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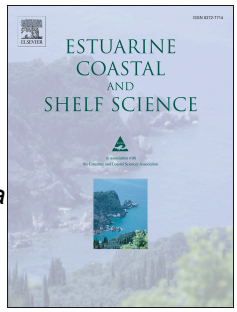

Salinity and growth effects on dimethylsulfoniopropionate (DMSP) and dimethylsulfoxide (DMSO) cell quotas of *Skeletonema costatum*, *Phaeocystis globosa* and *Heterocapsa triquetra*

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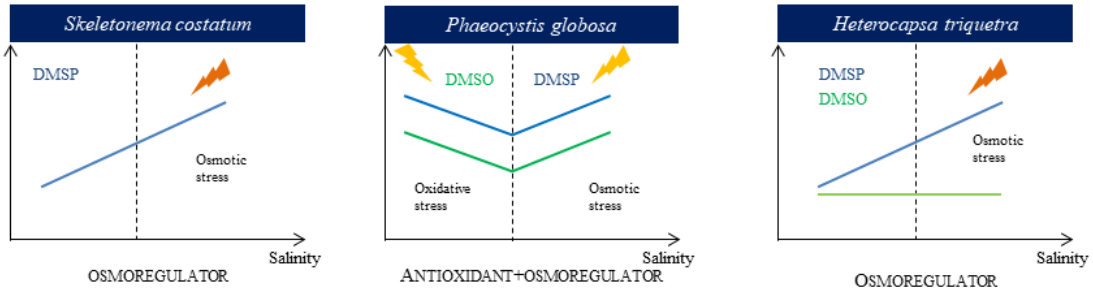
Skeletonema costatum
DMSP
Osmotic stress
Salinity
OSMOREGULATOR
Phaeocystis globosa
DMSO
DMSP
Oxidative stress
Osmotic stress
Salinity
ANTIOXIDANT+OSMOREGULATOR
Heterocapsa triquetra
DMSP
DMSO
Osmotic stress
Salinity
OSMOREGULATOR

Salinity and growth effects on dimethylsulfoniopropionate (DMSP) and dimethylsulfoxide (DMSO) cell quotas of *Skeletonema costatum*, *Phaeocystis globosa* and *Heterocapsa triquetra*

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Abstract The effects of growth stage and salinity on dimethylsulfoniopropionate (DMSP) and dimethylsulfoxide (DMSO) cellular content were investigated in laboratory batch cultures of three phytoplankton species (*Skeletonema costatum*, *Phaeocystis globosa* and *Heterocapsa triquetra*). DMSP and DMSO cell quotas of the three microalgae were measured at three salinities (20, 27, 35) and in three growth phases at salinity 35. DMSP and DMSO cell quotas varied along the growth for all species with an increase of DMSP for *S. costatum* and a decrease of the DMSP to DMSO ratio (DMSP/DMSO) for *P. globosa* and *H. triquetra* in late exponential-stationary phase. We hypothesized that the oxidative stress caused by light and/or nutrients limitation induced the oxidation of DMS or DMSP to DMSO. DMSP cell quotas increased with salinity, mostly in *S. costatum* and *H. triquetra*, for which DMSP is supposed to be an osmoregulator. In *H. triquetra*, DMSO cell quotas stayed constant with increasing salinity. DMSO was near detection limits in *S. costatum* experiments. In *P. globosa*, DMSP and DMSO concentrations increased at low and high salinity. DMSO showed higher increase at low salinity presumably as the result of a salinity-induced oxidative stress which caused DMSP oxidation into DMSO in hyposaline conditions. We concluded that DMSP acts as an osmoregulator for the three studied species and DMSO acts as an antioxidant for *P. globosa* at low salinity. In *P. globosa* and *H. triquetra*, DMSP/DMSO increase with salinity in response to salinity stress.

1. Introduction

Salinity can be one of the major limiting factor for growth and productivity of plants and algae depending on their salt-tolerance (Parida and Das, 2005; García et al., 2012). Salinity stress has many physiological effects on the cell such as ion toxicity, damage of photosynthetic apparatus, decreased growth, lower cell volume, increased respiration, disruption of enzyme activity causing shifts in metabolites, oxidative stress and changes in membrane permeability (Kirst, 1990; Sudhir et al., 2004; García et al., 2012; Lyon et al., 2016). Even the most euryhaline phytoplankton species are affected in their chemical composition (protein, lipid and carbohydrate contents) by low or high salinity levels (García et al., 2012).

The immediate effect of salinity changes on plant cells is rapid water fluxes due to osmotic gradients that lead to volume changes and severe disturbance of the metabolism due to changes in the cellular water potential. Cells respond to these immediate effects by processes of osmotic acclimation to maintain a constant cell turgor. It is done firstly by the

regulation of internal inorganic ions such as K^+, Na^+ and Cl^- ("non compatible" osmolytes), and secondly by the regulation of compatible solutes (osmolytes) such as DMSP, proline and glycine betaine (Stefels, 2000) which are characterized by similar structure (Vairavamurthy et al., 1985). DMSP can be accumulated or released from the cell in case of salinity up- or down-shock respectively (Kirst, 1996; Yang et al., 2011; Niki et al. 2007). Compatible osmolytes are mainly used in case of long term salinity upshock because the concentration of ions needed to counterbalance external hyperosmotic potential may be toxic for the cell (Kirst, 1996).

Effects of salinity stress on the induction of oxidative stress has been observed in plants and algae (Jahnke and White, 2003; Parida and Das, 2005; Liu et al., 2007; Tammam et al., 2011). Under environmental stresses including salinity stress, Calvin cycle activity is reduced causing an inhibition of nicotinamide adenine dinucleotide phosphate ($NADP^+$) regeneration and a consequent over-reduction of the electron transport chain. Under such conditions, excess electrons are transferred to oxygen generating reactive oxygen species (ROS), responsible of an immediate response of plants and algae (Apel and Hirt, 2004; Dring 2005; Tammam et al., 2011; Ahmad, 2014). To detoxify ROS, plants (including algae) possess low-molecular weight antioxidants (ascorbate, glutathione, phenolic compounds, tocopherols), antioxidant enzymes (superoxide dismutase (SOD), ascorbate peroxidase (APX) and catalase (CAT)) and compatible solutes with regulatory roles to alleviate damaging effects (Cavalcanti et al., 2007; Cuin and Shabala, 2007a; Sekmen et al., 2007; Tammam et al., 2011). A greater salinity tolerance and resistance to oxidative damage comes from high levels of antioxidants (Parida and Das, 2005; Tammam et al., 2011). Oxidative stress occurs when the accumulation of ROS exceeds the scavenging capacity of the cell (Apel and Hirt, 2004; Kreslavski et al., 2007; Tammam et al., 2011; Ahmad, 2014). In case of a long term stress, ROS cause damages in photosystems through desoxyribonucleic acid (DNA) mutation, protein denaturation, lipid peroxidation, chlorophyll bleaching and loss of membrane integrity (Leshem et al., 2007; Tammam et al., 2011).

The cellular content of dimethylsulfoniopropionate (DMSPp) and dimethylsulfoxide (DMSOp) in marine microalgae is specific and its regulation linked to their physiological functions. DMSP and DMSO productions are constitutively species-specific (Keller, 1989; Hatton and Wilson, 2007; Caruana and Malin, 2014) and even strain-specific (Shen et al., 2011). Dinophyceae, Prymnesiophyta and Chrysophyta being the major producers (Keller, 1989; Hatton and Wilson, 2007; Caruana and Malin, 2014); and Chlorophyta and diatoms low

producers with a few exceptions (Keller, 1989; Spielmeyer et al., 2011). Several authors have also shown that the intracellular DMSPp to DMSOp ratio (DMSPp/DMSOp) ratio decrease during the growth of *A.carterae* (dinoflagellate) from log phase to stationary phase as a result of an increase of the DMSO intracellular pool due to limiting conditions inducing oxidative stress (Simó et al., 1998; Hatton and Wilson, 2007). Other authors observed a DMSP and DMS increase until late stationary and senescent stages of growth in different taxa (dinoflagellates, prasinophytes, coccolithophorids and diatoms) with a positive correlation with cell density (Zhuang et al., 2011; Liu et al., 2014).

The DMS(P,O) content of algae are also affected by many abiotic and biotic variables, such as salinity, light, temperature, nutrients and growth phase. Several physiological functions have been attributed to DMSP and its derivatives (DMS, acrylate and DMSO). DMSP functions as an osmoregulator (Vairavamurthy et al., 1985), cryoprotectant (Kirst et al., 1991), methyl donor (Kiene et al., 2000), and potentially as a ballast mechanism in aflagellate phytoplankton (Lavoie et al., 2015; 2016). DMSP and its derivatives are grazing deterrent (Wolfe et al., 1996). Intracellular DMSO is exclusively formed by the oxidation of DMSP (or DMS) by ROS (Foote and Peters 1971; Amels et al. 1997; Sunda et al., 2002; Spiese, 2010) and only acts as an osmolyte in cold environments; it is a cryo-osmoregulator (Lee et al., 1999). In addition, these molecules take part of an intracellular antioxidant cascade as ROS scavengers (Sunda et al., 2002). Phytoplankton cells may probably use DMSP and derivatives for different functions, they are not necessarily exclusive (Harada and Kiene, 2011). Indeed, multifunctional osmolytes like DMSP are more likely to be selected by phytoplankton species (Welsh, 2000). For example, DMSP may serve a dual role for salinity stress tolerance in sea-ice diatoms; as compatible solute that lesser inhibits enzyme activity than equimolar concentration of NaCl (Gröne et al., 1991) and as part of the antioxidant cascade (Sunda et al., 2002;Deschaseaux et al., 2014) with DMSO, DMS, acrylate and methane-sulfinic acid.

An increase of DMSP and/or DMS production with salinity has been observed for different micro- and macroalgae species belonging to diatoms (including *Skeletonema costatum*), prymnesiophytes (including *Phaeocystis spp.*) and dinoflagellates (Vairavamurthy et al., 1985 ; Dickson et al., 1986; Karsten et al., 1992; Zhang et al., 1999; Zhuang et al., 2011; Yang et al., 2011; Kettles et al., 2014). The increase is exponential for the Haptophyceae *Phaeocystis sp.* (Stefels, 2000). Field studies have also revealed positive correlations between DMSP or DMS and salinity from the estuarine to the coastal and shelf

environments but with differences in the phytoplankton community with mainly diatoms in estuarine waters and Prymnesiophyceae in coastal and shelf waters (Iverson et al., 1989; Sciare et al., 2002). The accumulation or release of DMSP in response to the extreme environmental salinity gradients encountered by sea-ice diatoms is well documented (Lyon et al., 2016). They can accumulate DMSP in higher concentrations than their low DMSP producers temperate counterparts (Keller et al., 1989). Lyon et al. (2016) observed DMSO elevations and low ROS levels above and below 35-salinity controls supporting the dual role of DMSP.

Oxidation of DMSP (or DMS) by ROS produce DMSO, which seems to be exclusively formed this way. No direct biological pathways for DMSO synthesis are known so far (Kinsey et al., 2016). Therefore, concentrations of DMSO increase under oxidative stress with the increasing of ROS production (Sunda et al., 2002; Kinsey et al., 2016) and the intracellular DMSPp/DMSOp ratio is a good indicator of an oxidative stress (Hatton and Wilson, 2007). In some high DMSP producers, mainly dinoflagellates and prymnesiophytes such as *Phaeocystis spp.* (Keller et al., 1989; Stefels and van Leeuwe, 1998; Steinke et al., 1998; Hatton and Wilson, 2007), cellular DMSP is high enough to better control ROS levels than other antioxidants such as ascorbate and glutathione (Spiese, 2010). DMSO can act, in turn, as an antioxidant against hydroxyl radical but DMSP and DMS are more effective for that reaction because of their higher cellular concentrations with equivalent rate constants (Spiese, 2010; Kinsey et al., 2016). Salinity-induced oxidative stress in hyposaline conditions can lead to the increasing production of antioxidant enzymes and other antioxidant molecules in the macroalgae *Ulva prolifera* (Luo and Liu, 2011). This effect also exists in hypersaline conditions such as those encountered at low tide in the intertidal zone by macro- and microalgae (Rijstenbil, 2005; Liu and Pang, 2010; Luo and Liu, 2011; Kumar et al., 2011; Pancha et al., 2015).

In this study, we investigate cell quotas of DMSP and DMSO in acclimated batch cultures of *Phaeocystis globosa* (*P.globosa*, Prymnesiophyceae), *Skeletonema costatum* (*S. costatum*, Coscinodiscophyceae) and *Heterocapsa triquetra* (*H. triquetra*, Dinophyceae) in different growth phases and at different salinities. We test the hypothesis for these species that the intracellular DMSPp/DMSOp ratio is an indicator of a salinity stress potentially inducing an oxidative stress (Simó and Vila-Costa, 2006; Hatton and Wilson, 2007).

2. Materials and methods

2.1 Phytoplankton cultures

Strains of *S. costatum* (strain isolated from the Belgian Coastal Zone), *H. triquetra* (strain RCC4800 from Roscoff Culture Collection) and *P. globosa* (strain RCC1719 from Roscoff Culture Collection) were chosen for the important biomass they can reach in the North Sea (Rousseau et al., 1990) and for their high DMSP and DMSO production (Keller et al., 1989; Hatton and Wilson, 2007; Caruana et al., 2012).

S. costatum is a worldwide coastal diatom often dominant in spring blooms (Yang et al., 2011). Among diatoms, this species is known as a high DMSP and DMSO producer (see Table 2) (Keller et al., 1989; Hatton and Wilson, 2007; Yang et al., 2011). *S. costatum* is a coastal euryhaline species but its optimum salinity is around 25 (Brand, 1984).

H. triquetra is one of the most common coastal and estuarine dinoflagellate of the world. This species forms regular blooms in the German Bight of the North Sea (Litaker et al., 2002; Hoppenrath, 2004). It is a mesohaline species with an optimum salinity of 30 (Baek et al., 2011). As most dinoflagellates, this species is known for its high DMSP concentration (see Table 2) considered as an osmolyte and for its high DMSP-lyase activity (DLA) that cleaves DMSP into DMS and acrylate (Caruana et al., 2012; Caruana and Malin, 2014).

P. globosa is a prymnesiophyte known to form Harmful Algal Blooms (HAB) in temperate and tropical waters. It has a complex polymorphic life cycle in which flagellate and colonial cells express different properties but both produce high DMSP and DMSO concentrations (see Table 2) with high DLA (Liss et al., 1994; Stefels and Dijkhuizen, 1996; Schoemann et al., 2005; Hatton and Wilson, 2007). It is an euryhaline species with an optimum salinity of 29 (Peperzak, 2002).

2.2 Culture growth

All batch cultures were grown in a natural seawater F/2 medium and maintained at 12°C (*S. costatum* and *P. globosa*) and 15°C (*H. triquetra*) on a 12:12 dark:light cycle, at a photon flux density of 100 µmol quanta m^{-2} s^{-1} supplied by cool-white fluorescent lights. Antibiotics (Penicilin-G and Streptomycin) were added to the cultures to prevent bacterial growth. Different salinities medium (27, 20) were prepared by dilution of salinity 35 seawater with Milli-Q water. Gradual acclimation of the strains from salinity 35 took two weeks to

reach salinity 27 (i.e. at least 15 generations for *P. globosa* and *S. costatum* and 5 generations for *H. triquetra*) and four weeks to reach salinity 20 (i.e. at least 30 generations for *P. globosa* and *S. costatum* and 10 generations for *H. triquetra*). Cultures were inoculated with exponential phase acclimated cells and their growth was monitored daily by measuring cell density and Chl*a*-relative *in vivo* fluorescence (with a Turner Aquafluor handheld fluorometer).

2.3 Sampling

Samples were collected in triplicate at early, mid and late exponential growth phase for the three species at salinity 35 (the reference salinity) and only at mid-exponential for salinities 27 and 20. Triplicates come from the same cultures and are only representative of the variability in the culture. After collection, samples were stored for later analysis of dissolved and total DMS(P,O) (DMS(P,O)d and DMS(P,O)t) using established gas chromatographic (GC) techniques (See section DMS(P,O) analysis). Samples were microwaved until boiling after filtration in order to inactivate DMSP-lyase (Kinsey and Kieber, 2016), and avoid a possible bias of DMSP measurements (del Valle et al., 2011). Samples of bulk water and filtered were then preserved with the addition of H_2SO_4 (5 µl of 50% H_2SO_4 per millilitre of sample), as recommended by Stefels (2009). This allows to preserve the DMSP(O) content and removes DMS originally present in the sample. DMS(P,O)d analyses were done on filtered culture samples and DMS(P,O)t on bulk culture samples. Particulate DMSP or DMSO (DMS(P,O)p) concentration was calculated by subtracting DMS(P,O)d concentration on DMS(P,O)t concentration. We adopted the small volume gravity drip filtration procedure recommended by Kiene and Slezak (2006). Gentle filtration of maximum 20 ml was used, only the maximum first 10 ml of filtrate were collected.

2.4 DMS(P,O) analysis

DMS(P,O)d and DMS(P,O)t in diluted culture samples (dilution sufficient to get concentrations below 200nmol.L^{-1}) were analysed by GC coupled with a purge-and-trap after hydrolysis to DMS by 2 pellets of NaOH in a 10ml sample (Stefels, 2009). The DMS was purged with He during 20 min and trapped on a loop plunged in liquid N_2. The loop was then immersed into boiling water and the gas driven to the chromatographic column. We used an Agilent 7890B GC equipped with a Flame Ionization Detector (FID) and a Flame Photomultiplier Detector (FPD). The carrier gas (He, 2mL min^{-1}) was split between the two

detectors. Only the values derived from the FPD were used. The FPD was kept at 350°C with a H_2 flow of 72mL min^{-1}, a synthetic air flow of 72mL min^{-1} and a makeup (N_2) flow of 20mL min^{-1}. The oven was set at 60°C. DMSP for calibration was obtained from Research Plus. From this solid standard, a primary stock solution with a concentration of 2.23 mmol L^{-1} and working solutions with concentrations in the range of 10^{-7} or 10^{-8} mol L^{-1} were prepared successively by dilution in Milli-Q water. The same procedure was applied for DMSO from a 99.9% pure stock solution (Merck) to obtain working solutions of 10^{-7}–10^{-8} mol L^{-1} respectively. Six dilutions of the working solution in 10ml of Milli-Q water were realized in triplicate to obtain standards with concentrations from 0.5 to 200 nmol L^{-1} in 20ml serum vials sealed with gas tight PTFE coated silicone septa. Calibration curves were realized weekly for DMSP and DMSO by fitting a quadratic curve. Calibration was checked to be stable during a week, by comparing curves over successive weeks. The coefficient of variation was ± 10% between standards. NaOH pellets were added the day before analysis in standards and samples (at least 12h prior to analysis). Concentrations of total and dissolved DMSO (respectively DMSOt and DMSOd) were analyzed after total and dissolved DMSP (respectively DMSPt et DMSPd) in the same culture samples after acidification and reduction of DMSO into DMS using the $TiCl_3$ method (Kiene and Gerard, 1994). DMSP samples were analysed in triplicate but, due to analytical problems, part of DMSO measurements failed and were removed from the result analysis.

2.5 Chlorophyll-a and cell counts

Samples for Chl*a* measurement (10 to 50 mL) were gently filtered onto glass-fiber filters (GF/F) and extracted in 90% acetone, before applying the fluorometric method of Holm-Hansen et al. (1965). We measured Chl*a* fluorescence before (430nm in excitation and 664nm in emission) and after acidification with 100µL HCl 0.1N of a 3mL sample (410nm in excitation and 670nm in emission).

A few Chl*a* samples could have been damaged during storage as we measured high concentrations of Pheophytin *a* compared to Chl*a*. For these data (evidenced by a * in the Tables and Figures), we used a mean ratio of active Chl*a* estimated from the other samples of this study to retrieve consistent Chl*a* values.

Cell density was measured in triplicate by inverted microscopy using the Utermöhl method (Hasle, 1978) following fixation with 1% lugol-glutaraldehyde. For *P. globosa* samples, colonies were broken by agitation prior to cell counting. More than 400 cells were

counted on each sample to reach an error of 10% at a 95% level of confidence (Lund et al, 1958).

2.6 Nutrients

Nutrients were analysed by colorimetric methods with a spectrophotometer (UV/Vis Lambda 650S Perkin Elmer). Silicate and phosphate samples were prepared by using the molybdenum blue method (Koroleff, 1983b-c) and measured at 830nm and 885nm respectively. Nitrate plus nitrite samples were prepared by the reduction of nitrate by cadmium and then diazotization (Greiss reaction; Grasshoff, 1983) and measured at 540nm.

2.7 Growth rate

The specific growth rate (μ) was calculated from cell counts using the equation:

$$\mu = (\ln N_2 - \ln N_1)/(t_2 - t_1) \qquad \text{(Guillard, 1973)}$$

where $N_{1,2}$ are the cell densities (cell.L^{-1}) measured on cultures on days $t_{1,2}$ during growth.

2.8 Statistical analyses

We performed statistical analyses on Chl*a* contents for each species to detect any significant difference between salinity treatments and growth phases. Homoscedasticity has been assessed by Hartley tests. As variances were non-homogeneous for all species and both parameters (except for Chl*a* between growth phases of *P. globosa*), we performed non-parametric tests (Kruskal-Wallis).

3. Results

3.1 Growth curves and rates

Cultures of *S. costatum*, *P. globosa* and *H. triquetra* presented sigmoid-shaped growth curves in term of Chl*a* and cell density in the reference conditions (salinity 35; Fig.1). Growth curve of *S. costatum* comprised a latent phase of 4-5 days, an exponential phase from day 6 to 11 and a very short stationary phase (1 or 2 days) before senescence (Fig. 1a, d). *S. costatum* reached its maximum cell density and Chl*a* between days 11 and 14 (~9.10^8 cell/L, ~200 µg/L) and its maximal specific growth rate (SGR) on day 6-7 during exponential growth (1.29/day). Growth curve of *P. globosa* was identical to that of *S. costatum* in term of timing but reached a lower maximal Chl*a* (164 µg/L) value on day 11 associated to a lower maximum cell density (7.10^8 cell/L) and a lower SGR on day 6-7 (1.1/day) (Fig. 1b, e).

Growth curve of *H. triquetra* differs from those of other species only by an earlier exponential phase starting on day 5 and a longer stationary phase (from day 11 to 14) (Fig. 1c, f). *H. triquetra* reached the lowest maximum cell density (5.10^7 cell/L) with maximum cell density is 16-20 fold lower than both other cultures (Fig. 1f) but similar Chl*a* concentrations (Fig. 1c).

In all salinity treatments, growth curves were sigmoid-shaped for the three species (Fig. 2). Growth curves are shown in *in vivo* fluorescence as other growth parameters had only been measured at mid-exponential growth for salinities 27 and 20. Growth curves in Chl*a* (Fig. 1a, b, c) and *in vivo* fluorescence (Fig. 2) are similar for salinity 35. Maximum *in vivo* fluorescence and the timing of the different phases varied with salinity for all species. Growth curves of *S. costatum* had similar patterns with only 20% difference between maximum *in vivo* fluorescence. Growth curves of *P. globosa* were separated from the beginning of exponential phase with an increase of 46% in maximum *in vivo* fluorescence from salinity 20 to 35. Growth curves of *H. triquetra* were also separated from the beginning of exponential phase. For the three species, the time taken for reaching stationary phase decreased with salinity. Cellular Chl*a* content decreased during growth but non parametric statistical analyses (Kruskal-Wallis) showed no significant difference between salinity treatments for cellular Chl*a* content of *S. costatum*, *P. globosa* and *H. triquetra* (Table 1).

The growth of *S. costatum* was the least affected by the variation of salinity between 20 and 35 with only a 12.5% difference (not shown) between growth rates at salinity 20 (0.7 day-1) and 35 (0.8 day-1) and non-significant differences in cellular Chl*a* between salinity treatments. Among the three species studied, *S. costatum* was also the species reaching the highest cell densities at all salinities and growth phases. *P. globosa* and *S. costatum* had very close growth rates (0.85 and 0.79 day^{-1} respectively at salinity 35) but with greater variation between salinities for *P. globosa*. For *P. globosa*, the greatest growth rate was computed at salinity 27 reflecting its supposed optimum at salinity 29 (Peperzak, 2002). Similarly, Peperzak (2002) observed a decline of the *P. globosa* growth rate from a stable 1/day in the 25 to 35 salinity range to 0.8/day at salinity 20 and to cell death at salinity 15. Maximum cell density was also more variable between salinities (maximum at salinity 27) and growth phases (maximum on day 8) for *P. globosa* cultures than in *S. costatum*. *H. triquetra* had lower growth rates and in vivo fluorescence than the other two species. In our study, its growth rate did not vary much with salinity (0.2-0.3) but fluorescence was minimal at salinity 20 (Fig. 2) in accordance with its supposed optimum at salinity 30 (Baek et al., 2011).

The Chl*a*/cell ratios were highly variable between species and salinity treatments (Table 1) and characterized by a minimal value at salinity 27 for both *H. triquetra* and of *S. costatum* cultures. For *P. globosa*, an abnormally low Chl*a* (handling problem with samples) measured at salinity 35 decreased the Chl*a*/cell ratio below value measured at salinity 27 but we expected, as for the two others species, a minimal value at salinity 27. For all species, salinity 27 is the closest from their optimal salinity. As a result of the variation of Chl*a*/cell ratios with time and salinity, pattern of DMS(P,O)p/cell will differ from patterns of DMS(P,O)p/Chl*a* as a function of time or salinity.

3.2 DMS(P,O) cell quotas at mid-exponential growth

Among the three species studied, the highest cell quotas of DMSP (853±380 fmol/cell and 153±70 mmolS/gChla) and DMSO (47.7±18.5 fmol/cell, and 8.6±3.4 mmolS/gChla respectively) at mid-exponential growth for salinity 35 was found in *H. triquetra* (Table 2). *S. costatum* contained the lowest DMSPp and DMSOp cell quotas. DMSPp/DMSOp ratios were comprised between 57±72 (*P. globosa*) and 17.9±5.7 (*H. triquetra*) (not computed for *S. costatum* as DMSOp concentration was near the limit of detection of the GC and considered as 0) (Table 2).

3.3 DMS(P,O)p cell quotas at different growth stages

In *S. costatum* culture (salinity 35), DMSPp cell quotas were stable during early-mid exponential phase (~3.5 fmol/cell) and increased by 40% during late exponential phase (Fig. 3a). The same pattern is found for DMSPp per Chl*a* units (Fig. 3 d). Among the 3 species, *S. costatum* had the lowest cell quotas of DMSP during all growth phase.

In *P. globosa*, DMSPp per cell decreased along the growth (Fig. 3b) then DMSPp/Chl*a* was characterized by a minimal value at mid-exponential growth (Fig. 3e). DMSOp showed similar pattern (Fig. 3b, c). However, considering the high uncertainty no significant trend can be evidenced along *P. globosa* growth.

In *H. triquetra* cultures, DMSPp cell content decreased along the growth (Fig. 3c). The evolution is less clear for DMSPp/Chl*a* characterized by a maximal value at mid-exponential phase (Fig. 3f). DMSOp/cell showed the opposite evolution and increased with time in the *H. triquetra* culture (Fig. 3c). This trend is similar when considering DMSOp/Chl*a* but with higher uncertainties (Fig. 3f).

As a result, DMSPp/DMSOp ratios were maximal in mid-exponential phase of *P. globosa* and decreased along the growth for *H. triquetra* cultures but considering the high uncertainties no significant trend can be shown during growth experiment (Fig. 4).

3.4 DMS(P,O)p cell quotas at different salinities

In *S. costatum* cultures, DMSPp concentrations increased with increasing salinity in per cell or Chl*a* (Fig. 5a, d). In *P. globosa*, DMSPp and DMSOp were characterized by lowest values at salinity 27 and increased at salinities 35 and 20 (Fig. 5b, e). In *H. triquetra*, DMSPp increased with the increasing salinity then DMSOp stayed relatively constant whatever the salinity. *H. triquetra* was the highest producer of both DMSPp and DMSOp in all salinities (Fig. 5 c, f).

The average DMSPp/DMSOp ratio increased with salinity (Fig. 6). However, the high uncertainties do not permit to significantly differentiate values computed at salinities 27 and 35 for *P. globosa*.

4 Discussion and conclusion

4.1 DMS(P,O)p cell quotas at different growth stages

In *P. globosa* and *H. triquetra* culture, while DMSPp concentration decreased (or stayed relatively constant), DMSOp concentrations increased from mid- to late-exponential growth phase inducing a decrease of the DMSPp/DMSOp ratio (Fig. 4). In the late exponential phase of *P. globosa* and *H. triquetra* which possess DLA, DMSPp of senescent cells was quickly cleaved into DMS and then oxidized into DMSO whereas only DMSP can be oxidized into DMSO in DMSP-producing diatoms which do not possess DLA (Harada and Kiene, 2011). DMSP-lyase may be used to regulate DMSP concentration either for the greater efficiency of its by-products (DMS and acrylate) as antioxidants (Sunda et al., 2002) and grazing deterrents, or as a disposal of the over-accumulated DMSP for osmotic regulation and overflow mechanism (Stefels and van Leeuwe, 1998). Oxidative stress has been shown to upregulate DLA, supporting its role in the antioxidant cascade (Sunda et al., 2007). That would explain the coupling between DMSP decrease and DMSO increase in our experiments.

For S. *costatum*, an increase of DMSPp was observed along the growth in our experiment with no effect on DMSOp concentations. For species lacking DLA like *S.costatum*, DMSOp production could only be result from the direct oxidation of DMSPp.

This reaction seems to be insufficient in our experiment to induce an increase of DMSO cell content that was near the detection limit of the GC during the whole experiment.

Nutrient limitation can induce oxidative stress and increases the production of ROS (Berman-Frank et al., 2004). Sigaud-Kutner et al. (2002) observed different antioxidant strategies in the three studied species according to the limiting factors leading to senescence. In case of autoshading (*Tetraselmis gracilis*) due to a high cell density, pigment content increased, ROS production did not increased and SOD activity was not induced. In contrast, cultures of the diatom *Minutocellus polymorphus* are nutrient-limited enhancing ROS production and SOD activity. Finally, the dinoflagellate *Lingulodinium polyedrum* exhibited both antioxidant strategies. An oxidative stress induced by a limiting factor (light or nutrients) could also have caused the stationary growth phase and the increasing production of ROS and DMSO in our experiments.

Our results are in accordance to previous results that showed that diatoms (and *S. costatum* in particular) can increase their DMSP content under nutrient limitation (Sunda at al., 2002; Bucciarelli and Sunda, 2003; Sunda et al., 2007). In the case of *S. costatum*, this limiting factor could have been phosphate with only 0.9µmol/L left at day 11 (not shown). On the opposite, for species with high DMSP cellular content and the presence of DLA, increase of DMSP is not still measurable as for the coccolithophore *Emiliania huxleyi* (Sunda et al., 2007).

For *S. costatum, P. globosa and H. triquetra*, DMSPp cell quotas at salinity 35 and mid-exponential growth were similar to some previous studies (Keller et al., 1989; Keller et al., 1999; Caruana et al., 2012). DMSOp cell quotas were also similar to those measured by Hatton and Wilson (2007) for the three species (Table 2); the only study comparable for these species. DMSPp/DMSOp ratios seem highly variable between species in a same taxon and between studies for the same species but ratios computed in the present study were in the range of previously measured ratios (Table 2).

4.2 DMS(P,O)p cell quotas at different salinities

The three species showed different trends with salinity. In *H. triquetra* and *S. costatum* cultures, DMSPp was correlated with salinity. For those species DMSOp concentration stayed relatively constant (or below the detection limit for *S. costatum*) whatever the salinity. In *P. globosa*, DMSPp and DMSOp evolved in parallel with minimal

DMSPp and DMSOp values at salinity 27 near the salinity optimum. *P. globosa* has probably a higher DLA than *H. triquetra* (low among dinoflagellates, Caruana and Malin, 2014) and thus a higher part of DMS coming from the cleavage of DMSPp which can always be oxidized into DMSOp. In *H. triquetra*, DMSPp seemed to be cleaved to DMS intracellularly in hyposaline conditions as DMSPp decrease was neither accompanied by an increase in DMSOp nor by an increase in DMSPd (not shown). This result differs from those of Niki et al. (2007) who observed an enhanced DMSPd production in a salinity down-shock. It is possible that the short term reaction differs from the long-term reaction of an acclimated culture as excretion of DMSP could be done faster than the cleavage of DMSP in order to downregulate DMSP in the cell in case of rapid salinity downshock.

DMSPp seems to act as an osmoregulator in *S. costatum*, *H. triquetra* and *P. globosa* (for salinity > 27) with an increase of DMSPp with salinity. This had been previously observed by Zhuang et al. (2011) for dinoflagellates; by Yang et al. (2011) for *S. costatum*; and by Zhang et al. (1999) and van Bergeijk et al. (2003) for other diatoms. *S. costatum* is euryhaline and should than have evolved with different strategies to cope with long term salinity variations (antioxidant enzymes and osmoregulators). The strain we used, originated from the Belgian Coastal Zone (BCZ) is besides, dominant in Elbe and Scheldt estuaries (Muylaert and Sabbe, 1999). Brand (1984) reported an optimum salinity of 25 for this species but with a strain originating from the Peruvian upwelling. DMSP may also be an osmoregulator for *P. globosa* but with a more visible effect only for higher salinities (Stefels, 2000). Otherwise, other molecules could act as osmoregulators instead of DMSP in *P. globosa*. Niki et al. (2007) observed a DMSP excretion by *H. triquetra* cells quickly followed by its cleavage in salinity down-shock conditions. The same effect seems to have occured in our study, even with a decrease of DMSPd/DMSPp with the increase of salinity.

DMSOp seems to act as an antioxidant in *P. globosa* only to cope with the increase of ROS in hyposaline conditions. An oxidative stress leading to an increase of antioxidant enzymes (catalase, superoxide dismutase and glutathione reductase) and of other antioxidant molecules (ascorbate, glutathione and beta-carotenoid) had been reported in hyposaline conditions for the macroalga *Ulva prolifera* (Luo and Liu, 2011). This reflects the strategies to cope with salinity variations for a wide salinity range euryhaline species (growing in the 10 to 35 salinity range; Balzano et al., 2011) and the sensibility of *P. globosa* to low salinities (cell death at salinity 15; Peperzak, 2002) in comparison with another euryhaline species (*S. costatum*) and a meso- stenohaline species (*H. triquetra*).

In accordance, the DMSPp/DMSOp ratio (or only DMSPp concentration for *S. costatum* for which DMSOp was still near detection limits) increased with salinity for all species. Among the three DMS(P,O) producing species studied here, different patterns concerning the use of these molecules at different salinities and growth phase were observed. These patterns seem to depend on the strategies they have developed to adapt to their natural habitats. In particular, we assume that DMSP acts as an osmoregulator in *S.costatum* and *H. triquetra* and for *P. globosa* for salinities higher than 27, and DMSO acts as an antioxidant in *P. globosa* in hyposaline conditions. As salinity also induced an effect on growth parameters (cell and Chl*a*), we should use similar units when comparing salinity effect on DMSP and DMSO cell quotas between different studies.

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Table 1 : Cell Chlorophyll *a* content (pg/cell, mean ± standard deviation, * for Chl*a* estimated based on mean ratio of active Chl*a* from others samples) of the batch cultures during exponential growth of *Skeletonema costatum*, *Phaeocystis globosa* and *Heterocapsa triquetra* at salinity 35, 27 and 20.

Salinity	***S. costatum***	***P. globosa***	***H. triquetra***
35			
Day 5-6	0.37±0.13	0.52±0.12	11.2±2.54
Day 8	0.3*±0.17	0.24±0.01	5.56±2.91
Day 11	0.21*±0.1	0.22±0.04	6.99±6.42
Mean	0.29±0.13	0.33±0.08	7.92±3.96
27	0.13*±0.04	0.46±0.2	3.73±2.07
20	0.27±0.08	0.53±0.24	4.17±2.23

Table 2 : Dimethylsulfoniopropionate (DMSP) and dimethylsulfoxide (DMSO) cell quotas of *Skeletonema costatum*, *Phaeocystis globosa* and *Heterocapsa triquetra*. Data from the present study are in bold characters (mean ± standard deviation). References: [1]Keller et al., 1989; [2]Keller et al. (1999); [3]Hatton and Wilson (2007) (values for *H. triquetra* are means of several dinoflagellate species); [4]Yang et al. (2011); [5]Spiese et al. (2009) (values are means of several species of the same taxon); [6]Niki et al. (2000), [7]Stefels et al. (2007) (values are means of several species of the same taxon), [8]Bucciarelli et al. (2013) (measured on *T.oceanica*), [9]Simo et al. (1998) (measured on North Sea waters dominated by *Phaeocystis sp*.), [10]Simo et al. (1998) (measured on North Sea waters dominated by heterotrophic dinoflagellates).[11]Sunda et al. (2007).

Species name	**DMSP/ cell (fmol/ cell)**	**DMSO/ cell (fmol/ cell)**	**DMSP/ Chl*a* (mmolS/ gChl*a*)**	**DMSO/ Chl*a* (mmolS/ gChl*a*)**	**DMSP/ DMSO**
S. costatum	**3.5±1.4** 3.7[1] 1.5[2] 0.1[3] 0.35[4]	**N.D.** 0.02[3]	**11.8±6.7** 48 1.5-2.8[9] 5.5-10.1[11]	**N.D.** 0.40-0.46[8]	**N.D.** 12.2[3] 3.2[5] 3.7[8]
P. globosa	**17.7±4.5** 17.1[1] 1.1[3]	**0.31±0.41** 0.26[3]	**73.3±24.2** 52[8] 25.6[9]	**1.3±1.8** 1.2[9]	**57±72** 7.5[3] 306.3[5] 21.2[9]
H. triquetra	**853±380** 719[5] 127.3[3] 600[7]	**47.7±18.5** 35.7[3]	**153±70** 250[5] 111[8] 3.9[10]	**8.6±3.4** 2.9[10]	**17.9±5.7** 34.4[5] 6.9[3] 1.33[10]

Figure 1 : Growth curves of the batch cultures of *Skeletonema costatum*, *Phaeocystis globosa* and *Heterocapsa triquetra* at salinity 35 expressed as (a, b, c) *in vitro* (µg/L) Chlorophyll *a* (Chl*a*) fluorescence and specific growth rate (SGR) (/d) and (d, e, f) cell density (10^6 cell/L) (mean ± standard deviation).

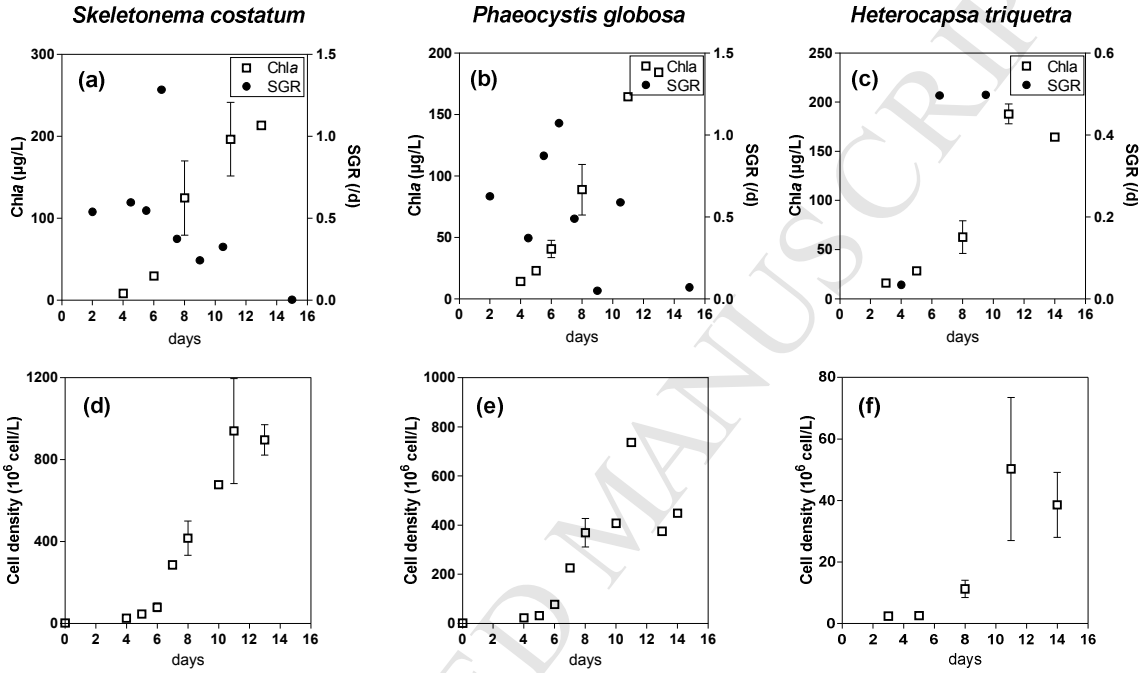


Figure 2 : Growth curves of the batch cultures of *Skeletonema costatum*, *Phaeocystis globosa* and *Heterocapsa triquetra* at 3 salinities (20, 27, 35) expressed as *in vivo* fluorescence *vs* time.

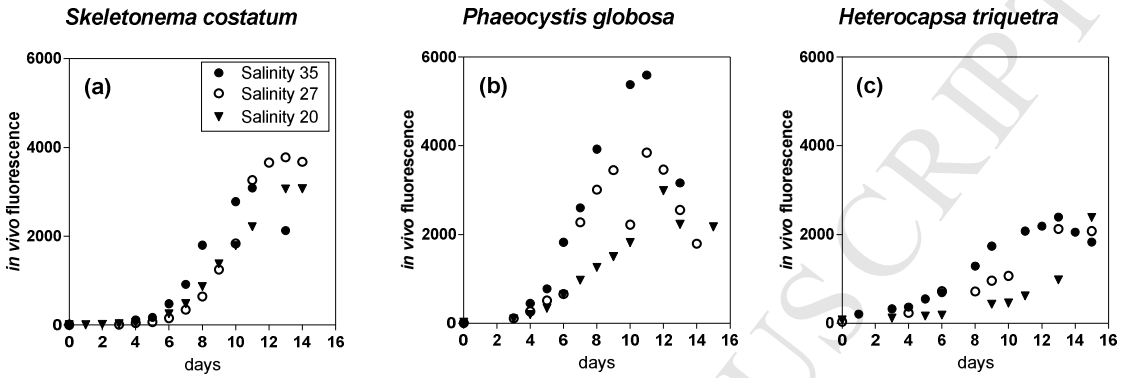


Figure 3 : Dimethylsulfoniopropionate (DMSP) and dimethylsulfoxide (DMSO) cell quotas of *Skeletonema costatum*, *Phaeocystis globosa* and *Heterocapsa triquetra* at salinity 35 expressed as (a-c) per cell (fmol/cell) and (d-f) per Chlorophyll *a* (mmol/gChl*a*) (mean ± standard deviation).

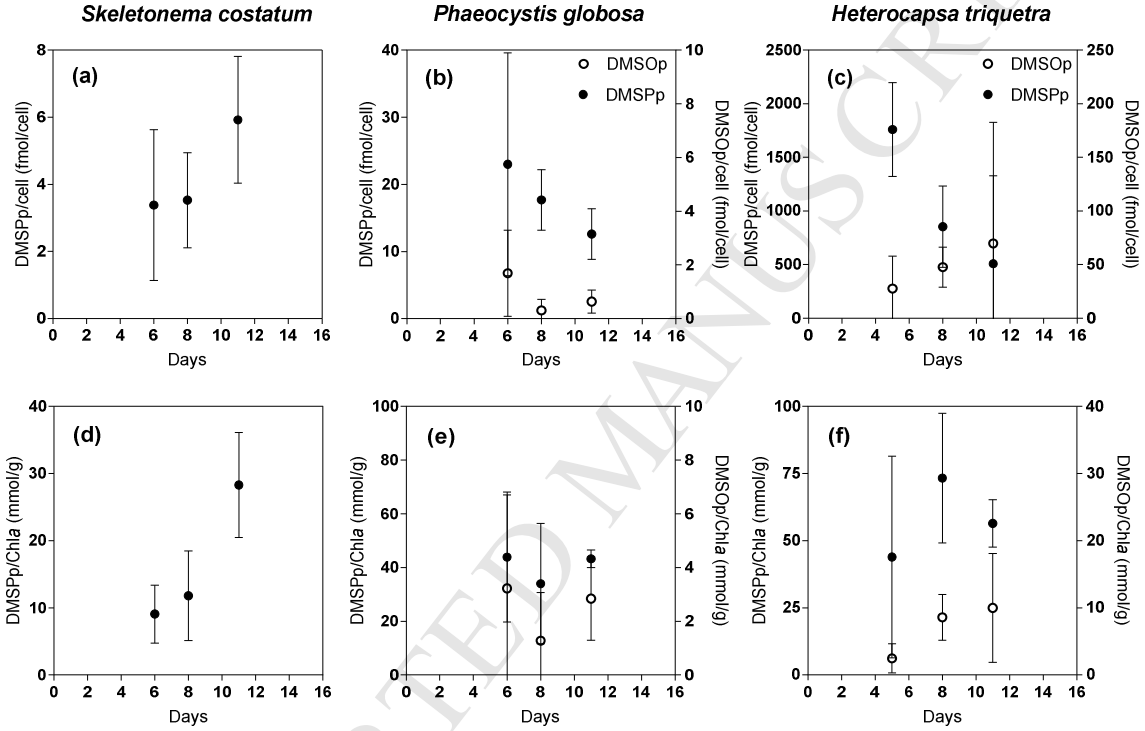


Figure 4: Intracellular dimethylsulfoniopropionate to dimethylsulfoxide ratios (DMSPp/DMSOp) of *Phaeocystis globosa* and *Heterocapsa triquetra* at salinity 35 during exponential growth phase (mean ± standard deviation).

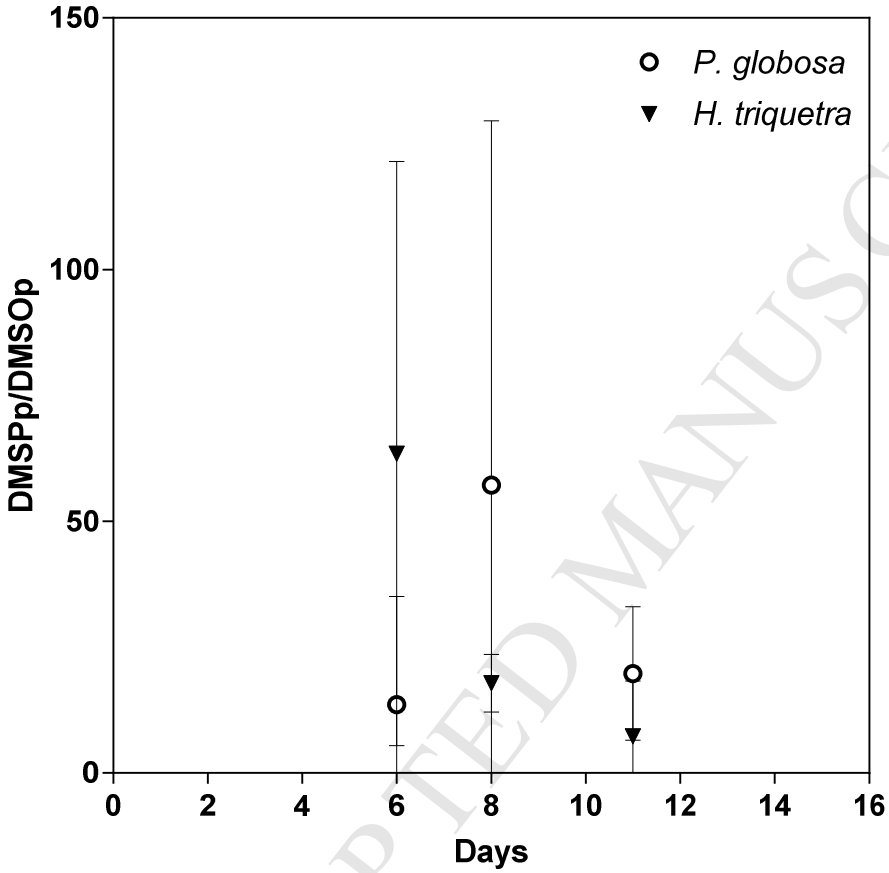


Figure 5 : Dimethylsulfoniopropionate (DMSP) and dimethylsulfoxide (DMSO) cell quotas of *Skeletonema costatum*, *Phaeocystis globosa* and *Heterocapsa triquetra* during mid-exponential growth in 3 salinities (20, 27, 35) expressed as (a-c) per cell (fmol/cell) and (d-f) per Chlorophyll *a* (mmol/gChl*a*) (mean ± standard deviation). Dotted lines indicate optimum salinity for each species.

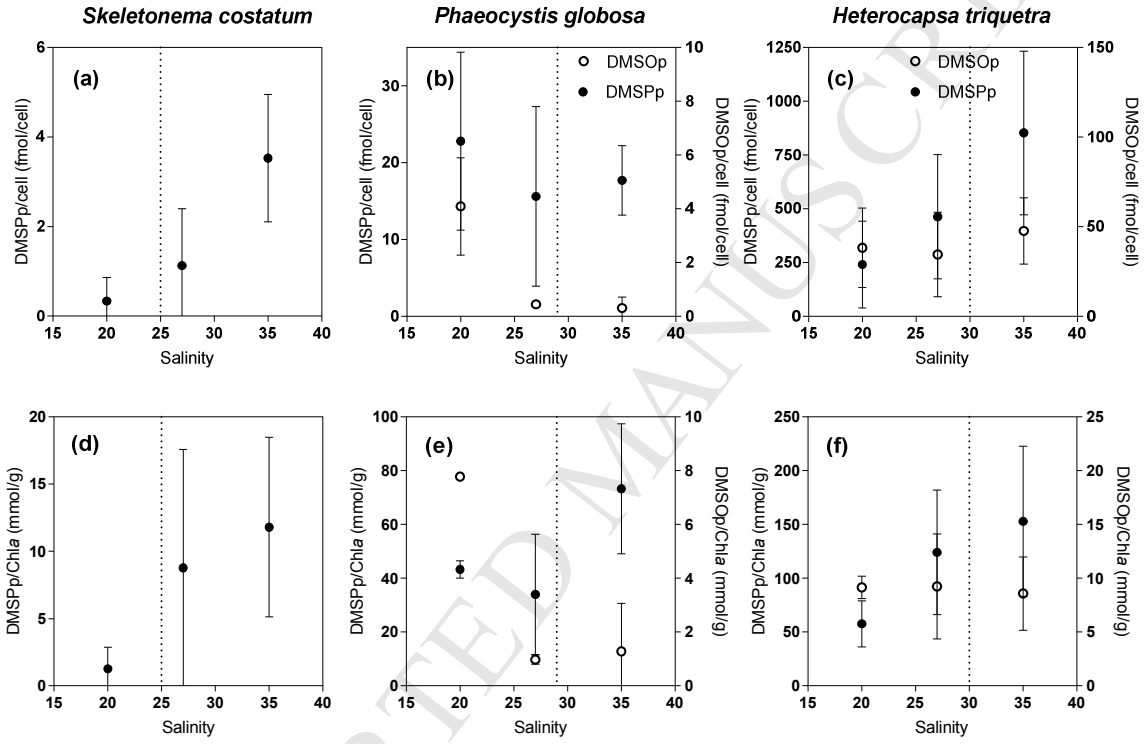
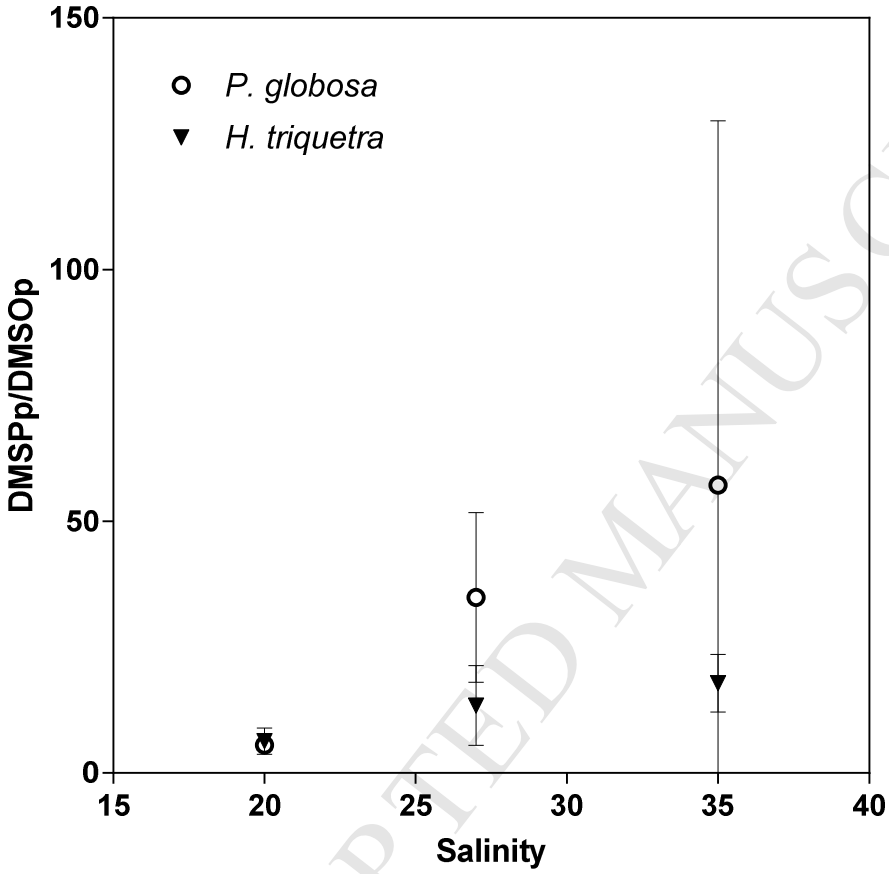


Figure 6: Intracellular dimethylsulfoniopropionate to dimethylsulfoxide ratios (DMSPp/DMSOp) of *Phaeocystis globosa* and *Heterocapsa triquetra* at 3 salinities (20, 27, 35) during mid-exponential growth phase (mean ± standard deviation).



Highlights

- DMSOp increase at the end of exponential growth phase for *Heterocapsa triquetra* and *Phaeocystis globosa*.
- DMSP is upregulated by increasing salinity for the 3 species.
- Salinity-induced oxidative stress causes DMSP oxidation into DMSO in *Phaeocystis globosa*.
- DMSP/DMSO decrease in case of oxidative stress (late exponential phase and salinity stress).