<sub>t</sub> [ $n = 3$ , 95% confidence interval]; i.e., less than 4%), indicating that DMSP<sub>d</sub> accounted for  $\sim 50\%$  of DMSP<sub>t</sub>. When taking into account the few measured values for DMSO<sub>t</sub>, dissolved DMSO concentrations were measurable only in some Fe-replete and moderately Fe-limited cells ( $1.3 \pm 0.2 \text{ mmol L}_{\text{cell}}^{-1}$  at  $\mu = 0.82 \text{ d}^{-1}$  [ $n = 2$ , 95% confidence interval] and  $12.5 \pm 1.2 \text{ mmol L}_{\text{cell}}^{-1}$  at  $\mu = 0.51\text{--}0.52 \text{ d}^{-1}$  [ $n = 4$ , 95% confidence interval]). DMSO in the dissolved phase can be generated by photooxidation of DMS, bacterial transformation of DMS, or diffusion of intracellular DMSO through phytoplanktonic cell membranes (Simo 2004). It is also possible that these anomalously high values were due to DMSO contamination.

For *T. erythraeum*, Fe limitation increased intracellular DMSP concentrations by 45-fold, but the concentrations were much lower than observed in *T. oceanica* (Table 3). In all *T. erythraeum* cultures, DMSP<sub>d</sub> represented a significant fraction of DMSP<sub>t</sub>, both under Fe-replete ( $48 \pm 8\%$ ,  $n = 4$ , 95% confidence interval) and Fe-limited conditions ( $40 \pm 5\%$ ,  $n = 4$ , 95% confidence interval), suggesting active exudation or leakage from cells during filtration. We cannot exclude the latter (i.e., that it is an analytical artifact), although the concentrations of DMSP<sub>d</sub> relative to the total pool were consistent among the four replicates and did not covary with the trichome concentration (which varied from one replicate to the other by up to 26%). A small-volume ( $< 3.5 \text{ mL}$ ) gravity drip filtration procedure is recommended to avoid artifactual release of DMSP from algal cells (Kiene and Slezak 2006). Such release of DMSP<sub>d</sub> during filtration was not an issue in the *T. oceanica* cultures because less than 5 mL was filtered and no DMSP<sub>d</sub> was measured except in one Fe-replete culture. However, cellular DMSP release during filtration could have artificially increased DMSP<sub>d</sub> concentrations in cultures of *T. erythraeum*. DMS, which was not measured during our *T. erythraeum* experiment, could also have contributed to the DMSP<sub>d</sub> pool in that species. But this is unlikely to be



significant, as Yassaa et al. (2006) reported less than 0.1  $\mu\text{mol}$  of DMS per g of Chl *a* in the same strain of *Trichodesmium*, which is much lower than our measured values of  $\text{DMSP}_d$  of 16–1700  $\mu\text{mol DMSP}_d$  per g of Chl *a*.

*DMSP: Chl a*, *DMSP: C*, and *DMSP: N*—Cellular DMSP normalized to cell C, N, and Chl *a* increased by 16-, 20-, and 40-fold, respectively, in the most severely Fe-limited cells of *T. oceanica* compared to the Fe-sufficient cultures (Table 2). Cellular carbon allocated to DMSP in this species increased from  $0.15 \pm 0.04\%$  in the Fe-replete cells to  $2.4 \pm 0.6\%$  under severe Fe limitation. In Fe-limited *T. erythraeum*, the same trends were apparent, with  $\text{DMSP}_p:\text{C}$ ,  $\text{DMSP}_p:\text{N}$ , and  $\text{DMSP}_p:\text{Chl } a$  increasing by 18-, 27-, and 145-fold, respectively (Table 3). Cellular carbon allocated to DMSP increased from  $0.002 \pm 0.001\%$  in the iron-replete cells to  $0.026 \pm 0.005\%$  in the severely iron-limited cultures.

## Discussion

To our knowledge, the effect of Fe limitation on intracellular DMSP concentrations of cultured marine phytoplankton has been studied previously only twice: in the Antarctic prymnesiophyte *Phaeocystis* sp. (Stefels and van Leeuwe 1998) and in the temperate coastal diatoms *Thalassiosira pseudonana* and *Skeletonema costatum* (Sunda et al. 2002). *Phaeocystis* sp. is considered to be a high-DMSP producer like other haptophytes (Keller et al. 1989). Under high light,  $\text{DMSP}_p$  increased by  $\sim 2$ -fold in Fe-limited *Phaeocystis* sp. compared to Fe-replete cells, while there was no difference between Fe-limited and Fe-replete cells under low-light conditions (Stefels and van Leeuwe 1998). However, as indicated by Stefels and van Leeuwe (1998), their Fe-replete cells under high and low light were likely to have been nitrogen limited. This may have increased intracellular DMSP concentrations of their Fe-replete, N-limited cultures and consequently decreased the observed effect of iron limitation on intracellular DMSP. Severely Fe-limited cells of *T. pseudonana* ( $\mu \sim 3\% \mu_{\text{max}}$ ) increased intracellular DMSP by  $\sim 20$ -fold (Sunda et al. 2002), similar to what we observed for *T. oceanica* ( $\sim 10$ -fold increase at  $3\% \mu_{\text{max}}$ ). For *S. costatum*, a 3-fold decrease in specific growth rate under iron limitation caused the cellular  $\text{DMSP}:\text{C}$  ratio to increase by 3-fold, from 1.6 to 4.7  $\text{mmol mol}^{-1}$ , or from 0.8% to 2.5% of the cellular carbon (Sunda et al. 2002). The latter value equals that observed here in *T. oceanica* under severe iron limitation.

No DMSO measurements have been previously reported in Fe-limited cultures of diatoms or phytoplankton in general. Hatton and Wilson (2007) measured intracellular DMSO concentrations of  $0.37 \pm 0.29 \text{ mmol L}_{\text{cell}}^{-1}$  ( $n = 6$ , 95% confidence interval) in six strains of diatoms grown under nutrient-replete conditions. Spiese et al. (2009) measured an intracellular DMSO concentration of  $0.86 \pm 0.23 \text{ mmol L}_{\text{cell}}^{-1}$  ( $n = 3$ , 95% confidence interval) in nutrient-replete *T. oceanica* cells (culture conditions: 14:10 LD cycle, 120  $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$ ,  $T = 22^\circ\text{C}$ ). Our DMSO values for Fe-replete *T. oceanica* cultures ( $0.81 \pm$

$0.07 \text{ mmol L}_{\text{cell}}^{-1}$ ,  $n = 3$ , 95% confidence interval) compare well with their values. Our  $\text{DMSP}_p:\text{DMSO}_p$  ratio in Fe-replete *T. oceanica* ( $4.2 \pm 1.2 \text{ mol mol}^{-1}$ ) is also consistent with the ratios determined in nutrient-replete *T. oceanica* by Spiese et al. (2009) ( $3.3 \pm 1.5 \text{ mol mol}^{-1}$ ) and in six other species of diatoms by Hatton and Wilson (2007) ( $4.3 \pm 4.1 \text{ mol mol}^{-1}$ ).

Under Fe-replete conditions, *T. erythraeum* had a low intracellular DMSP concentration and low  $\text{DMSP}_p:\text{C}$  and  $\text{DMSP}_t:\text{C}$  molar ratios (Table 3). Similarly, Keller et al. (1989) reported no detectable DMS +  $\text{DMSP}_t$  in nutrient-sufficient cultures of cyanobacteria, including five strains of *Synechococcus* and one of *Trichodesmium* sp. However, based on seawater mesocosm experiments, Wilson et al. (1998) suggested that *Synechococcus* spp. may be significant producers of DMSP. DMSP has never been measured in Fe-limited diazotrophic *Trichodesmium* or other cyanobacteria. Our study is thus the first to report an increase in  $\text{DMSP}_p$  and  $\text{DMSP}_t$  relative to biovolume, Chl *a*, and cell C and N under iron limitation in any species of cyanobacteria.

*The possible roles of DMSP and DMSO under Fe limitation*—Different hypotheses can be invoked to explain an increase in intracellular DMSP under Fe limitation. DMSP was initially proposed to play a role in osmoregulation (Vairavamurthy et al. 1985). Its increase under nitrate limitation can thus be explained by the replacement of N-containing osmolytes (e.g., glycine betaine and proline) by DMSP, an S-containing osmolyte (Keller et al. 1999). Since iron occurs in the active sites of enzymes needed to reduce nitrate to ammonium (nitrate and nitrite reductases) and to reduce dinitrogen to ammonium (nitrogenase) in  $\text{N}_2$  fixers like *Trichodesmium*, Fe limitation can induce nitrogen limitation, such that the cells become colimited by iron and nitrogen (Raven 1988; Kustka et al. 2003). Because N limitation can be induced by Fe limitation, Stefels and van Leeuwe (1998) hypothesized that increases in cellular DMSP under Fe limitation could be caused by a greater need for nitrogen-free osmolytes.

We observed a 20% higher mean C:N ratio with severe Fe limitation in *T. oceanica*. An even larger 60% increase in C:N was observed with severe Fe limitation of *T. erythraeum* (Table 3), which was cultured diazotrophically in the absence of added fixed nitrogen (Table 1). This higher effect may reflect the much larger 5-fold increase in cellular iron required for diazotrophic growth on  $\text{N}_2$  relative to that on ammonium (Kustka et al. 2003) compared to only a 50% increase in cellular iron needed for equivalent growth on nitrate vs. ammonium (Raven 1988). However, an intracellular DMSP concentration of  $\sim 2 \text{ mmol L}_{\text{trichome}}^{-1}$  under Fe limitation in *T. erythraeum* (Table 3) would not be high enough to make DMSP an effective osmolyte, given the osmolality of seawater ( $\sim 1 \text{ mol L}^{-1}$ ). Under severe Fe stress,  $\text{DMSP}_p$  and  $\text{DMSP}_t$ , respectively, represented only  $\sim 0.03\%$  and  $0.04\%$  of cellular C in this species. In these low-DMSP-producing species, the main role of DMSP does not seem to be to serve as an osmolyte, as may be the case for high-DMSP-producing phytoplankton like *Phaeocystis* sp.

Sunda et al. (2002) proposed that the increase in DMSP under Fe limitation is due to the antioxidant properties of this compound and its breakdown products (DMS, DMSO, methane sulfinic acid [MSNA], and acrylate). DMSP and DMSO have the ability to react efficiently with reactive oxygen species (ROS) (Sunda et al. 2002). ROS, like superoxide radicals, hydrogen peroxide, and hydroxyl radicals, result from the reaction of electrons with O<sub>2</sub>. When the flow of electrons within the photosynthetic electron transport chain is disrupted, ROS are produced that can damage lipids, proteins, and deoxyribonucleic acid (Lesser 2006). Because Fe is a major component of the photosynthetic apparatus, Fe limitation decreases the efficiency of photosynthetic electron transport and thereby increases ROS production and oxidative stress. This effect has been shown for the marine diatoms *T. pseudonana* and *T. weissflogii* (Sunda et al. 2002; Peers and Price 2004) and the cyanobacterium *Trichodesmium* spp. (Berman-Frank et al. 2004). The effect is all the more problematic for *Trichodesmium*, as ROS deactivate the nitrogenase enzyme and thereby impair N<sub>2</sub> fixation (Nuester et al. 2012). Important antioxidant enzymes, such as ascorbate peroxidase and superoxide dismutase (SOD), contain Fe, and thus their synthesis may be restricted under iron limitation, providing another mechanism for increased oxidative stress. Fe-limited *T. pseudonana* and *T. weissflogii* can replace Fe-containing SOD by Mn-containing SOD (Peers and Price 2004), and similar replacements of Fe-SOD, by Mn-, Ni-, and Cu-Zn-SODs can occur in *Trichodesmium* (Nuester et al. 2012). However, the replacement of Fe by other metals may only partially maintain enzymatic activity, and under Fe limitation the activity of SOD decreases in *T. weissflogii* (Peers and Price 2004).

DMSP and its breakdown products (DMS, DMSO, MSNA, and acrylate) can react with hydroxyl radicals within the cell with the same or greater efficiency as more conventional antioxidants, such as ascorbate and glutathione (Sunda et al. 2002). Under Fe limitation, they may react with increasing levels of ROS (e.g., hydroxyl radicals) and help compensate for the decrease in Fe-containing antioxidant enzymes. This hypothesis may help explain the increase in intracellular DMSP in both Fe-limited species and in intracellular DMSO in Fe-limited *T. oceanica*, as one likely mechanism for DMSO production is the oxidation of DMSP and DMS by OH radicals (Sunda et al. 2002). The DMSO produced then itself reacts with OH radicals at a 22 times higher specific rate than that for DMSP (Sunda et al. 2002). If DMSO is produced solely from OH radical oxidation of DMSP and is consumed at the same rate by reaction with OH radicals, then the DMSP:DMSO ratio in the cells at steady state should be 22, the ratio of the OH radical reaction rate constants for DMSO and DMSP. However, in our cultures the DMSP<sub>p</sub>:DMSO<sub>p</sub> ratios are 3 to 11, which has two important implications. One is that DMSO should be a more important antioxidant scavenger of OH radicals than DMSP by factors of 2- to 7-fold. The second is that there are likely other sources for DMSO than OH radical oxidation of DMSP, such as the oxidation of DMS by OH radicals or singlet oxygen (Sunda et al. 2002). The OH

reaction with DMS is highly favored, as OH reacts with DMS at 62 times higher specific rate than it does with DMSP and at an ~ 3 times greater specific rate than its reaction with DMSO (Sunda et al. 2002).

There is no evidence in *T. oceanica* for enzymatic lysis of DMSP to DMS (e.g., by a DMSP lyase), which makes DMS an unlikely net source of DMSO. To the contrary, DMSO is enzymatically reduced to DMS in *T. oceanica* and other algae, which can create a catalytic cycle between DMSO and DMS that efficiently removes OH radicals (Spiese et al. 2009). Since the more lipophilic DMS should diffuse more freely across cell membranes than the more polar DMSO, the cycling between DMSO and DMS should cause a net loss of DMSO from the cell (via its conversion to DMS), which could increase DMSP<sub>p</sub>:DMSO<sub>p</sub> ratios. Such an effect could be one reason for the higher DMSP<sub>p</sub>:DMSO<sub>p</sub> ratios in the Fe-limited *T. oceanica* cells relative to the Fe-sufficient cells (Fig. 4), as antioxidant systems and their enzymes are often up-regulated under increased oxidative stress, such as occurs under iron limitation (Sunda et al. 2002).

Somewhat different results were observed in batch cultures of the dinoflagellate *Amphidinium carterae*. Here intracellular concentrations of DMSO were higher during the nutrient- or CO<sub>2</sub>-limited stationary phase than during the exponential growth phase, again suggesting that this compound may be produced to fight oxidative stress induced by nutrient-limiting conditions (Simo et al. 1998; Hatton and Wilson 2007). However, in contrast to the current results with *T. oceanica*, where DMSP<sub>p</sub>:DMSO<sub>p</sub> ratios were up to 3-fold higher under iron limitation, the DMSP<sub>p</sub>:DMSO<sub>p</sub> ratios decreased substantially in the growth-limited *A. carterae* cells, from ~ 25 to 32 in the exponential phase of culture growth to 3 to 8 in the nutrient- or CO<sub>2</sub>-limited stationary phase (Simo et al. 1998; Hatton and Wilson 2007). The differences in the changes in DMSP<sub>p</sub>:DMSO<sub>p</sub> ratios under nutrient limitation of growth rate in the two species may be linked to differences in the activities of DMSP cleavage enzymes and DMSO reductase enzymes. *A. carterae* had an order-of-magnitude-lower DMSO reductase activity than *T. oceanica* (Spiese et al. 2009), which should greatly lower DMSO loss rates via its conversion to DMS and subsequent flux out of the cells. Moreover, unlike *T. oceanica*, *A. carterae* contains enzymes that cleave DMSP into DMS (Caruana et al. 2012), providing an additional potentially large source of DMSO under oxidative stress conditions via DMS oxidation by OH radicals (or other ROS). Increases in DMSP lysis to DMS has been observed under nitrogen limitation of growth rate in some species, such as the coccolithophore *Emiliania huxleyi* (Sunda et al. 2007), and such increased production of DMS could provide a large source of DMSO via DMS oxidation under nutrient-stressed conditions.

*Are diatoms and cyanobacteria overlooked in the DMS cycle?*—Stefels et al. (2007) estimated that DMSP<sub>p</sub>:C ratios of diatoms and cyanobacteria are  $0.86 \pm 1.26$  and  $0.0015 \pm 0.004$  mmol mol<sup>-1</sup>, respectively. This is much lower than mean DMSP<sub>p</sub>:C ratios of prasinophytes,

Table 4. Temporal variations in in situ concentrations of Chl *a*, DMSP<sub>p</sub>, and DMSP<sub>p</sub>:Chl *a* ratios during seven mesoscale iron fertilization experiments in three HNLC regions of the ocean. Chl *a* values are from <sup>a</sup> Martin et al. (1994), <sup>c</sup> Coale et al. (1996), <sup>d</sup> Boyd (2002), <sup>e</sup> Gervais et al. (2002), <sup>f</sup> Archer et al. (2011), <sup>g</sup> Marchetti et al. (2006), and <sup>i</sup> Nagao et al. (2009). DMSP<sub>p</sub> values are from <sup>b</sup> Turner et al. (2004), <sup>f</sup> Archer et al. (2011), <sup>h</sup> Merzouk et al. (2006), and <sup>i</sup> Nagao et al. (2009).

HNLC area	Fe fertilization experiment	Time (d)	Chl <i>a</i> ( $\mu\text{g L}^{-1}$ )	DMSP <sub>p</sub> ( $\text{nmol L}^{-1}$ )	DMSP:Chl <i>a</i> ( $\text{mmol g}^{-1}$ )
Pacific Equatorial	IronEx I <sup>a,b</sup>	0	0.24	30	125
	(Iron enrichment experiment I)	3	0.65	56	86
	IronEx II <sup>c,b</sup>	0	0.15	14	92
	(Iron enrichment experiment II)	6	2.98	36	12
Southern Ocean	SOIREE <sup>d,b</sup> (Southern Ocean iron release experiment)	0	0.25	27	108
		9	1.4	56	40
		12	1.8	34	19
	EisenEx <sup>e,b</sup>	0	0.5	45	90
		12	1.7	74	44
		19	1.95	47	24
Subarctic Pacific	SAGE <sup>f</sup> (Surface ocean lower atmosphere studies air-sea gas experiment)	0	0.4	32	80
		15.5	0.7	42	63
	SERIES <sup>g,h</sup> (Subarctic ecosystem response to iron enrichment study)	0	0.8	76	95
		12	1.6	25	16
		18	5.3	30	6
	SEEDS II <sup>i</sup> (Subarctic Pacific iron experiment for ecosystem dynamics study II)	2	0.8	20	25
	13	2.3	98	43	

haptophytes, chrysophytes, and dinoflagellates, which range from  $5 \pm 7$  to  $22 \pm 32$   $\text{mmol mol}^{-1}$  (Stefels et al. 2007). Diatoms and cyanobacteria are thus considered minor DMSP and DMS producers. The standard deviations on these ratios, however, are very high ( $\sim 1.4$  times the means), and these differences may represent not only interspecific but also intraspecific variability linked to environmental growth conditions. Our results clearly show that a diatom and a diazotrophic cyanobacterium significantly increased their DMSP<sub>p</sub>:C ratios under Fe limitation. Under severe Fe limitation, the cellular DMSP<sub>p</sub>:C ratio increased by an order of magnitude for both species. This increase places them above the range of DMSP<sub>p</sub>:C ratios generally attributed to diatoms and cyanophytes. However, even under severe Fe limitation, *T. erythraeum* could not be considered a significant DMSP producer based on its intracellular DMSP concentration or its DMSP<sub>p</sub>:C ratio. Severely Fe-limited *T. oceanica* in the present study and *S. costatum* in a previous study (Sunda et al. 2002) had DMSP<sub>p</sub>:C ratios of 4–5  $\text{mmol mol}^{-1}$ , close to the mean values reported for prasinophytes. Because Fe limitation also strongly decreases cellular Chl *a* content, DMSP<sub>p</sub>:Chl *a* ratios of severely Fe-limited *T. oceanica* (Table 2) and *T. erythraeum* (Table 3) are also much higher than the ones estimated by Stefels et al. (2007) for diatoms (4  $\text{mmol g}^{-1}$ ) and cyanophytes (0.008  $\text{mmol g}^{-1}$ ). Our results here suggest a possible role in DMSP production of seemingly minor DMSP producers (e.g., diatoms and cyanobacteria) in Fe-limited regions of the open ocean.

Fe limitation is proposed to limit phytoplankton growth in  $\sim 50\%$  of the open ocean, including high-nutrient, low-chlorophyll areas such as the Southern Ocean, where diatoms dominate the phytoplankton community (Boyd and Ellwood 2010). In mesoscale Fe enrichment experiments conducted in HNLC regions, concentrations of DMSP<sub>p</sub> generally increased when blooms developed. An

increase in DMSP<sub>p</sub> with increasing Fe concentrations was also observed along an Fe gradient in the HNLC subarctic Pacific (Royer et al. 2010). However, with the exception of the mesoscale Fe fertilization experiment “Subarctic Pacific iron experiment for ecosystem dynamics study II” (SEEDS II) and the above-mentioned study of Royer et al. (2010), the DMSP<sub>p</sub>:Chl *a* ratio generally decreased with alleviation of Fe limitation (Table 4). This ratio decreased by over 3-fold on average after 6–19 d during six mesoscale Fe fertilization experiments conducted in three different HNLC regions (from  $98 \pm 9$   $\text{mmol g}^{-1}$ ,  $n = 6$ , 95% confidence interval, to  $28 \pm 13$   $\text{mmol g}^{-1}$ ,  $n = 8$ , 95% confidence interval). This decrease is generally attributed to a shift from high-DMSP-producing species before Fe fertilization to low-DMSP-producing species (i.e., diatoms) during the bloom (Liss 2007). Our results, however, also show a decrease in the DMSP<sub>p</sub>:Chl *a* ratio in *T. oceanica* with the alleviation of iron limitation, from  $89.4 \pm 2.6$   $\text{mmol g}^{-1}$  in severely Fe-limited cells to  $1.5 \pm 0.1$   $\text{mmol g}^{-1}$  in Fe-replete cells (Table 2), which is due mainly to the decrease in cellular DMSP rather than to an increase in Chl *a*.

A similar observation can be made when comparing DMSP<sub>p</sub>:Chl *a* ratios in Fe-enrichment experiments where no significant change in the phytoplankton community is noted. For example, from day 12 to 18 during the mesoscale Fe fertilization experiment “Subarctic ecosystem response to iron enrichment study” (SERIES), the bloom was dominated by microalgae (i.e., diatoms), which made up 86% of total Chl *a* by day 17. In contrast, the Chl *a* contribution by picophytoplankton, nanophytoplankton, and dinoflagellates remained low and varied little (Marchetti et al. 2006). During this time, the DMSP<sub>p</sub>:Chl *a* ratio decreased from 16 to 6  $\text{mmol g}^{-1}$ . In the Peru upwelling system, Riseman and DiTullio (2004) also observed 2-fold increases in DMSP<sub>p</sub>:Chl *a* and DMSO<sub>p</sub>:Chl *a* ratios



between near-surface waters of a nutrient-replete, high-iron ( $1.8 \text{ nmol L}^{-1}$ ) inner-shelf station and waters of an adjacent nutrient-replete midshelf station with up to 10-fold lower dissolved iron concentrations ( $0.1\text{--}0.8 \text{ nmol L}^{-1}$ ). This occurred despite the presence of similar phytoplankton communities dominated by diatoms at both stations. Iron-enriched incubations of natural plankton communities in seawater samples from the Ross Sea in the austral summer also showed similar trends (Bertrand et al. 2007). In one of these experiments ( $76^{\circ}00'S$ ,  $178^{\circ}66'E$ ), the relative composition of the phytoplankton community was similar in the Fe-limited control and in the Fe-enriched incubation after 9 d (16% *Phaeocystis antarctica* and 84% diatoms vs. 13% *P. antarctica* and 87% diatoms, respectively). However, the  $\text{DMSP}_p:\text{Chl } a$  ratio was  $\sim 10 \text{ mmol g}^{-1}$  in the iron enriched treatment compared to  $\sim 47 \text{ mmol g}^{-1}$  in the control. In these two examples, particulate DMSP concentrations stayed nearly constant while Chl *a* and cell concentrations increased, indicating a decrease in DMSP per mg Chl *a* and per cell. In another incubation, conducted 8 d later at  $74^{\circ}60'S$ ,  $173^{\circ}20'E$ , the abundance of *P. antarctica* (a high-DMSP producer) increased from 14% to 24% on Fe addition, while that of diatoms decreased from 86% to 76% (Bertrand et al. 2007). However, the  $\text{DMSP}_p:\text{Chl } a$  ratio still decreased from  $\sim 28$  to  $8 \text{ mmol g}^{-1}$ .

All these observations suggest that the decrease in  $\text{DMSP}_p:\text{Chl } a$  and  $\text{DMSO}_p:\text{Chl } a$  that is generally observed in situ with alleviation of iron limitation is related not only to a species switch from high- to low-DMSP-producing species but also to a decrease in cellular  $\text{DMSP}:\text{Chl } a$  ratios in individual species, including diatoms. However, since  $\text{Chl } a:\text{C}$  ratios generally decrease with iron limitation of growth rate (this study; Sunda and Huntsman 1995), a portion of the decrease in  $\text{DMSP}_p:\text{Chl } a$  and  $\text{DMSO}_p:\text{Chl } a$  ratios is likely also caused by increasing cellular  $\text{Chl } a:\text{C}$  ratios associated with increasing algal growth rate following iron additions.

Based on a modeling study, iron availability is estimated to limit *Trichodesmium* growth in 45–75% of the global tropical and subtropical ocean (Moore et al. 2004), where temperatures are sufficient to permit the growth of this genus (La Roche and Breitbarth 2005). In oceanic waters where Fe is limiting, the density of *Trichodesmium* is generally lower than 300 trichomes  $\text{L}^{-1}$ , corresponding to a carbon concentration of  $\sim 1.3 \text{ } \mu\text{mol L}^{-1}$  ( $4.2\text{--}4.8 \text{ nmol C trichome}^{-1}$ ; La Roche and Breitbarth 2005). Based on our values of  $\text{DMSP}_t:\text{C}$  under Fe limitation (Table 3), 300 trichomes  $\text{L}^{-1}$  of Fe-limited *Trichodesmium* would not correspond to a significant amount of  $\text{DMSP}_t$  ( $\sim 0.11 \text{ nmol L}^{-1}$ ). In the eastern North Atlantic, for example, where Moore et al. (2009) demonstrated a positive correlation between Fe concentrations,  $\text{N}_2$  fixation rates, and *Trichodesmium* abundances, a maximum of 1040 trichomes  $\text{L}^{-1}$  was recorded during the Atlantic Meridional Transect 5 cruise (Tyrrell et al. 2003). It would correspond to a  $\text{DMSP}_t$  concentration of  $0.38 \text{ nmol L}^{-1}$  according to our Table 3, which is much lower than the mean in situ  $\text{DMSP}_t$  concentration of  $18.4 \pm 4.8 \text{ nmol L}^{-1}$  measured during the same cruise ( $n = 25$ , 95% confidence interval;

Bell et al. 2010). During bloom conditions, however, the density of *Trichodesmium* at the sea surface can be extremely high, with trichome concentrations varying from  $6 \times 10^3 \text{ L}^{-1}$  up to  $9.72 \times 10^6 \text{ L}^{-1}$  in mature blooms, corresponding to carbon concentrations of  $25\text{--}47 \text{ mmol L}^{-1}$  (La Roche and Breitbarth 2005). Using our values of  $\text{DMSP}_t:\text{C}$  (Table 3), we estimate that such blooms could contain  $0.15\text{--}280 \text{ nmol L}^{-1}$  of total DMSP under Fe-replete conditions and  $\sim 2.2$  to  $4100 \text{ nmol L}^{-1}$  of total DMSP under Fe-limiting conditions. *Trichodesmium* could thus contribute significantly to inventories of DMSP in surface seawater during dense blooms.

Such high variations in  $\text{DMSP}:\text{C}$  and  $\text{DMSP}:\text{Chl } a$  in major groups of oceanic phytoplankton species, as observed in our study, may thus have to be taken into account in studying the DMS cycle in oceanic environments. A significant increase in the intracellular concentration of DMSO under Fe limitation may also be important for the DMS cycle because of the potential importance of DMS and DMSP oxidation to DMSO under increased oxidative stress (Sunda et al. 2002) and the subsequent enzymatic reduction of DMSO to DMS (Spiese et al. 2009). The sensitivity to Fe limitation for other species belonging to high-DMSP-producing algal groups should also be studied not only for DMSP production but also for DMS and DMSO release and the activities of DMS-producing and DMSO-reducing enzymes. Indeed, in species where the DMSP concentration is constitutively high ( $200\text{--}400 \text{ mmol L}_{\text{cell}}^{-1}$ ), there may be no significant increase in intracellular DMSP but rather an increase in the activity of DMS-producing enzymes and in DMS release (and possibly DMSO production) on limitation by iron, as observed under N limitation in *E. huxleyi* (Sunda et al. 2007).

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