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Daytime, growth phase and nitrate availability dependent variations of dimethylsulfoniopropionate in batch cultures of the diatom *Skeletonema marinoi*

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ABSTRACT

Dimethylsulfoniopropionate (DMSP), a sulfur containing osmolyte and antioxidant, is suggested to take over the role of nitrogen-containing osmolytes under nitrate-depleted conditions. But other factors like time of day or limitation by nutrients can influence the cellular DMSP content as well. Especially in diatoms the complex dynamics of this central metabolite are still poorly understood. Therefore, we monitored the DMSP content in batch cultures of the diatom *Skeletonema marinoi* over the entire culture development, using a detailed sampling protocol on a diurnal basis to investigate the influence of the day/night cycle and limitation by nitrogen and other nutrients on cellular DMSP. Cultures were inoculated with initial high nitrate (N+) or low nitrate (N−) concentrations to evaluate the effects of nitrogen availability. The DMSP content per cell varied significantly with a 4-fold increase over the entire experiment. For N+ conditions a pronounced diurnal pattern was found with increasing DMSP contents per cell during the light and decreasing DMSP contents during the dark period. However, when DMSP was normalized to cell volume, the highest concentrations occurred near the beginning of the light period. For N− cultures no such pronounced pattern was found. DMSP values of N− cultures increased within a few hours after the medium became nitrate depleted, while no such connection between nitrogen-concentration and DMSP could be observed in the N+ cultures, where possibly an overflow mechanism or limitation of other nutrients such as Si might affect DMSP concentrations. Based on this data set we can conclude that DMSP formation in *S. marinoi* is under a pronounced diurnal control and depends in a rather complex manner on other factors such as growth limitation by nutrients.

1. Introduction

Dimethylsulfoniopropionate (DMSP), a sulfur containing metabolite, is produced by many macro and microalgae. Beside its physiological functions for the alga itself, DMSP is an important carbon and sulfur source for other heterotrophic organisms (Kiene and Linn, 2000; Simó et al., 2002; Zubkov et al., 2001). It is considered to be the most important precursor of the volatile dimethylsulfide (DMS), which serves as an infochemical between several trophic levels (Steinke et al., 2002). In addition, DMS emissions from the oceans contribute 13 to 37 Tg sulfur to the global sulfur cycle, which corresponds to ca. 50% of the biogenic sulfur emissions (Kettle and Andreae, 2000). Sulfate particles formed from DMS serve as cloud condensation nuclei, resulting in a climate relevant function of this gas (Bates et al., 1987; Charlson et al., 1987).

A number of physiological roles have been attributed to DMSP; for example, it can serve as an antioxidant, cryoprotectant and osmolyte in phytoplankton (Karsten et al., 1991; Kirst et al., 1991; Sunda et al., 2002). Concentrations of DMSP in algal cells can vary significantly from below 0.1 mM to about 400 mM (Keller et al., 1989). These variations not only depend largely on phytoplankton taxa, but also environmental conditions can lead to significant variability of the cellular DMSP content (Archer et al., 2010; Stefels and van Leeuwe, 1998; van Rijssel and Gieskes, 2002). Bucciarelli et al. (2007) conducted a 28 hour survey of cultures of the coccolithophore *Emiliania huxleyi* and showed that the cellular DMSP content varies considerably over a daily cycle. In addition, growth phase dependent variations of DMSP are observed as well. Stefels and van Boekel (1993) found for *Phaeocystis* sp. higher DMSP content per cell when batch cultures in stationary phase were compared to those in exponential phase.

Osmoregulation in algae depends not only on DMSP; other zwitterionic compounds such as glycine betaine (GBT) are discussed as alternative or additional osmolytes (Dickson and Kirst, 1986; Keller et al., 1999a, 1999b). Andreae (1986) hypothesized that under nutrient-depleted conditions DMSP might replace nitrogen-containing osmolytes like GBT. This picture is however complicated by several contradicting reports on the influence of nitrate on the DMSP content. Keller et al. (1999a and 1999b) analyzed the osmolytes DMSP and GBT in phytoplankton cultures (diatoms, prymnesiophytes, dinoflagellates) grown under nitrogen-repleted and nitrogen-limited conditions. They found no reciprocal link between both compounds

Abbreviations: DMS, dimethylsulfide; DMSP, dimethylsulfoniopropionate; GBT, glycine betaine.

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although GBT seemed to be correlated with nitrogen availability (Keller et al., 1999b). *Thalassiosira oceanica* showed higher DMSP concentrations when grown under nitrate limited conditions. However, in combination with increased UV radiation this effect was not observed (Harada et al., 2009). Sunda et al. (2007) showed that N-limitation also increased the cellular DMSP per unit of cell carbon in *Skeletonema costatum*.

In this study, we focus on diatoms that are generally considered to be low DMSP producers (Keller et al., 1989). However, some diatom species can produce considerable amounts of DMSP that reach values close to those of other dominant DMSP producers in the plankton (Spielmeyer et al., 2011). Given the fact that diatoms are distributed worldwide and contribute significantly to the global primary production in the sea (Nelson et al., 1995) it is important to understand their contribution to overall DMSP in the oceans as well. As in other phytoplankton members the DMSP content in diatoms is apparently also highly dynamic. Kasamatsu et al. (2004) found for cultures of the diatoms *Navicula* sp. and *Nitzschia* sp. increasing amounts of particulate DMSP (DMSPp) during the stationary phase while only small amounts of DMSPp were detected during the exponential phase. Bucciarelli and Sunda (2003) found not only increased intracellular DMSP concentrations in nitrate limited cultures of the diatom *Thalassiosira pseudonana*, but also phosphate, silicate or CO_2-limitation led to elevated DMSP cell contents. These results suggest that also in diatoms the DMSP content is under the influence of several regulating factors. Studies that only focus on one parameter risk not providing a comprehensive overview of changes of the DMSP cell content since overlying effects of other factors have to be considered. Here we undertake an effort to unravel the complex influences of nitrogen availability, the diurnal cycle and the growth phase on the cellular DMSP content in a marine diatom culture. We deliberately worked with batch cultures to monitor the continuous changes during the development of the manipulated cultures. The cosmopolitan diatom *Skeletonema marinoi* was selected as a study organism since it is widely distributed and abundant and since it is often used as a model species in the study of diatom physiology and ecology (Barofsky et al., 2010; Kooistra et al., 2008; Prince et al., 2008).

Nearly all previous studies on DMSP in phytoplankton relied on an indirect determination of this metabolite. DMSP is initially transformed to the volatile DMS using strong base (see e.g. Kiene, 1996). Since non-DMSP metabolites can release DMS under alkaline conditions as well (Spielmeyer et al., 2011), the indirect method may overestimate actual cellular DMSP concentrations. Thus, existing data sets include an unknown degree of inaccuracy if the detected variations in DMS concentrations are indeed due to variations of DMSP or rather of other metabolites. In contrast to previous investigations, we used a direct validated method for the quantification of DMSP that does not involve the base-mediated release of DMS (Spielmeyer et al., 2011). This allowed avoiding possible misinterpretations due to alternative DMS sources, which may be produced dependent on growth conditions and stages. The present study provides a comprehensive data set on the effect of the availability of nitrogen and other nutrients, cell growth and diurnal rhythm on the DMSP production in *S. marinoi*. Based on this data set we can conclude that DMSP formation is under a pronounced diurnal control and depends in a rather complex manner on other limiting factors as well.

2. Materials and methods

2.1. Cultivation

Unialgal, non-axenic cultures of *Skeletonema marinoi* (strain G4, University of Bergen, Department of Biology, isolated in the Raunefjord, Norway) were maintained in artificial seawater (Maier and Calenberg, 1994) at 15 °C under a 14:10 light/dark cycle. Light was provided from 6:00 h to 20:00 h from the side by Osram biolux lamps (PAR, 70 μmol quanta m^{-2} s^{-1}). Initial nutrient levels were 250 μmol L^{-1} silicate, 11.3 μmol L^{-1} phosphate and 630 μmol L^{-1} nitrate (cultures 1, 2, 3; high nitrate N+). For low nitrate (N−) cultures the nitrate content of the medium was adjusted to 63 μmol L^{-1} (cultures 4, 5, 6).

Cultures for inoculation were prepared from an exponentially growing stock culture (approx. 950,000 cells mL^{-1}); 75 mL of this culture was diluted with artificial seawater to a final volume of 600 mL. After two days of growth, the culture was divided and 300 mL were transferred into 1.5 L high nitrate or low nitrate medium, respectively. After three days of growth, each culture was divided into three aliquots (500 mL) which were used to inoculate 10.5 L of artificial seawater in polycarbonate bottles (nominal volume 10 L, Nalgene, Rochester, NY, USA), resulting in a density of 25,000 cells mL^{-1}. Bottle caps were equipped with an air inlet, an air outlet and an outlet for sampling. Sterile filtered (Hepa-Vent, Whatman, Florham Park, NJ, USA) air was continuously bubbled through the cultures. To prevent further culture contamination, samples were taken via a dripping chamber described by Vidoudez and Pohnert (2008). Briefly, the sampling outlet of the culture bottle was connected to a 2.5 mL syringe tube that was inserted leakproof into a 1 mL syringe tube. The 1 mL syringe was connected to silicone tubing that was closed via luer lock adapter sealing cones.

2.2. General parameters

Cell counts and cell volume determinations were conducted with unfixed samples. Depending on cell density, cells were counted in Fuchs-Rosenthal or Neubauer chamber using an upright microscope with phase contrast (DM2000, Leica, Heerbrugg, Switzerland). For cell volume determination, pictures were taken from respective N+ and N− cultures (Leica DFC280 system) and for each sampling point 10 to 20 cells were measured (software Leica IM50 (version 4.0)). The calculation of cell volume was based on the assumption of a cylindrical shape (Montagnes and Franklin, 2001).

For nutrient analysis, 10 to 15 mL filtrate (for details see Section 2.3) were collected and stabilized with 20 μL dichloromethane. Samples were stored at −20 °C before further preparation. Nitrate (Zhang and Fischer, 2006), silicate, phosphate and nitrite (Parsons et al., 1984) were determined spectrophotometrically at each sampling point using volume adapted methods (0.5 to 1 mL).

In vivo fluorescence was determined using a microplate reader (Mithras LB 940, Berthold Technologies, Bad Wildbad, Germany). The excitation wavelength was set to 430 nm, and emission was recorded at 665 nm. Samples were analyzed in triplicate in 96 well plates and 300 μL culture samples were used for determination.

2.3. Particulate DMSP (DMSPp)

Samples were taken in intervals of 4 h between 6:00 h and 22:00 h. The sample at 6:00 h was taken just before the light period started. On days 4 and 6 an additional sample was taken at 1 am. Cultures were gravity filtered on Whatman GF/C filters (filtration volume 16 to 96 mL, depending on cell density). Filters were transferred into 2 mL of methanol, containing 100 μL of a 200 μmol L^{-1} D_6-DMSP solution as an internal standard. The hydrochloride of D_6-DMSP was synthesized from D_6-DMS and acrylic acid (Sigma-Aldrich, Germany) according to Chambers et al. (1987). Derivatization of samples with 1-pyrenyldiazomethane and analysis was performed according to Spielmeyer et al. (2011). Samples taken at 14:00 h were additionally analyzed via GC/MS using base-mediated release of DMS for indirect determination of DMSP (for method details see Spielmeyer et al., 2011).