Uptake of dimethylsulphoniopropionate (DMSP) reduces free reactive oxygen species (ROS) during late exponential growth in the diatom *Thalassiosira weissflogii* grown under three salinities

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Abstract

Dimethylsulphoniopropionate (DMSP) is one of the most abundant and widespread organic sulfur molecules in the marine environment and has substantial physiological and ecological importance, from subcellular to global scales. Despite its diverse range of implications in the environment, little understanding of the physiological role of DMSP in the cell exists. Here, we report the physiological response of a non-DMSP-producing diatom *Thalassiosira weissflogii* grown at different salinities (15, 35 and 55 ppt) in the presence and absence of DMSP. Hypersaline conditions (55 ppt) negatively affected growth rate and hyposaline conditions (15 ppt) caused an increase in cell volume, yet no effect was observed on the photophysiological state of the algae, demonstrating a broad salinity tolerance in *T. weissflogii*. Addition of DMSP and subsequent uptake by *T. weissflogii* had no effect on the salinity-induced symptoms. Importantly, by using a non-DMSP producing diatom, we observed some of the first direct evidence of the intracellular role of DMSP as an antioxidant through the quenching of damaging reactive oxygen species (ROS), which based on its pattern, was likely due to the growth phase of the culture. This study confirms the utility of *T. weissflogii* as a model organism for DMSP-related physiological studies, with results revealing that DMSP accumulation reduces growth-related reactive oxygen in *T. weissflogii*.

Key words

Osmoregulation, oxidative stress, diatom, biogenic sulfur

Introduction

Dimethylsulphoniopropionate (DMSP) is a tertiary sulfonium compound that is abundant and widespread in marine ecosystems (Keller 1989; Kiene et al. 2000). Many phytoplankton in the marine environment produce DMSP in mass quantities, with global production reaching an estimated one billion tonnes per year (Curson et al. 2011; Johnston et al. 2016). In certain species of phytoplankton, DMSP has been reported to comprise 1 to 20% of the total cellular carbon (Matrai and Keller 1994; Sheehan and Petrou 2020; Matrai et al. 1995). Despite its ubiquity in nature, the unifying reason for the production and/or use of DMSP has yet to be confirmed.

The production of DMSP by marine phytoplankton has been shown to vary in response to a broad range of environmental factors (Stefels 2000). Among the multitude of physiological roles in marine systems, osmoregulation in response to changes in salinity is a widely attributed functional role of DMSP in microalgae (Stefels 2000; Welsh 2000; McParland et al. 2020). As a zwitterion, DMSP cannot cross cell membranes readily and therefore accumulates in the cell, making it a potential compatible solute in phytoplankton when exposed to osmotic stress. In high-DMSP producers, intracellular DMSP concentrations increase to alleviate hypersaline stress (Stefels 2000; McParland et al. 2020) with a recent study demonstrating that in high-DMSP producers, DMSP is likely to work exclusively as a compatible solute (McParland et al. 2020).

The plurality of factors that affect DMSP production suggests that DMSP, at least when in low or moderate concentrations, might not be directly related with a singular functional role in the cell (Stefels 2000; Petrou and Nielsen 2018; McParland et al. 2020). Instead, in addition to its suggested role as a compatible solute, DMSP may play a central role in response to general cellular stress via its demonstrated capacity to react freely with reactive oxygen species (ROS) (Sunda et al. 2002). The antioxidant theory has spawned broad interest in exploring the links between DMSP and antioxidant function in DMSP producers (Deschaseaux et al. 2014; Darroch et al. 2015; Gardner et al. 2016; Gardner et al. 2017a; Gardner et al. 2017b; Deschaseaux et al. 2019) with much of this work having been focussed on the microalgal symbionts of corals. In all cases, however, the presumed effect of

DMSP on ROS in the cell is formed from indirect evidence (purely correlative), showing a change in DMSP or dimethylsulfoxide (DMSO: the oxidation product after reacting with ROS) production with changes in antioxidant activity or ROS, under environmentally stressful conditions. The correlative nature of these results is due in large part to the inherent constraint imposed by using DMSP-producing organisms to elucidate the cellular function of DMSP. The absence of a DMSP synthesis inhibitor means that none of these studies were able to include a non-DMSP control, a methodological limitation that partly explains the impediment in developing a theoretical framework that can unequivocally explain the role of DMSP in the cell. As such, with the absence of a negative control (absence of DMSP in the control population), to our knowledge, no study to date has shown direct evidence that links intracellular DMSP concentrations with ROS quenching.

Recently, we proposed that using non-DMSP producing organisms that readily take up DMSP from the environment could overcome this experimental limitation (Petrou and Nielsen 2018). To date, only few studies have measured uptake of DMSP by non-DMSP-producing species (Yoch 2002; Vila-Costa et al 2006; Spielmeyer et al. 2011; Lavoie et al 2018; Petrou and Nielsen 2018), and consequently, little is known of the importance of such mechanism in the environment, or even whether organisms that take up DMSP from the environment use it in the same way as those that synthesise it. Nevertheless, understanding why and how DMSP is utilised by non-producers may be beneficial for investigating the more general role for DMSP in cell physiology and may additionally provide pointers to why some species produce DMSP in the first place. In this study, we use the diatom *Thalassiosira weissflogii* as a candidate non-DMSP producing alga. When tested for DMSP production (at a detection limit of 0.005 mM) *T. weissflogii* showed no detectable DMSP, even under known DMSP-inducing stress conditions (Petrou and Nielsen 2018). In the absence of genetic verification to conclusively determine whether *T. weissflogii* can produce DMSP, the evidence available suggests that DMSP does not accumulate to detectable levels in these cells. Therefore, for the purpose of this study we refer to *T. weissflogii* as a non-DMSP producer. Additionally, in light of its propensity to take up and accumulate DMSP from the

environment, *T. weissflogii* presents as an ideal candidate to investigate the role of DMSP in osmoregulation and the amelioration of salinity-induced oxidative stress.

Materials and Methods

Culture conditions, maintenance and experimental design

The centric diatom *Thalassiosira weissflogii* (CSIRO strain CS-871; synonym CCMP-1336) was obtained from the Australian National Algae Culture Collection (ANACC), Hobart. Non-axenic cultures of *T. weissflogii* were grown in autoclaved and 0.2 µm filtered seawater (FSW) enriched with f/2 nutrients (Guillard and Ryther 1962) and maintained at 20 ± 0.5 ˚C with illumination of ~ 55 µmol photons m^{-2} s^{-1} supplied under a 12:12h light: dark cycle, with constant stirring at 70 rpm. For the experiment, cultures of *T. weissflogii* were grown in quadruplicate (100 mL) at three salinities, to test both hyposaline (15 ppt), and hypersaline (55 ppt) stress, against the salinity of natural seawater (35 ppt). Salinity adjustments were made by either adding sterile milli-Q water (15 ppt) or dissolving NaCl (55 ppt) into the FSW. Salinity was determined by a digital refractometer (Hanna Instruments, Victoria, Australia) and cultures were transferred every two weeks under sterile conditions.

Following eight weeks of salinity acclimation (four transfers), cultures were duplicated on the last transfer and half of them (n=4 for each salinity treatment) were grown in the presence of DMSP (initial dose, final concentration 100 nM) from a freshly prepared 10 mM stock of DMSP-HCl (Tokyo chemical industry co. ltd., Tokyo, Japan) and left for 24 h under experimental conditions. Cultures grown in the absence of DMSP (controls *n*=4 for each salinity treatment) were set up with the same conditions, however the addition of DMSP (non-saline solution, < 50 µL) was substituted with the addition of an equal volume of sterile ultra-pure water. Following the first two subsampling time points (days 4 and 7), new additions of DMSP [100 and200 nM, respectively] were added to the treatment cultures, and all cultures (control and DMSP) topped up with 5 mL f/2 media at the respective salinity to make up for lost culture volume. The final concentration of DMSP (200 nM) was selected to replicate high environmental DMSPd concentrations experienced during blooms of DMSP-producing

phytoplankton (Yoch 2002). The experiment ran for 10 days, due to a power-outage on day 11. To assess the health of cells against hyposaline, natural seawater and hypersaline conditions during experimentation, all cultures were subsampled daily for cell counts, cell volume and growth rate determination, while measurements of maximum quantum yield of PSII (F_V/F_M) and reactive oxygen species (ROS) were made on cultures in post lag (day 4) mid- (day 7) and late exponential (day 10) phases. Samples for dissolved and particulate DMSP were taken on the same days as those for ROS, but we only measure detectable levels of DMSP during the late exponential (day 10) growth, when cell densities were high enough.

Cell counts, ROS, cell volume and photosynthetic health

Daily subsamples (1 mL) were fixed with glutaraldehyde (1% final concentration) for enumeration of *T. weissflogii*. Counts were performed on a CytoFLEX S flow cytometer (Beckman Coulter Inc, Indianapolis, USA), using side scatter (SSC) and chlorophyll-*a* fluorescence (laser/optical filter: 488/690 nm). Reactive oxygen species (ROS) concentrations were measured using a general oxidative stress indicator, CM-H_2DCFDA (Thermo Fisher Scientific Inc., Australia), at a final concentration of 5.77 µM (from a freshly prepared 1.0 mM stock made up with DMSO). CM-H_2DCFDA dye was added to live samples of *T. weissflogii,* which were incubated with the dye for 20 min in the dark before fluorescence was measured on a CytoFLEX S flow cytometer (Beckman Coulter Inc, Indianapolis, USA). Cells were excited at 488 nm and FITC fluorescence read at 525 nm. Using flow cytometry, we obtained single cell measurements of relative fluorescence units (RFU). Final fluorescence values were achieved by subtracting the fluorescence of an unstained sample from a CM-H_2DCFDA stained sample. Measurements for cell volume were taken using fixed subsamples of *T. weissflogii* throughout the experimental period (14 days). For each salinity treatment of acclimated cultures grown in the presence and absence of DMSP, the first 20 cells encountered in the light microscope were imaged using the software Infinity Analyze (version 6.5.5, Lumenera Corporation, Ottawa, ON, Canada). Approximate cell diameters (width) and length were measured using manual image analysis in Fiji (Schindelin et al.

2012), and the cell volume calculated assuming a cylinder-shaped cell, as per Hillebrand et al. (1999). Photosynthetic health was assessed using a pulse amplitude modulated (PAM) fluorometer (Water PAM, Walz GmbH, Effeltrich, Germany). Following 10 min dark-adaptation, minimum fluorescence (F_O) was obtained, before application of a saturating pulse of light (0.8 s, intensity 10) to determine maximum fluorescence (F_M). The maximum quantum yield (F_V/F_M) of photosystem II (PSII) was calculated according to Schreiber (2004).

Determination of DMSPd and DMSPp

To determine dissolved (DMSPd) and particulate DMSP (DMSPp) in the cultures, a 3 mL aliquot of culture was transferred onto a 25 mm Whatman GF/F filter (0.7 µm nominal pore size) and gravity filtered (taking care to minimise air exposure of the filter), to capture the cells while minimising cell rupture (Kiene and Slazek 2006). The filtrate (1 mL; DMSPd) was collected and added to an acid washed and autoclaved 15 mL glass vial containing 1 mL of sterile ultra-pure water. For DMSPp determination, the filter containing the cells was rinsed three times with 1 mL f/2 media of the same salinity to remove surface-bound DMSP, then placed face down into a glass vial containing 2 mL of ultra-pure water. To each vial, sodium hydroxide (NaOH) was then added (0.75M), stoppered immediately with a butyl rubber septum and sealed with an aluminium crimp cap, for the generation of DMS via the hydrolysis of DMSP. Prior to gas chromatography analysis, all samples were left in the dark for at least 24 h to ensure complete equilibration.

Analysis of DMSP was performed using a gas chromatograph (GC-2010 Plus, Shimadzu, Kyoto, Japan) coupled with a flame photometric detector (FPD) set at 180ºC with hydrogen and air flow rates of 40 and 60 mL min^{-1}, respectively. Samples were analysed via direct injection onto the column (DB1-Agilent) which was set at 120°C, using high purity helium as a carrier gas at 21 mL min^{-1} and a split ratio of five. Using a gas tight syringe, 500 µL of DMS in the headspace of each vial was extracted and injected directly into the GC. Injections were made on biological replicates, but in the instance of high variability between biological replicates (> 10 %), additional injections were performed

for verification. The integrated peak area was quantified against a calibration curve obtained from solutions prepared with known amounts of DMSP in ultra-pure water and hydrolysed with NaOH using the same method as for the samples. The absolute detection limit was 20 pmol of DMS per sample. Following analysis, all DMSPp data were normalised to cell density and average cell volume to quantify the internal concentration of DMSP taken up per cell.

Data analysis

Data were analysed using R (R Development Core Team 2018). Prior to analysis, data was tested for normal distribution and homogeneity of variances. A one-way analysis of variance (ANOVA) was used to test for a significant difference in the mean of biological replicates between control and DMSP treatments for specific growth rate and DMSP content for all salinities. A two factor ANOVA was carried out on cell volume, F_V/F_M and ROS comparing control and DMSP treatments between salinities. When significant differences were detected, Turkey's HSD multiple comparisons test were performed. Data were considered significant at $\alpha < 0.05$.

Results and Discussion

In this study, we explored the role of DMSP in microalgal physiology with respect to salinity changes, using a model diatom, *Thalassiosira weissflogii*, a species known to take up and accumulate DMSP from the environment to high intracellular concentrations (Petrou and Nielsen 2019). Our aim was to quantify DMSP uptake and accumulation in physiological acclimation to different salinities and determine whether this strategy confers an ecological advantage (such as improved growth or decreased ROS) via the proposed roles of DMSP as a compatible solute and antioxidant. We found that *T. weissflogii* was able to acclimate to all three salinity conditions with no effect on photosynthetic health (F_V/F_M; Table 1). The hypersaline conditions (55 ppt) did however, have a negative effect on growth rate ($F_{2,20} = 22.15$, $P < 0.001$; Figure 1a; Table 1), yet no change in cell volume when compared with the control (Table 1). These results are consistent with previous work that found *T. weissflogii* to be tolerant to large variations in salinity (25-50 ppt), but that higher salinities (> 40) negatively affected

cell density (García et al. 2012). *T. weissflogii* was able to cope with the hyposaline conditions (15 ppt) with minimal effect on growth rate (Figure 1a; Table 1), which was expected given that *T. weissflogii* is a known estuarine species (Radchenko and Il'yash, 2006). Also expected was an increase in cell volume, which more than doubled ($F_{1,37} = 279.82$; $P < 0.001$) in cells grown under hyposaline conditions (Table 1), indicating an osmotic response to the hyposaline conditions for maintaining turgor pressure (Kirst 1989). When cultures were provided with small, environmentally relevant pulses of DMSP, we saw no effect on the growth, cell volume or photosynthetic capacity of *T. weissflogii* (Figure 1a; Table 1), suggesting that the uptake and accumulation of DMSP inside the cell did not provide a physiological advantage to the cells under altered salinity. This was contrary to expectations, as we anticipated that the addition of DMSP would help buffer *T. weissflogii* when exposed to increased salinity by saving energy required to produce compatible solutes (Stefels, 2000). However, However, our results align with previous work on alphaproteobacterial *dysB* genotypes, where bacteria capable of synthesising DMSP displayed no physiological advantage under different salinities over a knockout strain unable to synthesise DMSP (Curson et al. 2017). Nevertheless, it cannot be ruled out that the amount of DMSP added may have been too low to induce an osmoregulatory function or equally, that the evolutionary response to an estuarine lifestyle means that *T. weissflogii* has an internal osmoregulatory system, and therefore cellular adjustment to changes in salinity is independent of the need for external osmolytes.

Cellular quotas of DMSP on day 10 differed between salinities (Table 1), with cells grown at a salinity of 55 ppt having double the amount as those grown at a salinity of 35 ppt ($F_{1,6} = 18.88$; $P = 0.005$) and cells grown at low salinity (15 ppt) containing more than three times the DMSP ($F_{1,6} = 7.00$; $P = 0.038$) than that of the control (Table 1). When normalised to cell volume, however, only cells grown at hypersaline conditions (55 ppt) had higher DMSP concentrations relative to the control ($F_{1,6} = 16.89$; $P = 0.006$; Table 1). With respect to hypersaline conditions, the higher DMSP content corresponds with studies that showed intracellular DMSP production to increase under hyperosmotic conditions (Dickson and Kirst 1986; Lyon et al. 2011; Kettles et al. 2014). The three-fold increase in DMSP in cells under hypo-osmotic conditions was unexpected, as uptake of DMSP under such

conditions would likely increase the water stress by increasing the internal osmolality. If DMSP was being used as a compatible solute, we would expect the uptake to be reduced under hyposaline conditions compared to the control. While this increase in DMSP content per cell under hyposaline conditions was likely a function of the increased cell volume, these results propose that DMSP may not be used for osmoregulation at high salinity by *T. weissflogii* at the concentrations and conditions of this study. This, however, does not preclude the possibility of DMSP having a role as a compatible solute if it were present in higher intracellular concentrations. A recent study showed changes to intracellular DMSP concentrations differed between low- and high DMSP-producers, where prolific producers seemed to use DMSP as a compatible solute (McParland et al. 2020), compared with low producers whose primary application is still unknown. This finding, combined with the need for compatible solutes to be present in high concentrations to be effective (Stefels 2000), suggests that the reason for DMSP accumulation and utilisation by *T. weissflogii* may differ depending on environmental condition. Concentrations of DMSPd were below detection in all samples (data not shown), demonstrating that most of the available DMSP was taken up by the cells and/or consumed.

Another functional role attributed to DMSP accumulation has been its potential value as a cellular antioxidant (Sunda et al. 2002). Numerous studies have observed increased DMSP production in conjunction with stress, suggesting DMSP to be a stress-response compound (Deschaseaux et al. 2014; Gardner et al. 2016; Deschaseaux et al. 2019). However, given the short lifetime of ROS (Sharma et al. 2012) and the inherent complexity of establishing unequivocal cause and effect in the cell, studies into ROS quenching in DMSP producing organisms have established only correlative links. As such, to date, no study has demonstrated directly that DMSP in the cell quenches ROS. Consistent with its purported role as an antioxidant, we hypothesised that DMSP would reduce the production of ROS, thereby enhancing the photosynthetic health (F_V/F_M) and growth of cells during salinity-induced oxidative stress. While we found no effect on F_V/F_M or growth between control and DMSP treatments at any of the salinities (Figure 1a; Table 1), we did find evidence for a DMSP-induced reduction of ROS (Figure 1b) and thus the first direct verification of DMSP functioning as an antioxidant in a cell.

At all three salinities, ROS was significantly reduced ($F_{1,21} = 44.39$; $P < 0.001$) in DMSP treated cells on day 10 (Figure 1b), corresponding with moderate amounts (54 - 99 μmol L^{-1}) of DMSP in the cells (Table 1). By plotting day-specific growth rate against ROS fluorescence we saw a significant correlation across all salinities in the absence of DMSP (Spearman's $\rho = -0.42$; $P = 0.011$), which showed that as growth rates slow towards stationary phase intracellular ROS increases, yet when DMSP was taken up by the cells, that relationship disappeared (Figure 2). Interestingly, the pattern of ROS production during the growth cycle (the increase in ROS at the onset of stationary phase; day 10) suggests that the ROS measured was likely related to the growth phase of the culture and thus the metabolic activity of the cells (Sharma et al. 2012) rather than salinity-induced physiological stress. The apparent positive effect of DMSP on reducing ROS with no change in F_V/F_M is congruent with a previous study that found no change to the photo-physiological state of *T. weissflogii* with the addition of DMSP, indicating a benefit elsewhere other than the photosystem (Petrou and Nielsen, 2018).

The regulation of DMSP in cells of marine phytoplankton has been widely studied, yet a unifying role for this important compound remains equivocal. Introducing DMSP to cultures of *T. weissflogii* we saw uptake and accumulation of DMSP at all salinities. As predicted, *T. weissflogii* accumulated DMSP to a higher concentration under hypersaline conditions, while maintaining cell volume. While no effect of salinity was observed on photosynthetic health, hypersaline conditions did reduce growth rate. Importantly, despite the low level of cellular stress observed in this study, addition of DMSP reduced free ROS in cells during late exponential growth at all salinities, indicating that the presence of biogenic DMSP in marine systems may be important for non-DMSP producers. While the potential role of DMSP as a compatible solute in *T. weissflogii* remains tenuous, by using a non-DMSP producer in our study (granting us a negative control), we present the first direct evidence of the ability of DMSP in reducing growth-related ROS in phytoplankton cells.

Conflict of interests. The authors declare that they have no conflict of interests

Authors' contributions. DAN, KP designed the experiments, AMT performed experiments, AMT, DAN and KP analysed data, AMT, DAN and KP wrote the manuscript.

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Table 1. Physiological parameters of *T. weissflogii* grown at three salinities in the presence and absence of DMSP. All measurements were done on *n*=4 biological replicates. Specific growth rate was calculated using cell densities on days 4 and 10. F_V/F_M and volume were averaged across days 1-10. In all samples, DMSPd concentrations (data not shown) were below the detection limit.

Salinity	**+/- DMSP**	**Growth rate**	**F_V/F_M**	**Cell volume**	**DMSP content (day**	**DMSP conc. (day 10)**
ppt		*day*$^{-1}$		*µm*^{3}	**10)**	*mM*
					fmol cell^{-1}	
15	-	0.623 ± 0.046	0.725 ± 0.006	1644 ± 218 ***	nd	-
35	-	0.616 ± 0.036	0.723 ± 0.009	795 ± 101	nd	-
55	-	0.528 ± 0.050 ***	0.713 ± 0.013	823 ± 127	nd	-
15	+	0.638 ± 0.029	0.720 ± 0.008	1692 ± 288 ***	0.137 ± 0.072 *	0.084 ± 0.044
35	+	0.645 ± 0.039	0.727 ± 0.008	757 ± 112	0.040 ± 0.015	0.051 ± 0.019
55	+	0.497 ± 0.042 ***	0.713 ± 0.015	851 ± 154	0.083 ± 0.012 **	0.101 ± 0.015**

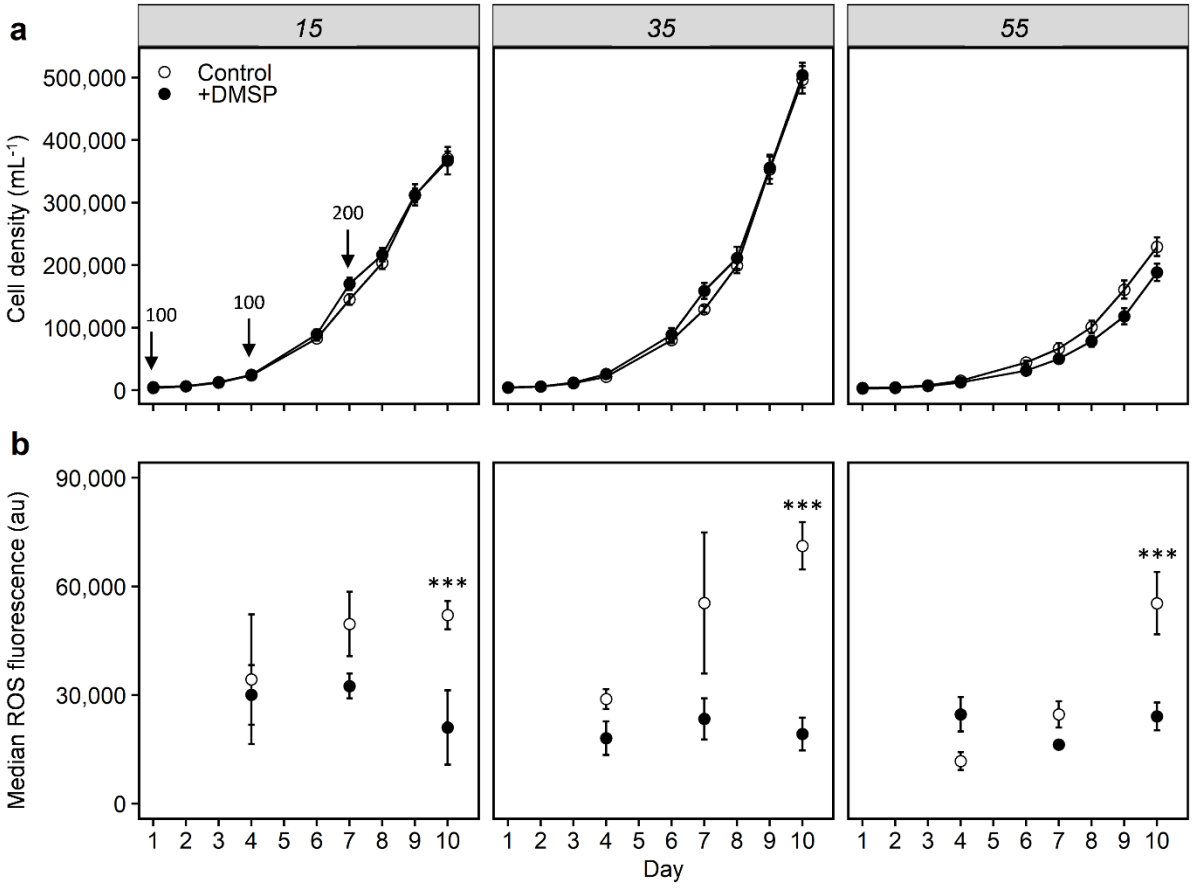
± Indicates +/- standard deviation of biological replicates; nd, not detected.

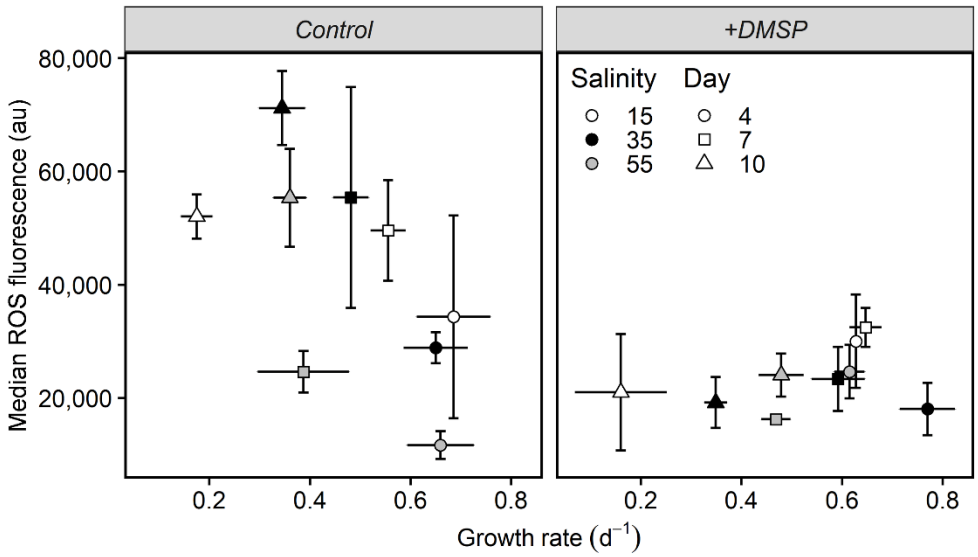
*, **, *** significance level for effect of 55 ppt or 15 ppt compared with control (35 ppt) [* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$]

Figure Legends

Fig. 1 Cell density and reactive oxygen fluorescence in *Thalassiosira weissflogii* under three salinities. a) Growth curves at salinities 15, 35, and 55 ppt, after acclimation to salinity treatment in the presence (open circles) and absence of DMSP (solid circles). The data are presented as cell density in cells mL^{-1} . Data represent mean cell density ± standard error (n = 4). Superscript numbers and arrows indicate concentration (nmol L^{-1}) and timing of DMSP additions. b) Reactive oxygen species (ROS) fluorescence per cell (RFU) for control (white) and DMSP (black) treatments of *T. weissflogii* at different salinities: 15, 35, 55. Data represent median fluorescence values of ROS (RFU) ± standard error (n = 3-4). Significant differences between treatments ($\alpha < 0.05$) are indicated by an asterisk (*)

Fig. 2 Relationship between day-specific growth rates (days 4, 7, 10) and ROS fluorescence per cell (RFU) in *T. weissflogii* grown at three salinities (15, 35, and 55 ppt) in the absence and presence of DMSP. Cross bars indicate standard error (n = 4)


a
15
35
55
Control
+DMSP
Cell density (mL-1)
500,000
400,000
300,000
200,000
100,000
0
100
100
200
b
Median ROS fluorescence (au)
90,000
60,000
30,000
0
***
***
***
1 2 3 4 5 6 7 8 9 10
Day


Control
+DMSP
Salinity
15
35
55
Day
4
7
10
Median ROS fluorescence (au)
80,000
60,000
40,000
20,000
0.2
0.4
0.6
0.8
Growth rate (d^{-1})