Uptake of dimethylsulphoniopropionate (DMSP) by the diatom *Thalassiosira weissflogii*: A model to investigate the cellular function of DMSP

Petrou K[1*] and Nielsen DA[1]

[1]School of Life Sciences, University of Technology Sydney, Sydney, NSW, Australia.

*Author for correspondence: Katherina Petrou

Phone: +61 2 95144159

Email: Katherina.Petrou@uts.edu.au

ORCID

Petrou: 0000-0002-2703-0694

Nielsen: 0000-0001-6678-5937

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Abstract

One of the most abundant organic sulphur molecules in the ocean, dimethylsulphoniopropionate (DMSP) has been implicated in numerous biochemical functions and ecological interactions, from osmotic and oxidative stress regulation within the cell, to the chemical attraction of bacteria, mammals and birds in the environment. Notwithstanding these varied and important discoveries, the primary role of DMSP in the cell remains elusive. In this study, we take a new approach to investigating the role of DMSP in cell physiology. Rather than utilising a known DMSP-producer, we instead exploit the propensity for the non-DMSP producing diatom *Thalassiosira weissflogii* to take up DMSP from its environment. We characterise the uptake and retention of the molecule under growth conditions and salinity stress with the aim to elucidate its utility as a model system for investigating the cellular function of DMSP. *T. weissflogii* showed concentration-dependent uptake of DMSP and complete retention within the cell for at least 6 h. Saturation of intracellular DMSP occurred at >87 mM, equivalent to some of the most prolific DMSP-producing species. Salinity shifts resulted in a reduction in DMSP uptake rate, but only at extremely low (17) or very high (45) salinities. These data demonstrate the potential for using *T. weissflogii* in physiological studies, providing a true (DMSP-free) control, as well as a DMSP-enriched version of the same strain. In this way, orthogonal experiments may be conducted with the aim to uncover the physiological purpose of DMSP in phytoplankton and potentially add key pieces to the enigmatic DMSP puzzle.

Introduction

Dimethylsulphoniopropionate (DMSP), is an abundant and ubiquitous organic sulphur compound in marine ecosystems, with more than a billion tonnes produced by marine phytoplankton annually (Johnston et al. 2016). Many marine phytoplankton produce and retain high amounts of DMSP, with cellular concentrations sometimes exceeding 400 mM (Stefels 2000). Once released into the surrounding environment, predominantly through cell lysis or grazing, DMSP is rapidly scavenged by other marine organisms, the most studied being heterotrophic bacteria (Kiene and Linn 2000; Moran et al. 2012). This lower level trophic interaction forms the engine of the marine sulphur cycle, whereby the bacteria selectively transform the DMSP into amino acids and proteins for growth or cleave it into dimethylsulphide (DMS), a volatile molecule that is readily fluxed into the atmosphere where it can nucleate to form cloud condensation nuclei, with the potential to influence climate (Charlson et al. 1987). Ecologically, both DMSP and DMS have been implicated in many marine trophic interactions, via their roles as a chemoattractant (Miller et al. 2004; Seymour et al. 2010), foraging cue (Nevitt and Bonadonna 2005; Savoca and Nevitt 2014; Lee et al. 2016) or through conversion of DMSP into a chemical deterrent (acrylate) against grazers (Wolfe et al. 1997).

Controls on DMSP production by marine phytoplankton have been linked to environmental drivers such as high light, temperature and UVB radiation (Stefels 2000), suggesting the molecule may play a role in cellular stress response (Sunda et al. 2002). Yet, its most commonly attributed role is osmoregulation (Malin and Kirst 1997; Stefels 2000; Welsh 2000), through its function as a compatible osmolyte. There is, however, considerable variability in DMSP regulation with respect to stressor (UV, temperature, salinity) and across species (see Stefels 2000). This variability underscores the intricate role of DMSP in phytoplankton physiology, but makes identifying the intended role(s) of this molecule in the cell inherently challenging. As such, despite its prevalence and purported importance in the chemical landscape of the ocean, the key functional role of DMSP remains unclear.

To date, research into understanding the functional role of DMSP in the cell has been focused overwhelmingly on DMSP-producing microalgae, where altered conditions cause a change in concentration or rate of production within the culture of interest. However, it has long been known that there are also phytoplankton species that take up and accumulate dissolved DMSP (Kiene et al. 1998), matching the removal rate of heterotrophic bacteria (Vila-Costa et al. 2006). Since then, to our knowledge, only three studies have corroborated the uptake of DMSP by non-DMSP producing microalgal species (Spielmeyer et al. 2011; Ruiz-Gonzalez et al. 2012; Lavoie et al. 2018), including the diatom *Thalassiosira weissflogii* (Spielmeyer et al. 2011). In this earlier

study, Spielmeyer and colleagues used isotopically labelled DMSP to probe the uptake and metabolism of DMSP in phytoplankton of high, low and no DMSP content. They found that the diatom *T. weissflogii* rapidly takes up and accumulate DMSP from the environment, with intracellular concentrations reaching levels similar to that of the prolific DMSP producer *Emiliania huxleyi*, but found no evidence for short- or long-term metabolism of DMSP (Spielmeyer et al. 2011). Here we propose that the propensity of *T. weissflogii* to take up and retain DMSP provides an optimal opportunity to investigate the physiological role of DMSP in the cell; providing the ability to test both DMSP-free and DMSP-enriched cultures of the same strain of organism. In this way, direct physiological comparisons can be made between stress responses in the presence and absence of DMSP. Whether these cells take up DMSP and/or utilise it for the same reasons as those that produce it remains a caveat to this model, however, DMSP uptake is an energy expending process, and it is thus unlikely that organisms would invest energy for no measurable gain or physiological advantage. In this study, we characterise DMSP uptake and utilisation in the centric diatom *Thalassiosira weissflogii*, with the aim to determine its utility as a model organism for investigating the role of DMSP in algal physiology.

Materials and Methods

Batch cultures of the centric diatom *Thalassiosira weissflogii* (CSIRO strain CS-871; synonym CCMP-1336) were grown in sterilised seawater amended with F/2 nutrients (Guillard and Ryther 1962) and maintained at 20°C. Light (cool white) was supplied at ~55 µmol photons m^{-2} s^{-1} (Hydra 52HD, Aquatic Illumination), programmed on a 12:12 h light:dark cycle. Cultures (200 mL) were grown in quadruplicate for 4-5 generations prior to experiments and all measurements were made on cells during exponential growth ($\mu = 0.49 \pm 0.06$ d^{-1}). Under culturing conditions no detectable levels of DMSP were found, verifying that this strain of *T. weissflogii* is not a DMSP producer.

Prior to experimentation, cultures were washed twice in sterile media (15 mL) via centrifugation (1800 rcf for 5 min), before being re-suspended into fresh sterile F/2 media, to minimise the influence of bacteria. Flow cytometry measurements showed that this procedure reduced non-attached bacterial counts by > 99% (data not shown), and fluorescence staining (SYBR green) of cultures, revealed minimal occurrence of attached bacteria. Both *T. weissflogii* and the bacterial consortia within the cultures were sampled for DMSP lyase activity, where a 5 mL aliquot of culture was filtered onto a 5 µm polycarbonate filter (MicroAnalytix, Taren Point, Australia) to collect the microalgal cells, after which the filtrate was re-filtered onto a 0.22 µm filter (MicroAnalytix, Taren

Point, Australia) for the bacterial component. Filters were flash frozen in liquid N_2 and stored at -80°C until analysis. Lyase activity was determined according to the methods of Harada et al. (2004). To ensure cells were not compromised after washing or during experimentation when stress was applied, photophysiological condition of the cells was assessed via variable chlorophyll *a* fluorescence using a pulse amplitude modulated fluorometer (Water PAM, Walz GmbH, Effeltrich, Germany). Briefly, following 10 min dark-adaptation, minimum fluorescence (F_O) was recorded before application of a saturating pulse of light (Duration = 0.8 s; Intensity =10), where maximum fluorescence (F_M) was determined and the maximum quantum yield of PSII calculated as $F_V/F_M = (F_M-F_O)/F_M$. Cultures were only used in experiments if the post-washing F_V/F_M values were $\gtrapprox$0.700. All uptake experiments were conducted under growth (temperature and light) conditions.

To characterise DMSP uptake in *T. weissflogii*, DMSP [500 nM] (from freshly prepared 10 mM stock of DMSP-HCl, Tokyo chemical industry co. ltd., Toshima, Kita-ku, Tokyo, Japan) was added to washed cultures (n = 4) and the cultures subsampled over time (0-6h) for both dissolved (DMSPd) and particulate (intracellular, DMSPp) DMSP. In a separate study, we investigated the retention of DMSP using washed cells that were pre-loaded with DMSP [500 nM] for 4 h under growth conditions. Following DMSP loading, cultures (n = 4) were washed to remove any remaining DMSPd from the medium and re-suspended in DMSP-free medium. Samples were taken immediately to measure the initial DMSPp and then again after 6 h to verify retention of DMSP. Additional uptake experiments were conducted as described above on culture filtrate containing just the bacterial fraction (< 5µm) to ensure that any responses observed could be solely attributed to *T. weissflogii*.

Tests for DMSP- [50, 100, 250, 500,1000 nM] and salinity- (17, 25, 35,45 psu at 500 nM DMSP) dependent responses were conducted via a series of rapid kinetic assays. Cultures (n = 3) of *T. weissflogii* were washed and then resuspended in 20 mL of fresh medium to a known cell density and placed under growth light. Each culture was subsequently adjusted to final DMSP concentration or salinity and cultures subsampled for DMSPp at set time points (0, 5, 10, 15, 20 min). The DMSP saturation point in *T. weissflogii* was determined by amending washed cultures (n = 3, ~10^4 cells mL^{-1}) with a range of DMSP concentrations [250-5000 nM] and incubating them for 4.5 h under growth conditions before subsampling for DMSPp. To test whether DMSP-rich *T. weissflogii* rid themselves of DMSP under lowered salinity, cells pre-loaded with DMSP (500 nM, 2h) were washed and re-suspended in DMSP-free media. After sampling for DMSPd and DMSPp (0 and 20 min) salinity of the medium was decreased to 25 using milliQ F/2 media and cells subsampled over time (20, 40, 60 min).

120 Sampling for DMSPd and DMSPp was done by gently filtering culture (2 mL) through 25 mm GF/F
121 filters using a low vacuum (< 5 mm Hg) hand pump to avoid cell rupture. Filtrate (1 mL) was transferred to a vial
122 containing 1 mL of milliQ water and the filter containing algal cells was washed three times with F/2 media before
123 being placed in a vial with 2 mL of milliQ water. A pellet of NaOH was added to each vial, immediately prior to
124 being stoppered and crimp capped. All samples were left in the dark for 24 h for equilibration to occur before
125 analysis. DMSPd and DMSPp were quantified as total DMS after conversion with NaOH and measured using a
126 gas chromatograph (GC-2010, Shimadzu, Kyoto, Japan) coupled with a flame photometric detector (FPD).
127 Samples (liquid and headspace) were purged with He (70 mL min^{-1} for 4 min) while cryo-trapped in liquid N_2 and
128 subsequently eluted onto a capillary column (DB-1, Agilent; injector: 120°C, column: 110°C, FPD: 150°C,
129 column flow: 2.1 mL min^{-1}). Samples with high concentrations of DMSPp (saturation experiment) were analysed
130 via direct injection of 500 µL of headspace (column flow: 3.66 mL min^{-1}). All DMSPp data were normalised to
131 cell density.

132 For the enumeration of *T. weissflogii* and bacteria cells, subsamples were fixed with glutaraldahyde (1%)
133 and counted on a Cytoflex S flow cytometer (Beckman Coulter Inc, Indianapolis, USA), using chlorophyll *a*
134 fluorescence (laser/collection: 488/690 nm) and forward scatter for *T. weissflogii* and SYBR Green nucleic acid
135 stain (1:10,000 dilution) for bacterial counts (laser/collection: 488/530 nm). Cell volume was estimated based on
136 microscopy measurements of the length and width of 20 cells and calculated assuming a cylinder-shaped cell as
137 per Hillebrand et al. (1999).

138 To test for a significant change in photophysiology or retention of DMSP over time, data were analysed
139 for statistical differences between treatments using ANOVA (IBM, SPSS, Statistics v24; IBM Corporation, New
140 York), with differences considered significant at $P < 0.05$. Prior to analysis, test for normal distribution and
141 Levene's test for homogeneity of variance were applied to the data. All uptake experiments comparing
142 concentrations or salinities were analysed using PERMANOVA, with a resemblance matrix based on Euclidean
143 distance. All *P* values obtained were based on Monte Carlo method. Analyses were carried out using Primer v6
144 statistical package (Primer-E, Plymouth, US; Clarke and Gorley, 2006) with the PERMANOVA+ add on
145 (Anderson et al, 2008). Following the test for main effects, pair wise comparisons were conducted for each
146 concentration or time point and significance denoted by superscript letters. For the concentration dependent uptake
147 experiment, Michaelis-Menten parameters were estimated from the raw data using the nls (nonlinear least squares)

function in R and the model v ~ Vm * S/(K+S). Starting parameters were: K = Vmax/2 and Vm = Vmax, where Vmax is the highest rate of uptake measured in any of the samples.

Results and discussion

Thalassiosira weissflogii has been shown previously to take up DMSP (Spielmeyer et al. 2011), however, this is the first study to characterise the uptake kinetics at varying concentrations and under different salinities. Uptake of DMSP by *T. weissflogii* (at 500 nM initial DMSPd concentration) resulted in intracellular accumulation of up to 17.7 ± 1.9 fmol $cell^{-1}$ within 4 h with a reciprocal decline in dissolved DMSP, where cells removed more than 97% of initial DMSPd (Figure 1a). Once taken up, *T. weissflogii* retained the DMSP, with no loss from the cells for at least 6 h (Figure 1b; ANOVA $F_{1,6} = 0.081$, $P = 0.786$). These data demonstrate that this species preferentially uses cellular energy to ensure intake of this molecule to maintain high concentrations of DMSP within the cell. Combined, the stoichiometric match between dissolved and particulate DMSP confirm uptake by the diatom cells as the dominant removal factor, as no uptake of DMSP by the culture-associated bacterial community and no lyase activity were detected in *T. weissflogii* or its associated bacteria (data not shown).

Rapid uptake kinetics revealed a concentration-dependent response (Figure 2a), resembling Michaelis-Menten kinetics (V_{Max} = 27.1 fmol $cell^{-1}$ h^{-1}, K_M = 632 nM) for concentrations between 50 and 1000 nM over 20 min (Figure 2b). The high K_M value relative to common oceanic DMSP concentrations (<10 nM) suggest that uptake of DMSP by *T. weissflogii* would mainly occur during bloom scenarios, where DMSP concentrations can increase >10-fold (Stefels et al. 2007). Longer incubations (4.5h) showed a linear relationship between initial DMSP concentration and final intracellular DMSP, until stabilising and saturating at ~87 mM (Figure 2c). The maximum accumulation of intracellular DMSP by *T. weissflogii* falls within the range of many major DMSP producing taxa, such as dinoflagellates, which range from 32 – 218 mM (Keller et al. 1989), or the prymnesiophyte *Phaeocystis* sp. of 71-161 mM (Stefels and Van Boekel 1993). In the present study, saturated cells reached an intracellular DMSP concentration of approximately 200 fmol $cell^{-1}$, which is two orders of magnitude higher than previously observed (Spielmeyer et al. 2011). The higher intracellular DMSP in our study can be explained by the lower cell densities and higher initial DMSP concentrations used, resulting in up to 100 times more DMSP available per cell.

Intracellular concentrations in DMSP-producers are known to vary depending on environmental condition and growth phase (Stefels 2000). However, in this study, the cells, which were maintained at non-stressful growth conditions, took up as much DMSP as was available, only saturating once intracellular concentrations reached >80 mM. This accumulation of DMSP in the absence of cellular stress would suggest a benefit to the cell in maintaining a large amount of DMSP that would at least equate to or offset the cost expended on taking it up. The fact that cells showed no change (ANOVA $F_{1,6} = 0.097$, $P = 0.766$) to their photophysiological state with addition of DMSP (0.737 ± 0.003 at initial time point and 0.736 ± 0.006 after 6 h) would indicate that this benefit is not perhaps to the photosystem or at least not under homeostatic growth conditions. The high DMSP uptake rate, intracellular saturation and retention support the idea that non-DMSP producing species like *T. weissflogii* may form a considerable sink for DMSP in the marine environment, particularly during bloom scenarios, invariably reducing DMSP available to other organisms and influencing the turnover of DMSP in ocean systems. These results clearly demonstrate that the potential influence of non-DMSP producing algae may be significantly more than recently suggested (Lavoie et al. 2018) and also offers up a new line of study for understanding the role of DMSP in cell physiology.

Salinity assays showed no change in the DMSP uptake rate at 25 compared with 35 psu, but did show a ~50% drop in uptake rate at very low (17) and high (45) salinities (Figure 3a; PERMANOVA Pseudo $F_{3,8} = 8.932$, P (mc) = 0.005). While no change in F_V/F_M was observed from 35 to 25 psu, corroborating no shift in uptake kinetics at these salinities, a minor, yet significant decline in F_V/F_M (ANOVA $F_{1,4} = 19.75$, $P = 0.011$) at the lowest (17) salinity from 0.706 ± 0.007 to 0.680 ± 0.008, was detected. Given the proposed osmoregulatory role of DMSP in cell physiology (Malin and Kirst 1997; Stefels 2000; Welsh 2000), the ~50% reduction in uptake rate with ~50% reduction in salinity was anticipated and is consistent with osmoregulatory theory, while the decline in F_V/F_M implies a change in cell physiology that may suggest cellular compromise. Contrary to our expectation, lower uptake rates were also measured at higher salinity (45), where a ~29% increase in salinity resulted in a halving of the uptake rate (Figure 3b), which does not corroborate the theory of osmoregulatory driven uptake. While the fluorescence data did not indicate any changes to cell photophysiology, this reduction may be explained by cellular changes that reduce ATP available for uptake. Indeed, previous work measured a significant negative effect on growth and cell volume in *T. weissflogii* at salinities above 40 (Garcia et al. 2012). When salinity was only reduced ~25%, no change was detected, suggesting a tolerance to shifts in salinity in this species. This result was corroborated by our test for salinity-based regulation of DMSP, where preloaded DMSP-enriched *T. weissflogii* did not rid themselves of intracellular DMSP when salinity was lowered by ~25% over time (Figure

206 3c), with no measureable change in DMSPp or DMSPd during the 60 min of observation (PERMANOVA Pseudo
207 $F_{4,12} = 0.766$, P (mc) = 0.545). Taken together, these data suggest that cellular processes were not obstructed or
208 affected by moderate changes in salinity, a finding congruent with a previous study that found *T. weissflogii* to
209 possess a relatively broad salinity tolerance range (25-50) with maximal growth rates at 25 psu (Garcia et al.
210 2012).

211 As one of the most abundant and important organic molecules in the ocean, the regulation and production
212 of DMSP has been studied extensively both at sea and in culture, resulting in many proposed physiological and
213 ecological functions for this one signature molecule. Yet, to date, no one functional role for this molecule is in
214 agreement across all studies and species. Using a non-DMSP producing diatom, *T. weissflogii*, we saw rapid
215 uptake and accumulation of DMSP that was retained by the cell. We found *T. weissflogii* exhibited concentration-
216 dependent uptake kinetics up to 1000 nM – much higher than is likely to occur in a natural environment—and that
217 intracellular concentrations saturated at around 87 mM. These data indicate that species not able to produce
218 DMSP, but instead take up available DMSP from the surrounding water, may constitute a major sink for DMSPd
219 in oceanic systems when DMSP concentrations are elevated, thus contributing to DMSP removal from the marine
220 environment. Further study into the utilisation of this molecule by non-producers, such as *T. weissfloggii*, may
221 help to uncover a primary role for DMSP in cell physiology and in doing so, reveal how the production and export
222 of DMSP into the water column, may make available the physiological or ecological advantage DMSP proffers
223 to non-producing members of the marine microbial community.

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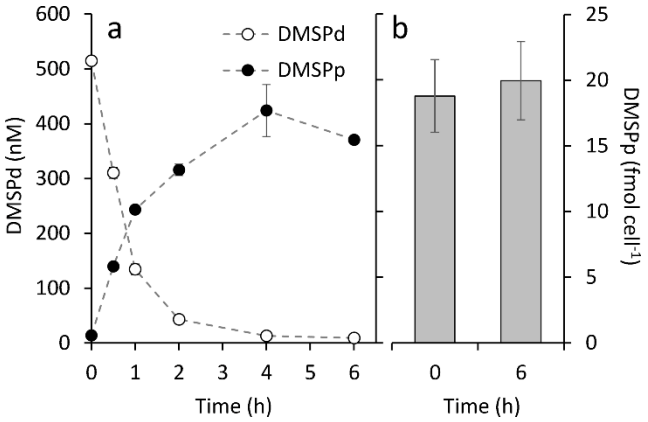
Figure captions

Fig. 1 Uptake and retention of DMSP by *T. weissflogii* a) drawdown and uptake of DMSP over 6 h, b) intracellular concentrations of DMSP in pre-loaded and washed cells at 0 and 6 hours. Data represent means ± SE (n = 4)

Fig. 2 Uptake kinetics and saturation of DMSP in *T. weissflogii* a) DMSP incorporation at five concentrations over 20 min b) uptake rate vs extracellular concentration fitted with Michaelis-Menten kinetics model c) intracellular concentrations of DMSP after 4.5 h at six different extracellular concentrations. Data represent means ± SE (n = 3-4). Letters denote statistical difference determined by PERMANOVA at $\alpha < 0.05$, b) Pseudo $F_{4,10}$ = 37.09, *P* (mc) = 0.001; c) Pseudo $F_{4,10}$ = 228.31, *P* (mc) = 0.001.

Fig. 3 Uptake kinetics and retention of DMSP in *T. weissflogii* exposed to different salinities a) DMSP incorporation at four salinities over 20 min b) DMSP uptake rate vs salinity c) intracellular DMSP concentrations of pre-loaded cells before (0, 20 min) and after lowering of salinity from 35 to 25 psu (dashed line). Data represent mean ± SE (n = 3-4). Letters denote statistical difference determined by PERMANOVA at $\alpha < 0.05$, b) Pseudo $F_{3,8}$ = 8.932, *P* (mc) = 0.005; c) Pseudo $F_{4,12}$ = 0.766, *P* (mc) = 0.545.

Figures: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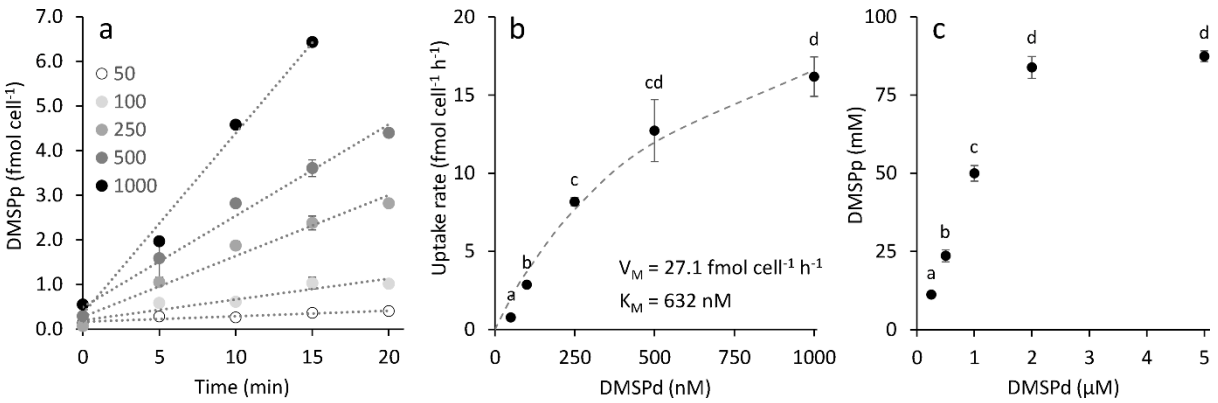
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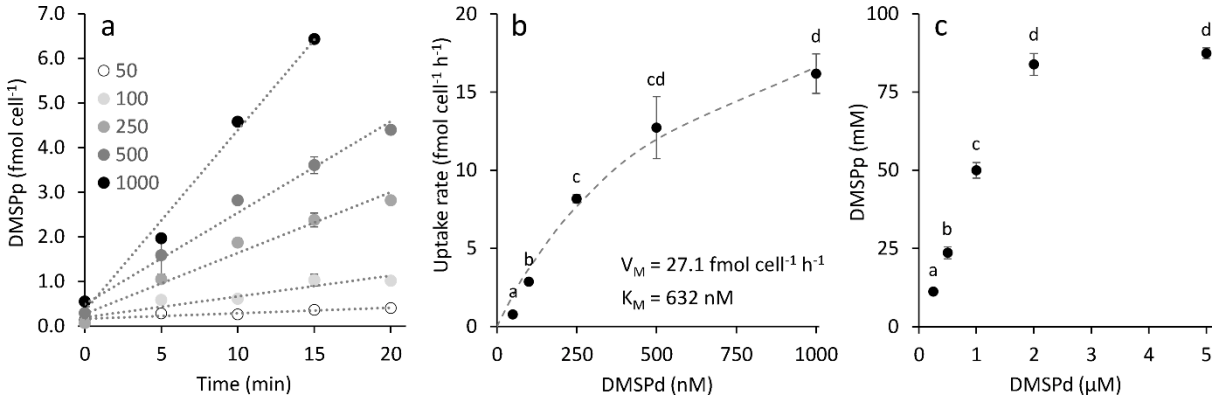

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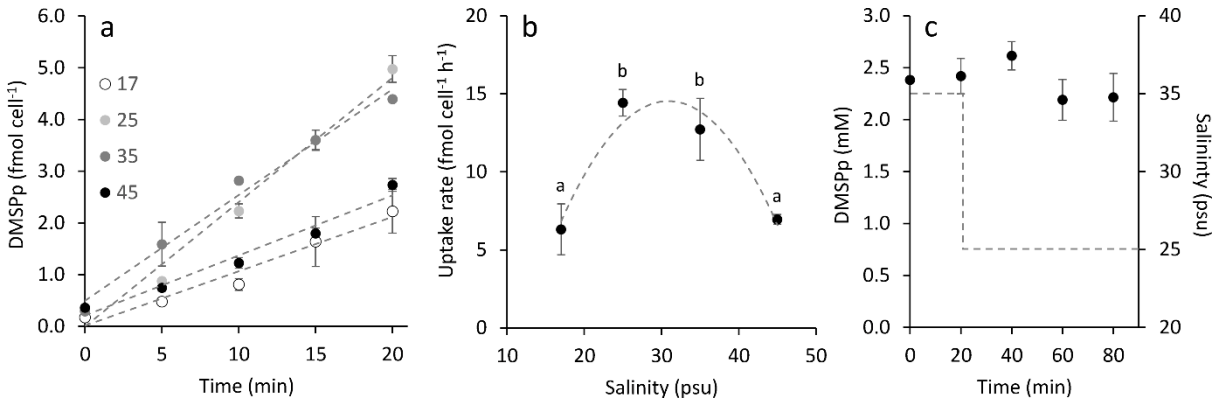
a
17
25
35
45
DMSPp (fmol cell-1)
Time (min)
b
Uptake rate (fmol cell-1 h-1)
Salinity (psu)
a
b
b
a
c
DMSPp (mM)
Salinity (psu)
Time (min)

346

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