Modulation of dimethylsulfoniopropionate (DMSP) concentration in an *Alexandrium minutum* (Dinophyceae) culture by small-scale turbulence: a link to toxin production?

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Abbreviations: DMSP, dimethylsulfoniopropionate; DMS, dimethylsulfide; PSP, paralytic shellfish poisoning

ABSTRACT

Some marine dinoflagellates produce important amounts of dimethylsulfoniopropionate (DMSP), a common compatible solute, and its cleavage product dimethylsulfide (DMS), a climatically active trace gas. In the field, dinoflagellate proliferations appear to be favored by calm weather and water column stability; indeed, small-scale turbulence is a physical factor that directly affects ecophysiological aspects of this phytoplankton group, including toxin production. Here we report the effect of experimentally generated turbulence on DMSP production by a paralytic shellfish poisoning (PSP) toxin producing strain of *Alexandrium minutum*, a widespread bloomforming dinoflagellate species. With respect to still conditions, the populations exposed to turbulence grew at a slower growth rate and yielded low cell numbers turbulence. Concurrently, the cellular DMSP concentration increased by ca. 20% (from 0.22 ± 0.01 to 0.27 ± 0.03 fmol μm^{-3} on a cell volume basis) in the shaken cultures. DMSP was preferentially synthesized during the light period in both treatments. During the night, a slowdown of the division process caused DMSP accumulation in the cells exposed to shaking. The study suggests the existence of a tight link between the dynamics of DMSP concentration and other cell processes entrained by circadian rhythms in dinoflagellates. The observed effects of small-scale turbulence on the DMSP dynamics supports the suggested role of this compound as an overflow mechanism in metabolically unbalanced cells. Furthermore, considering all the effects on the physiology of *A. minutum* exposed to the same experimental setup, we propose a possible link between the DMSP and the PSP metabolisms.

1. Introduction

Dimethylsulfoniopropionate (DMSP) is a major component of organic sulfur in the ocean and plays a significant role in the global sulfur cycle. Its cleavage produces dimethylsulfide (DMS), which is volatilized and adds gaseous sulfur to the atmosphere. Phytoplankton DMSP production is distributed among many phyla, although it appears to be species-specific (Keller et al., 1989, see review by Stefels et al., 2007). Haptophytes (=Prymnesiophytes, including coccolithophorids) and dinoflagellates have shown the highest intracellular levels of DMSP, which is synthesized as a compatible solute to reach high concentrations (up to 40-500 mM). Some of the algal species belonging to these groups bloom in vast areas, sometimes on intensive and/or recurrent proliferations. There, high amounts of DMSP are produced and circulated throughout the food web (Matrai and Keller, 1993; Simó, 2004) and, as a result, high amounts of DMS are released. It has been suggested that the emission of DMS to the atmosphere may affect cloud formation and brightness, and the backscatter of sunlight may reduce mean light intensity, and influence the phytoplanktonic bloom-formation (Charlson et al., 1987). Thus, the microalgae mediated dynamics of DMSP not only has important ecological implications, but a potential great influence in global sulfur biogeochemistry and climate (Simó, 2001).

The need to understand the DMSP dynamics has fostered a number of ecophysiological studies (reviewed in Stefels et al., 2007), although uncertainties still remain, for instance regarding its metabolic control and role in the cells. In nature, three different metabolic pathways for DMSP biosynthesis have been described in compositae (Hanson and Gage, 1996), gramineae (Kocsis et al., 1998) and chlorophyceae (Summers et al., 1998), all linked to the sulfur metabolism and to the protein synthesis through S-aminoacids

(cysteine and methionine). DMSP is released by algae through exudation or cell lysis, and acts as a chemical signal and a source of reduced sulfur for planktonic partners (Kiene et al., 2000; Simó, 2004; Seymour et al., 2010). Alternatively, DMSP is enzymatically cleaved to DMS and acrylate by DMSP lyases, a family of enzymes present in microalgae and bacteria (Kiene et al., 2000). In some cases, such cleavage would be induced by phytoplankton cells lysis (Wolfe and Steinke, 1996), with DMS and acrylate being subsequently released to the medium. As for the physiological functions of DMSP in the algal cell, it has been proposed (Kirst et al., 1991) that DMSP would act as an osmolyte and cryoprotectant. Sunda et al. (2002) suggested that DMSP and its breakdown products could form a cascade of radical scavengers that may serve as anti-oxidant system regulated in part by the enzymatic cleavage of DMSP. Furthermore, Stefels (2000) hypothesized that DMSP production would constitute an overflow mechanism for excess reduced sulfur under conditions of unbalanced growth (e.g., when carbon and nitrogen flows are out of tune). The three hypothesized roles are not mutually exclusive, but support to any of them requires to overcome methodological constraints and to continue detailed studies on the variability of DMSP concentration in phytoplankton.

In this sense, the available data suggest that the physiological state of the cells affects DMSP dynamics, as its concentration varies over the growth phases and diel cycles (Keller, 1991; Matrai and Keller, 1994; Bucciarelli et al., 2007). It has been also reported that environmental factors that modulate phytoplankton physiology in general, such as salinity, nitrogen availability, temperature, light or water motion, can also affect DMSP internal concentration in the cells (e.g. Vairavamurthy et al., 1985; Sheets and Rhodes, 1996; Gröne and Kirst, 1992; Wolfe et al., 2000; Sunda et al., 2002; see review by Stefels, 2000 and references there in). Until now, the results can be somehow

contradictory and thus hamper drawing clear trends. For instance, DMS production was stimulated by gas bubbling in *Phaeocystis pouchetii* colonies (Wolfe et al., 2000) and by both bubbling and mechanical agitation in *Alexandrium* spp. (Wolfe et al., 2002). However, while the *P. pouchetii* cells appeared to be physiologically damaged, massive destruction or rupture of the *Alexandrium* spp. cells was not detected.

With the aim to increase our understanding about how environmental factors modulate DMSP in phytoplankton, we explored whether a poor understood environmental factor, small-scale water motion, could affect DMSP dynamics in a dinoflagellate. As indicated earlier, dinoflagellates constitute one of the main DMSP phytoplankton producers, and have shown to be particularly sensitive to ~~mixing~~-natural hydrodynamics (e.g. Berman and Shteinman, 1998; Sullivan et al., 2003). In several dinoflagellate species, experimentally generated turbulence (e.g. listed in Berdalet et al., 2007; reviewed by Berdalet and Estrada, 2008) has caused deleterious effects, including alteration of cell division (e.g. Pollinger and Zemel, 1981, Berdalet, 1992), morphological changes (Zirbel et al., 2000), interference with organism swimming pattern (e.g. Karp-Boss et al., 2000; Berdalet et al., 2007) or cell disruption (e.g. White, 1976). In the case of the widespread bloom-forming and paralytic shellfish poisoning (PSP) producing species *Alexandrium minutum*, we found that shaking induced a decrease in its growth rate and biomass yield, along with lower PSP toxin concentration and ecdysal cyst production (Bolli et al., 2007). Turbulence also caused an immediate alteration of the diel timing and duration of the cell cycle phases, with a smaller fraction of the population proceeding through mitosis and an increase in the average DNA content per cell (Berdalet et al., 2007; Llaveria et al., 2009). Significant mortality did not occur, unless turbulence lasted for more than 5 days.

Here, we used the same strain and the same shaking conditions that caused the aforementioned responses in *A. minutum*. Changes in DMSP concentrations, cellular abundances and their corresponding biovolume were measured during 5 days of continuous exposure to turbulence. Given that dinoflagellates in general, and this species as well, show a clear circadian rhythm with cell division occurring mainly during the night period, we also examined the diel variation of DMSP concentrations. We hypothesized that if a DMSP light:dark cycle was observed, it could also be somehow altered by turbulence, in parallel to the responses observed in the cell cycle (Llaveria et al., 2009). Based on the observed changes in the DMSP pattern under turbulence, we discuss the possible roles of this compound in the dinoflagellate metabolism and suggest an eventual link to the PSP toxin metabolism. It should be noted that this and the above mentioned experimental studies performed by our research group, are aimed to unveil specific physiological responses to turbulence that cannot be separated from the rest of environmental forcings. Aware that the experimental conditions will never mimic nature, the studies are developed with a physiological scope with no aim to directly extrapolate to the field.

2. Materials and methods

2.1. Cultures. The *Alexandrium minutum* clonal strain employed in this study was VGO 651, kindly provided by the Vigo Oceanographic Center (Instituto Español de Oceanografía, Spain). Non-axenic stock and experimental unialgal cultures were maintained in a temperature controlled room at $20 \pm 1^\circ\text{C}$, $120 \mu\text{mol photon m}^{-2} \text{s}^{-1}$

irradiance provided by the combination of Grolux -58 W, Sylvania, Erlangen, Germany- and cool-white -58 W, Philips, Eindhoven, the Netherlands- fluorescent tubes, in a 1:1 proportion; 12:12 h LD cycle, light period starting at 00:00 local time. The microalgae were grown on f/2-Si, enriched seawater without silicate addition (Guillard, 1975); seawater (salinity 38) was obtained from Blanes Bay -NW Mediterranean-, 1 km offshore at 5m depth.

2.2. Experimental. Four 4 L Florence flasks (Pyrex spherical flasks with flat bottom) containing 3 L of medium were inoculated with 912 ± 81 *A. minutum* cells mL⁻¹). On day 3, immediately after sampling, two flasks (hereafter referred to as *Turbulent*) were randomly chosen to be subjected to continuous turbulence while two flasks were kept still as control (hereafter referred to as *Still*). Samples for the estimation of cell numbers, cell size and DMSP concentration were taken daily at 4:00 h local time (corresponding to 4 hours after the lights onset and 8 hours before the dark period) to track the effect of turbulence. Two days after the start of turbulence, on day 5, samplings were performed at regular time intervals of 2 hours (from 0:00 to 24:00 h local time, both included). To obtain representative and even samples of the *Still* flasks they were carefully and gently swirled to minimize disturbance.

2.3. Turbulence setup. Turbulence was generated by an orbital shaker operating at 120 rpm with a displacement of 30 mm. This corresponds to an average turbulent kinetic energy dissipation rate, ϵ , of 27 cm² s⁻³ ($2.7 \cdot 10^{-3}$ Watt Kg⁻¹, Bolli et al., 2007), as estimated by a miniaturised acoustic Doppler velocimeter. A scheme of the setup used to perform ϵ estimations can be found in Fig. 1 of Berdalet et al., 2007. A complete description of the calculations was provided by Guadayol et al., 2009. The experimental ϵ were large, both in intensity and persistence, compared to Mediterranean turbulence

events (Guadayol and Peters, 2006), although 4-day sustained events of $0.4 \text{ cm}^2 \text{ s}^{-3}$ do occur sometimes.

2.4. Measurements.

2.4.1. Cell abundances and cell volume. Cell abundances were estimated by microscopy with Sedgewick-Rafter counting cell slides after fixation with Lugol's iodine solution. A minimum of 400 cells were counted. Net exponential growth rates (μ , d^{-1} , as defined by Guillard, 1973), were calculated as the slope of the regression line of $\ln(N)$ versus time (t), where N was the estimated cell abundances by microscopic counting.

In vivo measurements of the population size spectrum were performed immediately after sampling using a Multisizer 3 Coulter Counter (Beckman Coulter, Fullerton, CA, USA). This instrument is provided with a $100 \text{ }\mu\text{m}$ aperture tube, with an effective particle-size range of 2-60 μm (spherical equivalent diameter), and a 300 channel particle-size analyzer.

2.4.2. DMSP concentration. Total DMSP concentration (dissolved and particulate forms) was determined as DMS following alkaline hydrolysis (Simó et al., 1996; Saló et al., 2010). Culture samples were extracted on 10 M NaOH in 10-mL glass vials. Vials were sealed immediately with Teflon-coated septa and stored in the dark. After overnight incubation, the alkaline hydrolysis of DMSP resulted in equimolar quantities of DMS, which was measured one week later using a Shimadzu GC-14A gas chromatograph equipped with flame photometric detection (GC-FPD). The carrier gas was helium delivered at a constant flow rate and volatiles were stripped during 4 min and cryo-trapped at the temperature of liquid nitrogen (-196°C). The trapped gases were desorbed with hot water (ca 70°C) and introduced into the gas chromatography system

for analysis. Calibration was carried out by injecting known amounts of gaseous DMS released by a permeation tube and diluted in helium flow (Simó, 1998). Interpolation on linear $\log(\text{peak area})/\log(\text{DMS mass})$ plots allowed quantification. Note that there was no filtration of the sample, so that particulate and dissolved DMSP, and dissolved DMS, were all measured at once. Quantitative distinction between the dissolved and the particulate pools of DMSP is problematic due to filtration artifacts that easily result in overestimation of the dissolved fraction and underestimation of the particulate fraction (Kiene and Slezak, 2006). The ‘total DMSP’ pool measured in this study can be approximated to particulate DMSP, and thus to intracellular DMSP, based on the fact that dissolved DMSP and DMS concentrations are generally very small with respect to particle-associated DMSP (Keller and Korjeff-Bellows, 1996; Hatton and Wilson, 2007), even under conditions of oxidative stress (Sunda et al., 2002).

2.4.3. Statistics. Statistical analyses were performed using Systat 11 for PC (Systat Software Inc., Point Richmond, CA, USA). The time course of the different parameters between treatments was compared using the non-parametric Kruskal-Wallis test (Motulsky, 2003) and the net growth rates by testing for the heterogeneity of slopes (analysis of covariance).

3. Results

3.1. Changes along the whole 7 days experiment. The *Still* population grew exponentially throughout the experiment (Fig. 1a), with a net growth rate of $0.37 \pm 0.005 \text{ d}^{-1}$ ($r^2 = 0.988$, $n = 12$). The development of the *Turbulent* population slowed

down after turbulence started on day 3 (t_3). The growth rate of the *Turbulent* cultures was $0.20 \pm 0.01 \text{ d}^{-1}$ ($r^2 = 0.95$, $n = 10$), i.e. a 55% than that of the *Still* ones. At the end of the experiment on day 7 (t_7), the final cell abundances in the *Turbulent* treatment ($5800 \pm 100 \text{ cells mL}^{-1}$) was ca. the 46% of the *Still* numbers ($12525 \pm 525 \text{ cells mL}^{-1}$). The average cellular volume (biovolume) increased by 38% (from $3124 \text{ }\mu\text{m}^3 \text{ cell}^{-1}$ to $4869 \text{ }\mu\text{m}^3 \text{ cell}^{-1}$) in both treatments before the application of turbulence (from t_0 to t_3 , Fig. 1b). This was probably due to cell acclimation after being transferred to the new culture medium. Later, the cells in the *Still* cultures decreased their mean biovolume, attaining values on day 6 similar to those of t_0 . Oppositely, the *Turbulent* cultures increased their cellular volume after one day of exposure to shaking, i.e. from t_4 to t_7 , and reached values near $5000 \text{ }\mu\text{m}^3 \text{ cell}^{-1}$ at the end of the experiment. However, such increase did not compensate for the marked decrease in cell abundances (Fig. 1a), and the biomass of the *Turbulent* cultures, expressed as total biovolume per mL of culture (Fig. 1c), was lower than in the *Still* flasks.

Over the experiment, the DMSP concentration in the cultures (DMSP mL^{-1}) increased exponentially (Fig. 1d). The DMSP measurements on t_0 and t_3 , i.e., before the application of shaking in the corresponding flasks, indicated that the temporal trends in the DMSP concentration were similar in the four experimental containers. During the shaking period (t_3 - t_7), the DMSP concentration increased at a rate of 0.34 d^{-1} , not significantly different from the rate, 0.32 d^{-1} , in the *Still* treatment ($p = 0.160$, $n = 10$). Normalization of DMSP concentration to cell counts, however, revealed clear differences (Fig. 1e). During the first three days, before the application of turbulence, slight increases of DMSP per cell were detected in all experimental flasks. Thereafter, it dropped suddenly from $967 \text{ fmol DMSP cell}^{-1}$ to $687 \text{ fmol DMSP cell}^{-1}$ from t_4 to t_6 under *Still* conditions, while in the *Turbulent* ones it increased since agitation started

and attained 1322 fmol DMSP cell⁻¹ at the end of the experiment. Because cell volumes had varied throughout the experiment (Fig. 1b), the changes in DMSP concentration per biovolume were also calculated and plotted (Fig. 1f). This parameter decreased from 0.26 ± 0.01 to 0.20 ± 0.01 fmol DMSP μm⁻³ between t₀ and t₃, as a result of biovolume enlargement. Over the rest of the experiment, the concentration of DMSP was almost constant under *Still* conditions (0.22 ± 0.01 fmol DMSP μm⁻³, n=10), whereas exposure to turbulence caused an immediate increase and a subsequent stabilization until the end of the experiment. Overall, the biovolume based DMSP concentration in the shaken cultures was about 0.27 ± 0.03 fmol μm⁻³ (from t₄ to t₇), significantly higher (Mann-Whitney U test = 1.000, p = 0.001, n = 16) than in the still controls.

3.2. Diel cycle. Under still conditions, the cell division of *A. minutum* occurred preferentially over the dark period (from 12:00 to 24:00 h, Fig. 2a), and cellular growth (increase in biovolume) took place during the light hours (from 0:00 to 12:00, Fig. 2b). Indeed, considering the sampled 24 h period, cell abundances increased by 77% (Fig. 2a), the average cell volume augmented from 3326 μm³ cell⁻¹ to 4152 μm³ cell⁻¹ during the first 12 h (Fig. 2b), and returned thereafter to the initial values. These patterns were altered by turbulence. Under agitation, cell numbers increased only a 35% of the initial value (Fig. 2a) over the sampled 24h period. During the light hours (0:00 to 12:00 h) the average cellular volume augmented from 3976 ± 39 μm³ cell⁻¹ to 4607 ± 43 μm³ cell⁻¹, and stabilized along the dark period, i.e. it did not return to the initial values as observed in the control cultures. The joint response of the cell abundances (Fig. 2a) and the cellular biovolume (Fig. 2b) resulted in a lower biomass of the shaken cultures expressed in terms of total cell biovolume (Fig. 2c).

Regarding the total DMSP concentration in the cultures, it was similar in the four flasks at the beginning of the intensive sampling, on day 5 at 0:00 h (Fig. 2d), after two days of stirring. Over the diel cycle, the estimated values were slightly but significantly larger in still conditions than under turbulence ($U = 219$, $p = 0.015$, $n = 52$). In both treatments, the concentration increased mainly during the light period, but at a slower rate in the *Turbulent* (ca. 0.050 h^{-1}) than in the *Still* cultures (ca. 0.036 h^{-1}). During the dark period, the DMSP production rates slowed down markedly. Expressed on a per cell basis, the DMSP concentration over the diel cycle (Fig. 2e) varied in parallel to the cell biovolume (Fig. 2b). When the intensive sampling began, the DMSP concentration per cell was clearly higher in cells under turbulent conditions than in those under stillness (Fig. 2e). It increased during the light hours in both treatments, attaining a maximum at sunset in the *Still* flasks ($1037 \pm 15 \text{ fmol cell}^{-1}$) and two hours before it in the *Turbulent* ones ($1404 \pm 71 \text{ fmol cell}^{-1}$). During the dark phase, the concentrations decreased and, at the end, the cells from the *Still* cultures had recovered their initial values (around $600 \text{ fmol cell}^{-1}$). However, the *Turbulent* cells maintained their DMSP content throughout the dark period until sunrise, although some variability was detected near the sunset (Fig. 2e, from 12:00 to 16:00). A similar pattern was observed when the DMSP was referred to cellular biovolume (Fig. 2f). Indeed, during the light period DMSP increased in parallel with the increase in cell biovolume (Fig. 2b), specially in *Still* treatment. Thereafter, during the darkness and coinciding with the cell division period the DMSP concentration per biovolume stabilized with some degree of variability.

4. Discussion

The aim of this study was to investigate whether the DMSP concentration in *Alexandrium minutum* cultures was influenced by turbulence, both, at a small temporal scale (over a 24h light:dark cycle) as well as over 4 days of exposure to experimental shaking. Our study showed two main findings. First, the populations exposed to turbulence grew at a lower growth rate, produced lower biomass yield and increased the cellular DMSP concentration (either expressed as per cell or per biovolume), compared to the still controls. Second, *A. minutum* displayed a circadian rhythm in the DMSP content that was altered by turbulence: while under stillness this compound was mainly produced during the light hours and it was distributed within the recently divided cells during the night, under turbulence DMSP accumulated in the cells during the dark period, likely because the cell division process could not proceed. To facilitate the interpretation of the results, we first focus on the dynamics of the DMSP under control (still) conditions over the intensive 24h sampling. Thereafter, we will discuss the modifications of such trends under turbulence and try to suggest the possible involved mechanisms. As noted at the Introduction, the findings and hypothesis drawn here have a physiological scope. Extrapolation to natural conditions would require adequate scaling that goes beyond the aim of present study.

Here we document in detail the existence of a light:dark related rhythm in the DMSP concentration of *A. minutum* cultures (under still conditions). In the cells, DMSP preferentially increased during the light hours and it decreased during the dark, down to the levels measured at the beginning of the previous light period. We assume that this trend is associated to the *A. minutum* light:dark entrained cell cycle, with cell division occurring during the night and cell growth during the day, as previously described for

this species (Llaveria et al., 2009) and confirmed here by the trends in the biovolume over the 24 h intensive sampling (see discussion below). Even though a non-axenic culture was used and bacteria can participate in the DMSP dynamics in nature (e.g. Ledyard and Dacey, 1994; de Souza and Yoch 1995a,b; Niki et al., 2000; Yoch 2002), there is no obvious mechanism through which bacterial activity could explain the observed DMSP variations along the diel cycle and/or tested treatments (including the responses to turbulence, discussed later). The observed DMSP rhythmicity, with concentration increasing mainly during the light hours, points to a tight link of DMSP biosynthesis to photosynthesis, and provides support to the role of DMSP as a protection against oxidative stress (Sunda et al., 2002) by scavenging hydroxyl radicals and other reactive oxygen species that form as byproducts of photosynthesis (Foyer, 1996).

Our data also revealed that DMSP constitutes an important component of the total carbon (C) pool of the cell. Specifically, DMSP-C accounted, on average and under still conditions, for the 36.4 ± 1.0 % (mean $\pm$ standard error of the mean; $n = 21$) of the total C pool along the experiment under still conditions. In our calculations, we included all biovolume estimations (by Coulter Counter) performed in the 6 experimental flasks under still conditions (i.e., including the data points on days 0 and 3, before the beginning of shaking in the turbulent flasks). The biovolume values were expressed as C based on the C to cell biovolume relationship described in dinoflagellates, $\text{pgC cell}^{-1} = 0.760 \times (\text{cell volume})^{0.819}$, by Mender-Deuer and Lessard (2000), and the conversion factor of 5 mols of C per DMSP molecule. The values are essentially the same when we used a fixed value, $0.04 \text{ pg C } \mu\text{m}^{-3}$, estimated in the clone A1 IV *A. minutum*, a long-extended culture in the Vigo Oceanographic Center grown also in f/2-Si (Frangópolis et al., 2000). This value is higher than the average proportion but falls near the high end of

the variability range for dinoflagellates, $11.1 \pm 15.8\%$, reported by Stefels et al. (2007).

Our finding suggests that DMSP should play important and, likely, diverse roles in dinoflagellates. We will speculate about it later, taking into account how the experimental turbulence modified the described trends.

As stated previously, the present study was performed with the same *A. minutum* strain and under the experimental conditions used in previous ones conducted by our research group (Berdalet et al., 2007; Bolli et al., 2007; Llaveria et al., 2009), which allows the direct comparison and common interpretation of the obtained results. Indeed, in terms of cell numbers, growth rate and cell biovolume, *A. minutum* responded to turbulence as earlier reported. These figures arise from the immediate alteration of the diel timing and the duration of the cell cycle phases induced by turbulence (Llaveria et al., 2009). For instance, the increase in the cellular biovolume under shaking would correspond to a longer G2/M phase and a shorter S phase. Concurrently, a smaller fraction of the shaken population entrained mitosis, compared to the still conditions. Altogether, these changes in the cell cycle pattern caused the lower net growth rate and cell numbers in the stirred populations. In the present experiment, cell mortality induced by turbulence was not expected to occur given that this physical forcing lasted only for 4 days (Llaveria et al., 2009).

In the present study, the direct effect of turbulence on the cell cycle and population dynamics was accompanied by differential diel and long term trends in the DMSP concentration. In the *A. minutum* population exposed to turbulence, the DMSP levels per cell reached at the end of the photosynthesis period remained stable, in contrast to the decrease observed in the unshaken cultures. In other words: DMSP accumulated in the cells whose cell division cycle was arrested under stirring, and it continued to increase

along the experiment. Such DMSP accumulation could be the result of the blockage of the protein synthesis pathways associated to the production of new cells from mitosis. Protein and DMSP are linked by a common precursor, methionine (Fig. 3; Stefels, 2000 and references there in), that also acts as a catalyst accompanied by a recycling system in which methionine is regenerated (Giovanelli, 1987). It has been suggested that DMSP production can constitute an overflow mechanism when growth is unbalanced and there is a need for dissipating metabolic energy or progressing towards alternative metabolic pathways (Stefels, 2000). Gröne and Kirst (1992) also argued that a build-up of the pool of methionine under stress (*senso lato*, i.e., all conditions that reduce growth) resulted in an increase of DMSP production. In line with these studies, we suggest that DMSP biosynthesis increased in *A. minutum* as a response to stress induced by turbulence, leading to DMSP accumulation. During the shaking period, the DMSP-C constituted a $45.4 \pm 0.9 \%$ ($n=17$) of the estimated total C of the cells. This value was 20% higher than the estimated under still conditions ($36.4 \pm 1.0 \%$). The implications of such increase in a wider ecological context should be taken with caution. As already stated, is it still too soon to scale up this value from small (experimental, physiological) to high (ecologic, oceanic) scales.

The tight coupling between the dynamics of the DMSP cell content, cell division and growth under stillness, and the way how all these physiological processes were altered by turbulence, support a possible implication of DMSP in the circadian rhythm regulation of several physiological process in dinoflagellates (Nakamura et al., 1993; Nakamura et al., 1997; Miller 2004; Ratti and Giordano 2008). In particular, DMSP participates in the metabolic pathway that transfers the methyl group from methionine to gonyauline and gonyol in *Gonyaulax polyedra* (Nakamura et al., 1992; Nakamura et al., 1997). Gonyauline (*cis*-2-(dimethylsulfonio)cyclopropane carboxylate) is a

zwitterionic S-compound involved in the regulation of the bioluminescent circadian rhythm in *G. polyedra* (Roenneberg et al., 1991; Nakamura et al., 1992). Gonyol (3S-5-dimethylsulfonio-3-hydroxypentanoate), identified in several dinoflagellates (Nakamura et al., 1993; 1997) would also play a role in circadian rhythm regulation, including cell division.

Our study was conducted with a multiple PSP toxin (saxitoxins and analogs) producing dinoflagellate. Interestingly, the saxitoxins levels (GTX1, GTX4, C1, C2) in shaken *A. minutum* and *A. catenella* cells significantly decreased under turbulence concurrently to the already mentioned slowdown of cell division processes (Bolli et al., 2007). Several other studies have also reported variations in the toxin dynamics related to the cell division cycle (e.g. Taroncher-Oldenburg et al., 1997; Cho et al., 2011) although the clear role of toxins in the dinoflagellate cells physiology has not been clarified yet. The saxitoxins produced by *A. minutum* and *A. catenella* include N-sulfocarbamates and sulfoxides in their structure, and their biosynthetic pathway (yet not definitively established) seems to involve methionine (Shimizu, 1986; 1990; Shimizu et al., 1990). Methionine, in turn, participates in the biosynthesis of DMSP (Stefels, 2000), as illustrated in Fig. 3. Based on the overall results observed in *A. minutum* exposed to turbulence, we hypothesize here a possible link between DMSP and toxin production via a common precursor: methionine. When turbulence blocked the physiological processes that led to toxin production, maybe linked to the slowdown of cell division (Bolli et al. 2007), the precursor methionine could be deflected towards DMSP production that can be excreted (overflowed) and thus excess S (and C) could be palliated (as discussed above). Testing this hypothesis requires sophisticated physiological and biochemical research, but it points to the link between two metabolic

pathways, DMSP and PSP toxin, that definitively play relevant yet not understood roles
in the biology and ecology of dinoflagellates.

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7. Legends

Figure 1: Temporal changes along the whole experiment in the different parameters estimated in the two treatments *Still* and *Turbulent*. a) Cell abundances. b) Biovolume. c) Total cellular volume per mL of culture. d) DMSP concentration in the culture. e) DMSP per cell. f) DMSP per biovolume. Vertical bars indicate the standard error of the mean ($n = 2$), and "T" indicates the beginning of turbulence in the corresponding flasks on day 3. Inside squares indicate the period where the intensive 24 hours sampling was performed (see results in Figure 2).

Figure 2: Temporal changes in the different parameters estimated over the diel cycle in the two treatments *Still* and *Turbulent*. a) Cell abundances. b) Biovolume. c) Total cellular volume per mL of culture. d) DMSP concentration in the culture. e) DMSP per cell. f) DMSP per biovolume. Vertical bars indicate the standard error of the mean ($n = 2$). The shaded bar represents the dark period during the diel cycle.

Figure 3. Schematic representation of the processes involved in the biosynthesis of DMSP (based on Figures 1, 2 and 4 in Stefels, 2000) and paralytic shellfish poisoning toxins (PSPs, based on Figure 2 in Plumley, 1997) in marine algae. APS: Adenosine-5'-phosphosulphate; SAM: S-adenosylmethionine, SAH: S-adenosylhomocysteine. "T" and the double bars indicate possible pathways bloqued somehow and indirectly by turbulence.

Figure 1

Figure 2

Figure 3