Responses to Reviewers' comments

We would like to thank both Reviewers for their comments and suggestions, which have helped improving the wording and reasoning of this manuscript. It is now better acknowledged throughout the manuscript that although a 12°C increase in temperature represents an extreme scenario of Marine Heat Waves (MHWs), this temperature treatment presented an opportunity to investigate the physiological and biochemical response to thermal stress of an ecologically relevant dinoflagellate in the context of MHWs. The choice of temperatures is now more thoroughly justified in the method section. Uncertainties that led to speculative comments are now better acknowledged throughout the manuscript. It is also now better acknowledged that the shifts in microbiome structure at 32°C could be linked to either the physiological and biochemical response of *A. minutum* to thermal stress or by the presence of other solutes that were not measured in this study. Figures have also been modified to reflect the true timeline of this study. We believe that this revised version will now fit with the scope and quality of Biogeosciences and look forward to receiving your feedback.

Responses to Reviewers' comments appear in blue throughout the document.

Interactive comment on "Shifts in organic sulfur cycling and microbiome composition in the red-tide causing dinoflagellate Alexandrium minutum during a simulated marine heat wave" by Elisabeth Deschaseaux et al.

Anonymous Referee #1
Received and published: 5 January 2019

General comments:
The manuscript by Deschaseaux et al presents a study of how two different levels of temperature change affected 1) the growth and physiological state of the cultured dinoflagellate Alexandrium minutum. 2) the concentrations of the phytoplankton osmolyte DMSP and its degradation products, DMS and DMSO in the cultures, and 3) the taxonomic composition of the bacterial community associated with the cultures, over a six-day period after the temperature shifts. The goal was to assess how temperature increases that might be representative of marine heat waves would affect the phytoplankton and the associated sulfur biogeochemistry and microbial ecology. Marine heat waves are certainly a topic worthy of study, and their effects need investigation.

The authors chose as their control temperature, 20 oC and acclimated the Alexandrium cultures to that temperature before shocking them with +4 and +12 oC increases. The authors don't really justify the choice of their temperatures very well, and their relevance to potential changes in the natural habitats where Alexandrium minutum is found is not evident. The +4 degree temperature shift caused little effects. The +12 degree shift caused effects but what is the environmental relevance of a sudden 12 degree shift? It seems doubtful that a heat wave of that magnitude in a marine system would happen in a short period, if at all. The choice of control temperature of 20 deg was unfortunate. It seems it should have been higher and perhaps the temperature upshift less dramatic. That would have been more realistic.
The 20°C control was chosen based on average summer temperatures at the site where this strain of *Alexandrium* was found (Port River, South Australia). The amplitude of the temperature increase was dictated by preliminary experiments conducted at 20°C, 24°C, 28°C, 30°C and 32°C. The physiology

of this strain was found to be highly robust to these temperature increases, with only the 12°C increase in temperature (32°C) leading to a physiological stress response. This may be an adaptation of this strain to shallow coastal environments characterised by dynamic temperature regimes. While a 12 °C increase in temperature might be rare in the environment (which we now acknowledge more clearly on **lines 143-146**), this treatment presented an opportunity to investigate the physiological, biochemical and microbial consequences of thermal stress on this relevant phytoplankton in the context of extreme MHWs. We have now aimed to better justify this aspect of the study **on lines 141-143 and 390-393, 404-406**.

While there was a clear response of the +12 deg temperature on growth, Fv/Fm and cellular ROS, the effects on DMSP, DMS and DMSO were less clear. There were just a few points with significant differences - not very convincing that it was experimental effect. Most of the discussion is speculation in trying to explain the odd points of higher or lower parameters at particular time points.
Because DMS(P)(O) turnover in seawater can occur very quickly (Simo et al 2000), it is perhaps not surprising that changes in concentrations occurred only over 1 or 2 time points. However, a clear cascading stress response was still evident with our results, which provides useful information regarding the manner with which biogenic sulfur compounds may play a role in thermal stress tolerance in this relevant dinoflagellate. However, in response to the Reviewer's concerns, this is now better acknowledged in the discussion (**see lines 494-498**).

In my opinion, the changes in the microbiome were not particularly informative for interpreting the DMS/P/O data. It seems the authors can only speculate on what drove the changes; the MDS analyses are not very convincing for firm conclusions. I know they replicated the treatments in this experiment, but to be really convincing that temperature effects microbiome shifts reproducibly, the entire experiment should be repeated.
We understand the Reviewer's concern, however, the MDS clearly shows a significant difference in the microbiome between the 20°C and 32°C treatments, which corresponded with significant changes in the DMS(P)(O) data. We agree with the Reviewer that the link between the shift in microbiome and DMS(P)(O) concentrations cannot be directly established in this study, and the speculative aspects of the discussion regarding these potential links have now been scaled-back (**see lines 504-505, 540-542, 547-551**). It is now acknowledged that "These shifts in microbiome structure are likely to have been driven by either the changing physiological state of *A. minutum* cells, shifts in biogenic sulfur concentrations, the presence of other solutes, or a combination of all." (**lines 41-43**).

Also, the bacterial populations would respond to dissolved materials released from the phytoplankton, but there were no measurements aimed at quantifying those releases, making interpretations difficult.
The Reviewer makes a fair point that the microbiome composition will be dictated by a range of biochemical factors, and as stated above we now acknowledge this point on **lines 41-43, 504-505, 540-542 and 547-551.** However, without performing a full metabolomic analysis of the samples, which was beyond the scope and focus of this study, it is not possible to make a priori assessments of the range of chemicals to monitor. Given that *A. minutum* is a prolific DMSP producer, and it is widely hypothesized that DMSP is a key currency in the chemical exchanges between phytoplankton and bacteria, we focused on the role of Sulphur compounds.

Overall, I feel that the manuscript does not make a substantial contribution as it is, primarily because of the extreme temperature used to produce effects.
The use of laboratory conditions to exactly mimic environmental processes is typically highly challenging from a number of perspectives, and accommodations for environment – laboratory

variability often need to be made. Our main goal here was to examine how the heat-stress response of *A. minutum* was reflected in changes in biogenic sulphur cycling and interactions with the microbiome. The temperature range used here was based on substantial pilot studies (described above) that revealed the large shift in temperature that was required to invoke a physiological stress response in the dinoflagellate species in question. Without increasing the temperature to this level we did not observe a marked physiological response in the dinoflagellate. We now more clearly point out the reasons for the choice of temperature used in the study (see **lines 141-146**) and feel that our observations provide valuable new insights into how the stress response of dinoflagellates can influence biogenic sulphur cycling in coastal habitats.

Specific comments:

Title. They really didn't study sulfur cycling so I suggest changing the wording.
In response to the authors concerns we have now changed the title to "Shifts in dimethylated sulfur concentrations and microbiome composition in the red-tide causing dinoflagellate Alexandrium minutum during a simulated marine heat wave."

In Figure 4, the DMSP per cell (0.5 to 1.6 pmol per cell) for Alexandrium minutum is much lower than you report in Introduction for A. minutum (14.2 pmol/cell; line 68). Is there an explanation for that?
We thank the Reviewer for this comment and have added a whole new paragraph **on lines 395-406** to discuss this point. This paragraph now clearly states that : "... DMSP concentrations reported in this study were a degree of magnitude lower (0.42 ± 0.04 to 1.63 ± 1.70 pmol cell^{-1}) than that previously reported for *A. minutum* (14.2 pmol cell^{-1}; Caruana and Malin, 2014;Jean et al., 2005). This is potentially because this culture of *A. minutum* had been isolated from free-living *A. minutum* for a long time (1988) or because culturing conditions failed to mimic the natural biochemical conditions in which this strain of *A. minutum* usually grow. This biochemical difference could potentially reflect that this strain of *A. minutum* in culture is more robust than free-living dinoflagellates of the same species, thereby potentially justifying the need of a 12°C increase in temperature to induce thermal-stress."

L90. When mentioning the 2016 Marine Heat Waves associated with El Nino, give some indication of the temperature increases that occurred.
We have now added this information: "The 2016 MHW that was associated with El Niño Southern Oscillations resulted in an 8°C increase in sea surface temperature leading to the mass coral bleaching of more than 90% of the Great Barrier Reef (Hughes et al., 2017)" (**see line 89-91**).

L131. Julabo, country??
We thank the Reviewer for noticing this omission and have added information has follows: "Temperature and light control was achieved using circulating water heaters (Julabo, USA) and programmable LED lights (Hydra FiftyTwo, AquaIllumination, USA)" (**see lines 133-136**).

L178. 10 ul of H2O2. Give the concentration of H2O2 added and the final concentration in the sample.
We thank the Reviewer for picking that up and have added information as follows: "A positive (+ 10 µL of 30% H2O2, final concentration 97mM) and negative (no ROS added) control of PBS were run to ensure that detected cell fluorescence was completely attributable to the ROS probe." (**line 187**).

L185. The DMS samples were unfiltered. Were they purged for analysis or did you do static headspace? The static headspace would have a relatively high detection limit.
Please provide that value.
Due to the very high DMS concentrations in the Alexandrium cultures, it was possible to analyse DMS concentrations using simple headspace injections as indicated in the methods. The detection limit is now provided as follows: "Detection limit was 50 nM for 500µL headspace injections" (**lines 212-213)**.

L188. From the description, the "DMSP" samples would include DMS that was already in the sample. Was this subtracted from the total DMS after the NaOH?
The reviewer makes a good point and we have now corrected our DMSP values to account for the presence of DMS, and have included this extra detail in the methods (**lines 213-215**). Furthermore, Figure 4 and result section (**lines 318-320**) have been amended accordingly. It is important to note that because DMS values corresponded to less than 5% of DMSP values, this amendment did not lead to any substantial change.

L192. The transition here to "after the experiment DMSP samples were opened:" is awkward because they didn't describe yet how the DMSP samples were measured.
They did this by headspace analysis, which is described further down. I suggest reorganizing to make it clearer.
The Reviewer makes a good point and we have now reorganized this whole paragraph accordingly (**see lines 192-229**).

It should be mentioned in methods that all the sulfur compounds were normalized to cell number.
We thank the Reviewer for pointing out this omission and this details has now been added as follows: "All dimethylated sulfur compounds were normalised to cell density, which best reflects biogenic production." (**lines 228-229**)

But normalizing these parameters to the cells may be misleading. While most of the DMSP will be in the cells, the DMS is most certainly not in the cells. The DMSO has an unknown dissolved and particulate partitioning in their cultures. Referring to them as "cellular" concentrations is not correct.
We agree with the Reviewer that DMS and DMSO concentrations should not be referred to as "cellular" since they are most likely not contained within the algal cells. We have thus modified this accordingly throughout the manuscript. However, normalising DMS(P)(O) concentrations to cell numbers remains the most accurate and realistic way to normalise these biogenic sulfur compounds as expressing them in nM without taking algal growth into account would lead to an overestimation of their net production (**see lines 228-229**). It is to be noted that DMS and DMSO are commonly expressed per cell (Hatton and Wilson, 2007; Steinke et al., 2011) or per Chl a concentration (Harada et al., 2009; Bucciarelli et al. 2013) in the literature, which is a very similar approach.

L225. The description of which samples were sequenced is a little vague. They sayvthey sequenced the three highest DNA samples from each treatment at time zero (so 6 samples) and at T=120 h (6 samples). So, a total of 12 samples were sequenced. Isvthat correct? By choosing the three samples with the highest DNA could that bias thevresults?
Yes, the Reviewer's interpretation is correct. By using this approach, we had 6 samples at time 0 (all confirmed to have very similar microbial composition), and 6 samples at time 120 (3 from the 24°C and 3 from the 32°C treatment). Samples with the highest DNA quantity (for which DNA extraction was the most successful) were chosen to ensure cost-effective and successful sequencing. However, this approach should not lead to any inherent bias, as the relative abundance of associated microbes should

be similar across all replicated samples from the same treatment, regardless of the DNA concentrations. It is also to be noted that the sequence provider normalises the samples according to the DNA concentrations to ensure sufficient reads from all samples.

They filtered 400 ml onto a 0.22 _m filter, so this would capture both prokaryote and eukaryote DNA. Any interference from all the phytoplankton DNA? They mention removing the chloroplast DNA sequences later on. If the focus here is only the bacteria then the description should be clarified.
We used a bacterial specific 16S rRNA primer set, which will specifically target bacterial DNA, so there should be little influence of the eukaryotic DNA in our sequencing results. Chloroplast sequences were indeed removed, further limiting any influence of the eukaryotic DNA.

L248. I am not an expert in statistics so I can't comment on the approaches used here.
But I will say that it wasn't clear to me whether the relative abundance of bacterial groups in each independent replicate was averaged to obtain an error term.
We were not entirely sure of what the Reviewer was asking here, but a two-way PERMANOVA with Bray-Curtis takes the response variable of each replicate and the error term is derived from the full data set.

L287 Add word : : :compared to the 20_C CONTROL at all time points: : :
We have now amended this text in-line with the Reviewer's comments (**see line 301**)

L 289. You say the 32 deg cultures increased to close to those of the control, but were they still significantly lower?
The Reviewer is correct and we have now clarified this detail in the manuscript, where we state: "However, on days 5 and 6, the FV/FM of cultures kept at 32°C recovered to values (0.72 ± 0.008) close to those of the control (0.75 ± 0.004) (Fig. 2B), although it remained significantly lower than at 20°C ($p < 0.01$ and $p < 0.001$ on day 5 and 6, respectively." (lines 302-305)

L396. It should be a negative correlation, not positive.
We thank the Reviewer for noting this typo. We have now changed this text on **line 425-426**.

L436. The statement that algal DMSP lyases seem to be exclusively extracellular, is not correct. The Stefels paper is the only one that reported extracellular lyase activity, and that study might have methodological issues that led to that conclusion. Evidence against extracellular lyase in Phaeocystis (the same genus studied by Stefels) was presented in del Valle et al (2011, Marine Chemistry, 124: 57-67). Admittedly, few studies have looked at this directly, but even from the bacterial side, most of the evidence from natural water samples (algae and bacteria present) points to intracellular degradation of DMSP. This is based on the fact that an inhibitor of DMSP uptake (e.g. glycine betaine), which does not inhibit DMSP lyases, is nearly 100% effective at blocking DMSP degradation (e.g. Li et al. 2016, Environ. Chem. 13: 266). If extracellular lyases were important, DMSP degradation would not be blocked by glycine betaine. Furthermore, the bacterial taxa that were identified to have an extracellular lyase (Alcaligenes sp), and its lyase type (dddY), are not prevalent in marine systems (Moran et al Ann Rev Marine Sci, 2012, 4: 523).
We thank the Reviewer for his/her comment. We have now modified this paragraph accordingly: "Although sporadic, the increases in DMS and DMSO observed in the 32°C treatment may have resulted from enhanced intracellular DMSP cleavage by phytoplankton (Del Valle et al., 2011) or enhanced DMSP exudation from phytoplankton cells during cell lysis (Simó, 2001), resulting in an

increasing pool of dissolved DMSP made readily available to both bacteria and phytoplankton DMSP-lyases (Riedel et al., 2015;Alcolombri et al., 2015;Todd et al., 2009;Todd et al., 2007)..." (**see lines 459-470**).

L534. In this conclusion section the authors need to make it clear that the effect was with the extreme 12-degree upshift.
We thank the Reviewer for this suggestion and this change has now been made (**see line 559-564**): "Here, we hypothesized that a very acute increase in temperature, mimicking extreme coastal MHWs, would trigger both a physiological and biochemical stress response in the DMSP-producing dinoflagellate *A. minutum*. This response was indeed observed following a 12°C-increase in temperature, with evidence for impaired photosynthetic efficiency, oxidative stress, spikes in DMS and DMSO concentrations, a drop in DMSP concentration and a shift in the composition of the *A. minutum* microbiome."

Figures 1 and 2. If you are going to connect the data points as a time trend, you should plot them on a linear x-axis rather than a categorical axis, as presently done. The categorical axis gives a misleading impression of the time trend.
We thank the Reviewer for this suggestion and have modified the x-axis throughout Figures 1, 2, 4 and 5.

Figure 3. The x scale is screwed up. Fv/Fm should be less than 1. It seems they have multiplied it by 100. Please fix.
We thank the Reviewer for noting this. We have now amended Figure 3 accordingly.

Interactive comment on "Shifts in organic sulfur cycling and microbiome composition in the red-tide causing dinoflagellate Alexandrium minutum during a simulated marine heat wave" by Elisabeth Deschaseaux et al.

Anonymous Referee #2

Received and published: 30 January 2019

The manuscript reports an experiment where a cultured strain of the dinoflagellate Alexandrium minutum was exposed to temperature increases of 4_C and 12_C. Growth rate, photosynthetic efficiency, oxidative stress, dimethylated sulfur compounds and bacterial community composition were measured over several days. The objective of the experiment was to study if an expected decline in growth rate resulting from impaired physiology was accompanied by up-regulated levels of dimethylated sulfur compounds, and if this matched changes in the microbiome that could be related to sulfur-utilizing bacteria. The environmental context for the lab work is the effects of marine heat waves on coastal ecosystems, including harmful algal blooms.
Even though the idea behind the experiments is timely and interesting, the experimental conditions chosen generate a little concern, and the actual results are only partially convincing. Perhaps the authors can provide further convincing arguments with the data at hand.

I will give my comments following the order of the manuscript:

L55: The role of DMSP as a grazing deterrent is, at the least, debatable. It is true that the works of Wolfe et al. and Strom et al. suggested deterrence, but more recent work by one of the authors and others (Seymour et al.) indicated DMSP may be more an attractant than a deterrent.
The Reviewer makes a fair point and in fact, it is the cleavage of DMSP to DMS and acrylate that is believed to have strong deterrent properties for grazers, most likely through the presence of acrylate at high concentrations. We have now changed this sentence to read: "Many marine phytoplankton produce the organic sulfur dimethyl sulfoniopropionate (DMSP) (Zhou et al., 2009;Berdalet et al., 2011;Caruana and Malin, 2014), for which it can function as an antioxidant, osmolyte, chemoattractant and currency in reciprocal chemical exchanges with heterotrophic bacteria (Stefels, 2000;Sunda et al., 2002; Kiene et al., 2000;Seymour et al., 2010)." (lines 52-56)

L80: acute temperature increases – should you say also "ephemeral"?
The Reviewer makes a fair point and we have now made this change (see lines 79 and 387).

L343-349: I do not like the use of the word "driven" here. Should it be "aligned"? What the MDS analysis shows is that, in the 32_C treatment, differences in the microbiome we aligned with elevated ROS, but that the latter drove the former is just a hypothesis.
The same applies to the microbiome composition and abundances in the control, and to the subsequent comparison of variables.
We agree with the Reviewer's comments and have amended this term accordingly throughout the Results section (**see lines 357-364**).

L374: In the case of the San Francisco Bay, MHW were characterized by "increases in temperature of about 8_C above the yearly average". Was it +8_C of the yearly (annual?) average or of the monthly climatological temperatures? +8_C above the annual average would not be too impressive.
The 8°C increase in temperature referred to here was indeed above the monthly average, whereby the MHW occurred during September, with surface water temperatures reaching 22.6°C, while the average temperature for this month is ~ 14°C. We have now clarified this statement on **lines 389-390**, where we now state: "Large increases in temperature of about 8°C above the monthly climatological average led to red-tides of exceptional density in San Francisco Bay (Cloern et al., 2005)".

I mention this because one of my concerns is with the experimental conditions chosen. +12_C seems quite a dramatic treatment. Is there a record of MHW in the S Australian coast where the strain was

isolated from? Or perhaps this is not relevant – in any case, what are the temperature shift records of MHW in Australian coasts and elsewhere? More 20_C to 24_C, or 20_C to 32_C?
We agree with the Reviewer and in fact, the next sentence of this paragraph acknowledges this point: "While a 12°C increase in temperature constitutes an extreme scenario of MHWs, even for coastal habitats, this experimental temperature was selected with the intention to induce thermal stress in *A minutum*."
The amplitude of the temperature increase was dictated by preliminary experiments conducted at 20°C, 24°C, 28°C, 30°C and 32°C, with only a 12°C increase in temperature (32°C) leading to a physiological stress response in this strain of *Alexandrium* in culture. Although an increase in temperature of this magnitude might be rare in coastal marine systems (which is now acknowledged **on lines 143-146**), this presented an opportunity to investigate the biochemical and microbial consequences of thermal stress on this relevant phytoplankton in the context of MHWs. It is also to be noted that cultured *A. minutum* could be more robust than their free living relatives, and in fact they present biochemical differences that are now acknowledged in the manuscript (**see lines 395-407**).

L396: The correlation is negative, not "positive".
We thank the Reviewer for noting this typo. We have changed this **on line 425-426.**

L421-426: I may understand, as a working hypothesis, that optimal growth (hence less physiological stress) could be associated with lower DMS/P/O concentrations per cell.
But it is harder to understand that sulfur concentrations (per culture volume) decreased during the experiment, even with A. minutum being in exponential growth.
The Reviewer is correct and we believe that this interpretation is due to our initially unclear description of the data. What we meant was that the DMS(O) concentrations were significantly lower than in the 20°C control, rather than that the concentrations decreased. We now clarify this point on **lines 449-454**, where we now state: "This temperature optimum was associated with lower DMS and DMSO concentrations than in the 20°C control, although this was only evident 24h after the start of the experiment. Since algal stress responses often result in increased cellular sulfur concentrations in dinoflagellates (McLenon and DiTullio, 2012;Berdalet et al., 2011), it is perhaps not surprising that DMS and DMSO concentrations were lower under what appear to have been more optimal growth temperature conditions."

L434-438: Why do you say that algal DMSP lyases are exclusively located extracellularly? This is definitely not the case in, e.g., Emiliania huxleyi (works by Steinke, Wolfe, Alcolombri).
The Reviewer is correct and we have now modified our text to reflect this, where we now state on **lines 459-464**: "Although sporadic, the increases in DMS and DMSO observed in the 32°C treatment may have resulted from enhanced intracellular DMSP cleavage by phytoplankton (Del Valle et al., 2011) or enhanced DMSP exudation from phytoplankton cells during cell lysis (Simó, 2001), resulting in an increasing pool of dissolved DMSP made readily available to both bacteria and phytoplankton DMSP-lyases (Riedel et al., 2015;Alcolombri et al., 2015;Todd et al., 2009;Todd et al., 2007)."

L446-451: There always is a difficulty when trying to explain and provide experimental evidence for the role of DMS in scavenging ROS: what is first, the decline in DMS or the decline in ROS? It is probably a matter of time scales and potential upregulation by metabolic synthesis. The arguments you provide here carry some assumption that must be explicated.
The Reviewer makes a good point and we are now acknowledging the level of uncertainties in this paragraph by saying: "In contrast, 24h after the start of the experiment, increased ROS coincided with an abrupt decline in DMS and DMSO, perhaps suggestive of serial oxidation via active ROS scavenging of both DMS to DMSO and DMSO to methane sulfinic acid (MSNA) (Sunda et al., 2002), although it is always difficult to confidently link DMS(O) and ROS dynamics unless using tracing techniques." (**see lines 475-479**).

L492: I would replace DMSP metabolism with DMSP catabolism.
The Reviewer makes a fair point and we have now amended this terminology in line with their suggestion (**see line 515**).

The bacterial community composition characterization was not very informative or illustrative with respect to the cycling of sulfur compounds. Very few of the OTUs that increased their abundances under warming had relatives with genes for sulfur compound transformations. I do not find it any surprising – I think it was too naïve to expect that the bacterial community associated with stressed algae relies mainly on sulfur compounds. Instead, I would expect e.g. opportunistic bacteria. So, I agree with what you say in L513-515. However, I do not agree with your statement in L509-512, at least with the wording used. Quick conversion of DMSP to DMS and oxidation of DMS to DMSO is not a reflection of preferential growth of sulfur-consuming bacteria. Actually, DMSP-to-DMS and DMS-to-DMSO are two processes that do not consume sulfur; if anything, they consume carbon or provide energy. Demethylation of DMSP does lead to sulfur consumption and utilization, and this is a competing process to DMSP cleavage.

The Reviewer is correct and we have reworded this section to clarify our point, which we agree was unclear (**see lines 549-559**). What we meant by "…quick conversion of DMSP to DMS and oxidation of DMS to DMSO…" was that "…rapid changes in DMS and DMSO concentrations were potentially caused by (or led to) a shift in microbiome composition towards the preferential growth of sulfur-consuming bacteria." It is now acknowledged that: "the change in microbial abundance could have also been triggered by a range of other parameters that were not measured in this study." (**Lines 541-543**)

Also, you should not base your explanation of the dynamics of the sulfur compounds on the bacterial community alone. There is a potential large role of the dinoflagellate itself: arrest of methionine synthase activity under growth arrest, DMSP cleavage to DMS by the algal lyases, etc.

We agree with the Reviewer and have now included discussion of these potential processes by stating:

"Although sporadic, the increases in DMS and DMSO observed in the 32°C treatment may have resulted from enhanced intracellular DMSP cleavage by phytoplankton (Del Valle et al., 2011) or enhanced DMSP exudation from phytoplankton cells during cell lysis (Simó, 2001), resulting in an increasing pool of dissolved DMSP made readily available to both bacteria and phytoplankton DMSP-lyases (Riedel et al., 2015;Alcolombri et al., 2015;Todd et al., 2009;Todd et al., 2007)." (**lines 459-464**)

"Since this decrease in DMSP at 96h was not coupled with an increase in DMS, this could alternatively be indicative of a decrease in methionine synthase activity (McLenon and DiTullio, 2012) or assimilation of DMSP-sulfur by bacterioplankton for *de novo* protein synthesis (Kiene et al., 2000), with this demethylation pathway often accounting for more than 80% of DMSP turnover in marine surface waters." (**lines 466-470**)

From the figures: The (opposite) patterns of ROS and FvFm are pretty consistent.
Conversely, the patterns of sulfur compounds are less convincing. The fact that the two controls (20_C) show remarkable differences makes one wonder what would have been the results from repeated perturbations. You may need an extra effort to persuade the readers/reviewers of the robustness of the observed responses with respect to the sulfur compounds.

As described in the method, both experiments were conducted at different times and it was thus not to be excluded that the 2 controls kept at 20'C could present some physiological (Fig. 1 & 2) and biochemical (Fig. 4) differences, which perhaps reflected inherent heterogeneity in biological systems. However, the significant differences that were observed between temperature treatments in each experiment were clearly driven by the increase in temperature since both temperatures (control and experimental) were tested at the same time, on the same culture, and under the exact same experimental conditions of light and GSe medium in each experiment.

Because the turnover of DMS(P)(O) in biological systems can occur very quickly (Simo et al 2000), measured changes in DMS(O) concentrations can seem to occur sporadically. However, a clear cascading stress response emerged from these results, which is worth reporting and discussing.

We have now acknowledged variability and uncertainties **on lines 495-499**, which reads: "Because the turnover of DMS, DMSP and DMSO in biological systems can occur very quickly (Simo et al 2000),

DMS and DMSO concentrations can change rapidly, which sometimes makes it difficult to clearly establish cause-effect relationships between physiological stress and the biogenic sulfur response."

L531: Only the "very acute" treatment elicited a response.
This section has been amended based on comments from both Reviewers and it now reads as follows: "Here, we hypothesized that a very acute increase in temperature, mimicking extreme coastal MHWs, would trigger both a physiological and biochemical stress response in the DMSP-producing dinoflagellate *A. minutum*."

References: the reference Simó 2001 is repeated.
We thank the Reviewer for picking this up. This has been amended.

Figure 4b: The difference between treatments is essentially one time point.
We agree with the Reviewer, however, the fact that differences in sulfur concentration between treatments rely on rapid changes in DMS(O)(P) concentrations, reflective of a quick turnover of DMS(P)(O) in biological systems (Simo et al 2000) is now better acknowledged and explained in the discussion (see **lines 495-499**)

Shifts in ~~organic~~dimethylated sulfur ~~cycling~~concentrations and microbiome composition in the red-tide causing dinoflagellate *Alexandrium minutum* during a simulated marine heat wave

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Abstract

The biogenic sulfur compounds ~~dimethylsulfide~~dimethyl sulfide (DMS), ~~dimethylsulfoniopropionate~~dimethyl sulfoniopropionate (DMSP) and ~~dimethylsulfoxide~~dimethyl sulfoxide (DMSO) are produced and transformed by diverse populations of marine microorganisms and have substantial physiological, ecological and biogeochemical importance spanning organism to global scales. Understanding the production and transformation dynamics of these compounds under shifting environmental conditions is important for predicting their roles in a changing ocean. Here, we report the physiological and biochemical response of *Alexandrium minutum*, a dinoflagellate with the highest reported intracellular DMSP content, exposed to a 6~~-~~day increase in temperature mimicking mild and extreme coastal marine heatwave conditions (+ 4°C and + 12°C). Under mild temperature increases (+ 4°C), *A. minutum* growth was enhanced, with no measurable physiological stress response. However, under ~~an~~a very acute increase in temperature (+ 12°C), *A. minutum* growth declined, photosynthetic efficiency (F_V/F_M) was impaired, and enhanced oxidative stress was observed. These physiological responses indicative of thermal stress were accompanied by increased DMS and DMSO concentrations followed by decreased DMSP concentrations. At this ~~higher~~ temperature extreme, we observed a cascading stress response in *A. minutum*, which was initiated 6h after the start of the experiment by a spike in DMS and DMSO concentrations and a rapid decrease in F_V/F_M. This was followed by an increase in reactive oxygen species (ROS) and an abrupt decline in DMS and DMSO on day 2 of the experiment. A subsequent decrease in DMSP coupled with a decline in the growth rate of both *A. minutum* and its associated total bacterial assemblage coincided with a shift in the composition of the *A. minutum* microbiome. Specifically, an increase in the relative abundance of OTUs matching

the genus *Oceanicaulis* (17.0%), *Phycisphaeraceae* SM1A02 (8.8%) and *Balneola* (4.9%) as well as a decreased relative abundance of *Maribacter* (24.4%), *Marinoscillum* (4.7%) and *Seohaeicola* (2.7%), were primarily responsible for differences in microbiome structure observed between temperature treatments. These shifts in microbiome structure are likely to have been driven by either the changing physiological state of *A. minutum* cells, shifts in biogenic sulfur concentrations, the presence of other solutes, or a combination of ~~both. We~~all. Nevertheless, we suggest that these results point to the significant effect of heatwaves on the physiology, growth and microbiome composition of the red-tide causing dinoflagellate *A. minutum*, as well as potential implications for biogenic sulfur cycling processes and marine DMS emissions.

Keywords: DMS, DMSP, DMSO, oxidative stress, ~~temperature~~thermal stress

1. Introduction

Many marine phytoplankton produce the organic sulfur ~~dimethylsulfoniopropionate~~dimethyl sulfoniopropionate (DMSP) (Zhou et al., 2009;Berdalet et al., 2011;Caruana and Malin, 2014), for which it can function as an antioxidant, osmolyte, ~~grazing deterrent~~chemoattractant and currency in reciprocal chemical exchanges with heterotrophic bacteria (Stefels, 2000;Sunda et al., 2002;~~Wolfe et al., 1997;~~ Kiene et al., 2000;Seymour et al., 2010). Phytoplankton-derived DMSP is in fact a major source of sulfur and carbon for marine heterotrophic bacteria (Kiene et al., 2000), which in turn play a major role in the cycling and turnover of organosulfur compounds in the ocean (Todd et al., 2007;Curson et al., 2011). The subsequent cycling of DMSP into other biogenic sulfur molecules including ~~dimethylsulfide~~dimethyl sulfide (DMS) and ~~dimethylsulfoxide~~dimethyl sulfoxide (DMSO) by a suite of microbial transformation pathways (Kiene et al., 2000;Sunda et al., 2002) and physical drivers (Brimblecombe and Shooter, 1986) have important ecological and biogeochemical implications spanning from cellular to global scales (Sunda et al., 2002;Charlson et al., 1987;DeBose et al., 2008;Van Alstyne et al., 2001;Knight, 2012;Nevitt et al., 1995).

Among DMSP-producing phytoplankton, the dinoflagellate *Alexandrium minutum*, has the highest recorded DMSP cell content, with an average concentration of 14.2 pmol cell^{-1}, compared with less than 1 pmol cell^{-1} in most other dinoflagellates (Caruana and Malin, 2014). Blooms of *A. minutum* occur from the Mediterranean Sea to the South Pacific coast in sea surface waters within temperature ranges of 12°C to 25°C (Laabir et al., 2011). Notably, some strains of *Alexandrium*, including *A. minutum*, produce saxitoxins, which lead to paralytic shellfish poisoning (PSP) and are responsible for the most harmful algal blooms in terms of magnitude, distribution and consequences on human health (Anderson et al., 2012).

A minutum commonly inhabits shallow coastal and estuarine waters (Anderson, 1998), which are globally experiencing substantial shifts in environmental conditions, including increases in sea surface temperature (SST) associated with climate change (Harley et al., 2006). Although generally less studied than chronic temperature rises associated with global climate change (Frölicher and Laufkötter, 2018), acute ephemeral temperature increases known as marine heatwaves (MHWs) (Hobday et al., 2016) have recently been demonstrated to be becoming more frequent and persistent as a consequence of climate change (Oliver et al., 2018). Increases in MHW occurrence are anticipated to become particularly frequent within the shallow coastal

and estuarine waters, where *A. minutum* blooms occur (Ummenhofer and Meehl, 2017;Anderson, 1998).

Coastal MHW events have recently had dramatic impacts on coastal environments. ~~MHW events~~MHWs in Western Australian (2011) and the Northeast Pacific (2013-2015) resulted in significant ecosystem shifts with increases in novel species at the expenses of others (Frölicher and Laufkötter, 2018). The 2016 MHW that was associated with El Niño Southern Oscillations resulted in an 8°C increase in sea surface temperature leading to the mass coral bleaching of more than 90% of the Great Barrier Reef (Hughes et al., 2017). While it is clear that MHWs can have severe consequences on a variety of systems and organisms, their effects on marine microbes and the biogeochemical processes that they mediate have rarely been investigated (Joint and Smale, 2017).

While there is evidence that increases in seawater temperature can lead to increased DMSP and/or DMS concentrations in phytoplankton (McLenon and DiTullio, 2012;Sunda et al., 2002), it is not clear how a shift in DMSP net production by phytoplankton under acute temperature stress will alter the composition and function of their associated microbiome and how, in turn, this will influence biogenic sulfur cycling processes within marine habitats. There is therefore a pressing need to understand the physiological and biogeochemical consequences of thermal stress on phytoplankton-bacteria interactions within the context of events such as MHWs. This is particularly important, given that a shift in the composition of the phytoplankton microbiome could potentially dictate atmospheric DMS fluxes depending on whether the bacterial community preferentially cleave or demethylate DMSP (Todd et al., 2007;Kiene et al., 2000).

The aims of this study were to investigate how acute increases in temperature, such as those associated with MHW events, alter the physiological state and biogenic sulfur cycling dynamics of *A. minutum* and determine how these changes might influence the composition of the *Alexandrium* microbiome. We hypothesized that an abrupt increase in temperature would lead to physiological impairment (Falk et al., 1996;Robison and Warner, 2006;Iglesias-Prieto et al., 1992;Rajadurai et al., 2005) and oxidative stress (Lesser, 2006) in *A. minutum*, leading to an up-regulation of DMSP, DMS and DMSO production (McLenon and DiTullio, 2012;Sunda et al., 2002) in this high DMSP producer, which could ultimately lead to a shift in the composition of the *A. minutum* microbiome.

2. Methods

2.1.Culturing and experimental design

Cultures of *Alexandrium minutum* (CS-324), isolated from Southern Australian coastal waters (Port River, Adelaide, 11/11/1988, CSIRO, ANACC's collection) were grown in GSe medium at 18°C and 50 µmol photons m^{-2} s^{-1} under a 12:12 light:dark cycle. One month before the start of each experiment, *A. minutum* cultures were acclimated over four generations to 20°C (average summer temperature at Port River, IMOS) and 200 µmol photons m^{-2} s^{-1} ~~(~~using a 14:10 ~~hour~~h light:~~ ~~dark cycle~~).~~ mimicking summer conditions. Light intensity was comparable to that used in Berdalet et al. (2011) for *A. minutum* and conveniently allow to grow other algae cultures using the same facilities. Cultures were grown to a cell concentration of ~60,000 mL^{-1} before cells were inoculated into fresh GSe medium. Six days prior to the start of ~~experiments~~each experiment, 20 L of GSe medium was inoculated with a cell concentration of 1,140 mL^{-1} (experiment 1, April 2016) and 680 mL^{-1} (experiment 2, June 2016) and aliquots of 500 mL were transferred into 40 individual 750 mL sterile tissue culture flasks. Culture flasks were incubated in four independent water baths (10 flasks in each) and maintained under control conditions of 20°C and 200 µmol photons m^{-2} s^{-1}. Temperature and light control was achieved using circulating water heaters (Julabo, ~~country~~USA) and programmable LED lights (Hydra FiftyTwo, ~~Company, Country~~AquaIllumination, USA). All cultures were mixed twice daily to keep cells in suspension by gentle swirling.

On Day 1 (T_0), five culture flasks from each 20°C water bath were transferred to four new water baths for exposure to experimental treatment temperatures (either 24°C experiment 1; or 32°C, experiment 2), so that each control and experimental water bath contained five flasks. Experimental temperatures were carefully chosen based on preliminary experiments conducted at 24°C, 28°C, 30°C and 32°C, where only a 12°C increase in temperature (32°C treatment) led to a physiological stress response in this strain of *A. minutum* in culture. Although an increase in temperature of this magnitude might be rare in coastal marine systems, this presented a unique opportunity to investigate the consequences of MHW-induced thermal stress on this relevant phytoplankton. One culture flask from each tank was immediately sampled for baseline measurements of: DMS (2 mL), DMSP and DMSO (1 mL) concentrations, photochemical efficiency (3 mL), algal and bacterial cell counts (1 mL), ROS quantification (1 mL) and DNA extraction (~470 mL). The dissolved DMSP fraction was not determined because preliminary investigations showed that gravity filtration was too time

consuming, potentially due to clogging of filters by the large *A. minutum* cells (30 µm diameter), leading to filtration artefacts for DMSP analysis, as have previously been mentioned by Berdalet et al. (2011). At 18:00 on Day 1 (T_6), 12:00 on Day 2 (T_{24}), 12:00 on Day 5 (T_{96}) and 12:00 on Day 6 (T_{120}), one flask from each of the eight water baths was removed from the incubation conditions and sampled as described above.

2.2.Photosynthetic efficiency measurements

Subsamples for measurement of photosynthetic efficiency were dark adapted for 10 min under aluminium foil and transferred to a quartz cuvette for Pulse Amplitude Modulated (PAM) fluorometric analysis using a Water PAM (Walz GmbH, Effeltrich, Germany). Once the base fluorescence (F_0) signal had stabilized (measuring light intensity 3, frequency 2s), a saturating pulse (intensity 12, Width 0.8s) was used to measure the maximum quantum yield (F_V/F_M) of photosystem II (PSII). As base fluorescence is dependent on cell density, the photomultiplier gain was adjusted and recorded to maintain F_0 at a level of 0.2 a.u. before saturating the photosystem. Samples were kept in suspension during measurements via continuous stirring at minimal speed inside the quartz cuvette to avoid cells settling.

2.3.Microalgal and bacterial cell counts

Subsamples for bacterial cell counts were stained with SYBR Green at a final concentration of 1:10,000 and incubated in the dark for 15 min (Marie et al. 1997). Subsamples for microalgal cell counts and stained subsamples for bacterial cell counts were diluted 1:10 and 1:100 respectively into sterile GSe medium prior to analysis with a BD Accuri C6 Flow Cytometer (Becton Dickinson). Phytoplankton cells were discriminated using red auto-fluorescence and side scatter (SSC), whereas bacterial populations were discriminated and quantified using SYBR green fluorescence and SSC.

2.4.Reactive oxygen species measurements

The presence of reactive oxygen species (ROS) was detected within cultures using the fluorescent probe 2,7-dichlorodihydrofluorescein-diacetate (CM-H2DCFDA; Molecular Probes), which binds to ROS and other peroxides (Rastogi et al., 2010). The reagent was thawed at room temperature for 10 min and activated using 86.5 µL of DMSO, with 5 µL of activated reagent added to each sample (final concentration 5 µM). Samples were vortexed for 5 sec and incubated at room temperature for 30 min. Samples were then centrifuged at 2,000 g for 2 min, the supernatant with reagent dye was discarded, and stained cells were resuspended

in 1 mL of PBS, prior to quantification of fluorescence by flow cytometry. Mean green fluorescence was quantified from cytograms of forward light scatter (FSC) against green fluorescence. A positive (+ 10 µL of 30% H_2O_2, final concentration 97mM) and negative (no ROS added) control of PBS were run to ensure that detected cell fluorescence was completely attributable to the ROS probe.

2.5.Sulfur analysis by gas chromatography

The preparation of all blanks and samples used in the dilution steps described below were prepared with sterile (0.2 µM filtered and autoclaved) phosphate-buffered saline (PBS, salinity 35ppt) to avoid cell damage from altered osmolarity and to maintain similar physical properties as seawater during headspace analysis by gas chromatography. Aliquots for DMS analysis were transferred into14 mL headspace vials that were immediately capped and crimped using butyl rubber septa (Sigma Aldrich Pty 27232) and aluminum caps (Sigma Aldrich Pty 27227-U), respectively. DMSP aliquots were 1:1 diluted with sterile PBS and DMSP was cleaved to DMS by adding 1 pellet of NaOH to each vial, which was immediately capped and crimped. Samples were incubated for a minimum of 30 min at room temperature to allow for the alkaline reaction and equilibration to occur prior to analysis by gas chromatography (Kiene and Slezak, 2006). ~~At the end of the experiment, alkaline samples used for DMSP analysis were uncapped and left to vent overnight under a fume hood. On the next day, samples were purged for 10 min with high purity N_2 at an approximate flow rate of 60 mL min^{-1} to remove any remaining DMS produced from the alkaline treatment. Samples were then neutralized by adding 80 µL of 32 % HCl and DMSO was converted to DMS by adding 350 µL of 12 % $TiCl_3$ solution to each vial, which was then immediately capped and crimped (Kiene and Gerard, 1994;Deschaseaux et al., 2014b). Vials were then heated in a water bath at 50°C for 1h and cooled down to room temperature prior to analysis by gas chromatography.~~

DMS~~, DMSP~~ and ~~DMSO~~DMSP samples were analyzed by 500 µL direct headspace injections using a Shimadzu Gas Chromatograph (GC-2010 Plus) coupled with a flame photometric detector (FPD) set at 180°C with instrument grade air and hydrogen flow rates set at 60 mL min^{-1} and 40 mL min^{-1}, respectively. DMS was eluted on a capillary column (30 m x 0.32 mm x 5 µm) set at 120°C using high purity Helium (He) as the carrier gas at a constant flow rate of 5 mL min^{-1} and a split ratio of five. A six-point calibration curve and PBS blanks were run by 500 µL direct headspace injections prior to subsampling culture flasks using small volumes of concentrated DMSP.HCl standard solutions (certified reference material WR002, purity 90.3

± 1.8% mass fraction, National Measurement Institute, Sydney, Australia) that were diluted in sterile PBS to a final volume of 2 mL. Detection limit was 50 nM for 500µL headspace injections. Concentrations obtained in vials treated with NaOH accounted for both DMS and DMSP. Consequently, DMSP concentration in each sample was obtained by subtracting the corresponding DMS concentration.

Following DMS and DMSP analysis, alkaline samples used for DMSP analysis were uncapped and left to vent overnight under a fume hood. On the next day, samples were purged for 10 min with high purity N_2 at an approximate flow rate of 60 mL min^{-1} to remove any remaining DMS produced from the alkaline treatment. Samples were then neutralized by adding 80 µL of 32 % HCl and DMSO was converted to DMS by adding 350 µL of 12 % $TiCl_3$ solution to each vial, which was then immediately capped and crimped (Kiene and Gerard, 1994;Deschaseaux et al., 2014b). Vials were then heated in a water bath at 50°C for 1h and cooled down to room temperature prior to analysis by 500 µL direct headspace injections on the GC-FPD as described above. A 5-point calibration curve was run prior to DMSO analysis using DMSO standard solutions (Sigma Aldrich Pty, D2650) diluted in PBS to a final volume of 2 mL and converted to DMS with $TiCl_3$ in the same manner as the experimental samples. PBS blanks treated with NaOH and $TiCl_3$ were also run along with the calibration curves. All dimethylated sulfur compounds were normalised to cell density, which best reflects biogenic production.

2.6.DNA extraction

Following sub-sampling for the physiological and biogenic sulfur measurements described above, the remaining 400 mL within each culture flask was filtered onto a 47 mm diameter, 0.22 µm polycarbonate filter (Millipore) with a peristaltic pump at a rate of 80 rpm to retain cells for DNA analysis. The filters were subsequently stored in cryovials, snap frozen with liquid nitrogen and stored at -80°C until extraction. DNA extraction was performed using a bead-beating and chemical lysis based DNA extraction kit (PowerWater DNA Isolation Kit, MoBio Laboratories) following the manufacturer's instructions. DNA quantity and purity were checked for each sample using a Nanodrop 2000 (Thermo Fisher Scientific, Wilmington, DE, USA). Three replicate samples with the highest DNA quantity and purity from the control and treatment tanks, collected at the beginning (T_0) and end (T_{120}) of the experiment, were subsequently sequenced.

2.7.16S rRNA amplicon sequencing and bioinformatics

To characterize the bacterial assemblage structure (microbiome) of *A. minutum* cultures, we employed 16S rRNA amplicon sequencing. We amplified the V1-V3 variable regions of the 16S rRNA gene using the 27F (AGAGTTTGATCMTGGCTCAG, Lane, 1991) and 519R (GWATTACCGCGGCKGCTG, Turner et al., 1999) primer pairing, with amplicons subsequently sequenced using the Illumina MiSeq platform (Ramaciotti Centre for Genomics; Sydney, NSW, Australia) following the manufacturer's guidelines. Raw data files in FASTQ format were deposited in the National Center for Biotechnology Information (NCBI) Sequence Read Archive (SRA) under the study accession number PRJNA486692.

Bacterial 16S rRNA gene sequencing reads were analysed using the QIIME pipeline (Caporaso et al., 2010;Kuczynski et al., 2012). Briefly, paired-end DNA sequences were joined, de novo Operational Taxonomic Units (OTUs) were defined at 97% sequence identity using UCLUST (Edgar, 2010) and taxonomy was assigned against the SILVA v128 database (Quast et al., 2012;Yilmaz et al., 2013). Chimeric sequences were detected using usearch61 (Edgar, 2010) and together with chloroplast OTUs were filtered from the dataset. Sequences were then aligned, filtered and rarefied to the same depth to remove the effect of sampling effort upon analysis.

2.8.Statistical analysis

Repeated measures analysis of variance (rmANOVA) models were fitted to the data to quantify the effects of temperature and time (fixed factors) on all response variables measured in this experiment (cell density, F_V/F_M, ROS, DMS, DMSP and DMSO concentrations) using IBM SPSS Statistics 20. Assumptions of sphericity were tested using Mauchly's test. In cases where this assumption was violated, the degrees of freedom were adjusted using the Greenhouse-Geisser correction factor. Bonferroni adjustments were used for pairwise comparisons. Each variable was tested for the assumption of normality and log, ln or sqrt transformations were applied when necessary.

For sequencing data, alpha diversity parameters of the rarefied sequences and Jackknife Comparison of the weighted sequence data (beta diversity) were calculated in QIIME (Caporaso et al., 2010). A two-way PERMANOVA with Bray-Curtis similarity measurements was performed on abundance data of taxonomic groups that contained more than 1% of total generated OTUs (represent 90.23% of the data) using PAST (Hammer et al., 2008). In addition, PAST was used to perform non-metric multidimensional scaling (nMDS)

analysis and isolate the environmental parameters (normalised as follows: (x-mean)/stdev) that contributed the most to the differences between groups using the Bray-Curtis similarity measure. SIMPER analysis performed with the White *t*-test was used to identify the taxonomic groups that significantly contributed the most to the shift in bacterial composition in *A. minutum* cultures over time and between temperature treatments.

3. Results

3.1. Algal growth and physiological response

A. minutum cell abundance exponentially increased over time in both the control (20°C) and 24°C temperature treatment, but a significantly faster growth rate ($p = 0.001$, *t*-test) occurred at 24°C (2.66 ± 0.01 d^{-1}; average ± SE) compared to the 20°C control (2.57 ± 0.01 d^{-1}), resulting in significantly greater cell abundance at 96h ($p = 0.007$) and 120h ($p < 0.001$) (rmANOVA, **Table 1, Fig. 1a**). On the other hand, the 32°C treatment resulted in decreased growth rates (2.40 ± 0.02 d^{-1} versus 2.58 ± 0.02 d^{-1}; *t*-test) and significantly lower cell abundance, relative to the 20°C control, at all time points from 6h after the start of the experiment ($p \leq 0.03$; rmANOVA, **Table 1**, **Fig. 1b**). *A. minutum* abundance demonstrated a marked decline on day 5 in the 32°C treatment.

No significant difference in the maximum quantum yield (F_V/F_M) of *A. minutum* cultures occurred between 20°C and 24°C until 120h after the start of the experiment, where a significantly lower F_V/F_M occurred in the 24°C treatment ($p = 0.01$; rmANOVA, **Table 1**, **Fig. 2a**). In contrast, F_V/F_M was significantly lower in *A. minutum* cultures maintained at 32°C compared to the 20°C control at all time points from 6h after the start of the experiment ($p \leq 0.01$~~) (~~; rmANOVA, **Table 1**, **Fig. 2b**). However, on days 5 and 6, the F_V/F_M of cultures kept at 32°C recovered to values (0.72 ± 0.008) close to those of the control (0.75 ± 0.004) (**Fig. 2B**~~).~~**2B**), although it remained significantly lower than at 20°C ($p < 0.01$ and $p < 0.001$ on day 5 and 6, respectively.

3.2. Reactive oxygen species (ROS)

Significantly lower concentrations of ROS were measured at 24°C than at 20°C at 96h ($p = 0.003$) and 120h ($p = 0.03$) (rmANOVA, **Table 1, Fig. 2c**). In contrast, significantly greater concentrations of ROS were measured at 32°C than at 20°C ~~at~~ 24h ($p < 0.001$), 96h ($p = 0.001$) and 120h ($p = 0.01$) after the start of the experiment (rmANOVA, **Table 1**, **Fig. 2d**). In-line

with the recovery in measured F_V/F_M, ROS concentrations in cultures kept at 32°C started to decline to values closer to those of the control on days 5 and 6 of the experiment (**Fig. 2d**). A significant negative correlation between F_V/F_M levels and ROS concentrations was observed under the 32°C temperature treatment ($R^2 = 0.623$; $p = 0.02$, $n = 18$; **Fig. 3**).

3.3. Biogenic sulfur dynamics

~~Cellular~~Biogenic concentrations of DMSP, DMS and DMSO ranged from ~~444 ± 33~~424 ± 35 to ~~1681 ± 175~~1629 ± 170 fmol cell^{-1}, from 13 ± 1.02 to 87 ± 5 fmol cell^{-1} and from 9 ± 1.41 to 94 ± 24 fmol cell^{-1}, respectively, over both experiments (**Fig. 4**). Concentrations of all three sulfur compounds slowly decreased over time in all *A. minutum* cultures regardless of the temperature treatment. No significant difference in DMSP concentration was recorded between 20°C and 24°C throughout the experiment ~~1~~ ($p > 0.05$; rmANOVA, **Table 1**, **Fig. 4a**), whereas significantly less DMSP was measured in cells at 32°C than in the 20°C control at 96h ($p = 0.02$; rmANOVA, **Table 1**, **Fig. 4b**).

Significantly lower DMS concentrations were measured at 24°C compared to 20°C at 24h ($p < 0.001$) and 120h ($p = 0.002$) (rmANOVA, **Table 1**, **Fig. 4c**). In contrast, DMS was significantly higher at 32°C than 20°C 6h after the start of the experiment ($p = 0.008$; rmANOVA, **Table 1**, **Fig. 4d**). A similar pattern was observed for DMSO, where relative to the controls, it was significantly lower at 24°C 24h after the start of the experiment ($p = 0.001$; rmANOVA, **Table 1**, **Fig. 4e**) and significantly greater at 32°C after 6h and 24h ($p < 0.05$, **Fig. 4f**).

3.4. Bacterial abundance and composition

Bacterial cell abundance exponentially increased over time at both 20°C and 24°C (**Fig. 5a**). Bacterial abundance was significantly greater at 24°C than at 20°C 120 h after the start of the experiment ($p = 0.05$; rmANOVA, **Table 1**, **Fig. 5a).** However, no significant difference ($p > 0.05$, *t*-test) in bacterial growth rate was observed between 20°C (4.15 ± 0.05 d^{-1}) and 24°C (4.18 ± 0.01 d^{-1}). In contrast, bacterial growth rate was significantly lower at 32°C than ~~at~~in the 20°C control (4.05 ± 0.01 d^{-1} versus 4.23 ± 0.02 d^{-1}; $p < 0.001$, *t*-test) (**Fig. 5b**), resulting in significantly lower bacterial cell densities at 24h ($p = 0.002$), 96h ($p = 0.002$) and 120h ($p < 0.001$) relative to the control (rmANOVA, **Table 1**, **Fig. 5b**).

The composition of the initial (T_0) *A. minutum* microbiome was consistent across all samples, but then diverged significantly with time and between temperature treatments (**Fig. 6a-b;** Bray-Curtis similarity measurement, Shepard plot stress = 0.0587). A significant temporal shift in bacterial composition occurred at both 20°C and 32°C, with dissimilarities in community composition between T_0 and T_{120} of 27% and 42% occurring respectively (SIMPER analysis). Notably, bacterial communities at 32°C differed significantly (two-way PERMANOVA; $p < 0.05$) to 20°C at T_{120}, with 32% dissimilarity in community composition. These differences were primarily driven by increased relative abundance of bacterial Operational Taxonomic units (OTUs) within the *Oceanicaulis* (17%), *Phycisphaeraceae SM1A02* (8.8%) and *Balneola* (4.9%) genus along with a decline in the relative abundance of OTUs matching *Maribacter* (24%), *Marinoscillum* (4.7%) and *Seohaeicola* (2.7%) (*Rhodobacter* family) in the 32°C treatment (White test, **Fig. 6c**), with all taxa cumulatively contributing to 63% of the OTU differences between temperature treatments at T_{120} (SIMPER analysis). In the 32°C treatment, differences in microbiome composition between T_0 and T_{120} were ~~driven by~~aligned with the elevated levels of ROS, while in the control (20°C) the community shift was principally ~~driven by~~aligned with differences in bacterial and algal cell abundance (**Fig. 6a**; MDS analysis). Similarly, the elevated concentration of ROS as well as the lower F_V/F_M, lower algal and bacterial cell abundance and lower DMSP, DMS and DMSO concentrations ~~in the~~at 32°C ~~drove~~were aligned with the differences in microbiome composition between the temperature treatments (**Fig. 6b**; MDS analysis)

4. Discussion

Climate change induced shifts within marine ecosystems are predicted to fundamentally alter the physiology of planktonic organisms and the biogeochemical transformations that they mediate (Finkel et al., 2009;Tortell et al., 2008;Hallegraeff, 2010). Rising seawater temperatures are one of the major impacts of climate change on marine ecosystems (Harley et al., 2006), and can be manifested both as long-term gradual increases (IPCC, 2007, 2013) or intense episodic marine heatwaves (Frölicher and Laufkötter, 2018;Hobday et al., 2016). Although less examined to date than chronic temperature increases, MHWs are predicted to become more frequent and severe (Oliver et al., 2018) and have been proposed as a mechanism for triggering toxic algal blooms (Ummenhofer and Meehl, 2017). Against this ~~back-drop~~backdrop of changing environmental conditions, microbial production and cycling of dimethylated sulfur compounds could be particularly relevant because they simultaneously

play a role in the stress response of marine phytoplankton (Berdalet et al., 2011;Deschaseaux et al., 2014a;Sunda et al., 2002;Wolfe et al., 2002;Stefels and van Leeuwe, 1998) and have been predicted to have biogeochemical feed-back effects that are relevant for local climatic processes (Charlson et al., 1987).

This study investigated the biogenic sulfur cycling dynamics of *A. minutum*, and its microbiome, in response to an intense, short-term thermal stress event, akin to the marine heat-wave events occurring with increasing frequency within coastal habitats (Oliver et al., 2018). Indeed, MHWs have been defined as an abrupt and ephemeral increase in temperature of at least 3 to 5°C above climatological average that lasts for at least 3 to 5 days (Hobday et al., 2016). Large increases in temperature of about 8°C above the ~~yearly~~monthly climatological average led to red-tides of exceptional density in San Francisco Bay (Cloern et al., 2005). While a 12°C increase in temperature constitutes an extreme scenario of MHWs, even for coastal habitats, this experimental temperature was selected after preliminary investigations with the intention to induce thermal stress in this strain of *A minutum* in culture.

A. minutum has been targeted in this study as 1) an ecologically relevant phytoplankton responsible for some of the most harmful algal blooms (Anderson et al., 2012) and 2) as biochemically relevant for containing the highest DMSP concentrations ever reported in marine dinoflagellates (Caruana and Malin, 2014). However, it is to be noted that DMSP concentrations reported in this study were a degree of magnitude lower (0.42 ± 0.04 to 1.63 ± 1.70 pmol cell^{-1}) than that previously reported for *A. minutum* (14.2 pmol cell^{-1}; Caruana and Malin, 2014;Jean et al., 2005). This is potentially because this culture of *A. minutum* had been isolated from free-living *A. minutum* for a long time (1988) or because culturing conditions failed to mimic the exact same biochemical conditions in which this strain of *A. minutum* usually grow. This biochemical difference could potentially reflect that this strain of *A. minutum* in culture was more robust than free-living dinoflagellates of the same species, thereby potentially justifying the need of a 12°C increase in temperature to induce thermal-stress.

4.1.Effects of thermal stress on A. minutum growth, physiology and ROS production

A 4°C increase in temperature resulted in faster algal growth and lower oxidative stress, indicating that 24°C was close to a temperature optimum for this strain of *Alexandrium*. This

is perhaps not surprising considering that *Alexandrium* species are capable of growing under a wide range of temperatures from 12°C to 25°C (Laabir et al., 2011). In contrast, a 12°C increase in temperature resulted in a rapid and clear cascade of physiological responses, indicative of an acute thermal stress response in *A. minutum*. Overall, *A. minutum* cells exposed to 32°C immediately exhibited slower growth relative to the 20°C control, suggesting that a 12°C increase in temperature rapidly led to either an increase in cell death rate or a decrease in cell division (Rajadurai et al., 2005;Veldhuis et al., 2001). The slower growth rate at 32°C was coupled with a drop in photosynthetic efficiency and an increase in ROS concentrations, which are both common stress responses to thermal stress in marine algae (Lesser, 2006;Falk et al., 1996;Robison and Warner, 2006;Iglesias-Prieto et al., 1992). In fact, these two physiological responses are often interconnected as increased ROS production generally occurs in both the chloroplast and mitochondria of marine algae exposed to thermal stress, causing lipid peroxidation and ultimately leading to a loss in thylakoid membrane integrity (Falk et al., 1996) and a decrease in the quantum yield of PSII (Lesser, 2006). This was reflected in the ~~positive~~negative correlation observed between the maximum quantum yield of PSII and ROS concentrations.

Although photosynthetic efficiency remained impaired and ROS concentrations remained high under 32°C until the end of the experiment, both biomarkers of stress started to return to values closer to those of the 20°C control by day 5 and 6 of the experiment. This was most likely at the expense of a decline in algal abundance since slow growth often coincides with concurrent cellular repair and photosystem activity recovery (Robison and Warner, 2006). The differential physiological response between 24°C and 32°C indicates that although cultures of this strain of *A. minutum* appear to be highly resistant to temperature changes, an abrupt increase in temperature of 12°C simulating an extreme marine heatwave led to a ~~prolonged (4 day)~~clear stress response. ~~It could~~The physiological pattern at 32°C also ~~suggest~~suggested an acclimation period necessary for such an abrupt shift in temperature, especially since recovery (in F_V/F_M and ROS levels) was observed towards the end of the experiment.

4.2.Biogenic sulfur cycling as a response to thermal stress in A. minutum

Biogenic organic compounds are key compounds in the stress response of phytoplankton, with evidence they can be used in responses to changes in temperature (Van Rijssel and Gieskes, 2002;Stefels, 2000). An up-regulation of the biogenic sulfur yield was expected as a stress

response to increased temperature in *A. minutum*, through either an increase in cellular DMSP concentrations, or an increase in DMS via the cleavage of DMSP (McLenon and DiTullio, 2012;Berdalet et al., 2011;Wolfe et al., 2002;Sunda et al., 2002). No significant change in DMSP concentrations was observed between the control and 24°C treatment, where, as described above, physiological responses converged to indicate that 24°C was in fact a more optimal growth temperature for this organism. This temperature optimum was generally associated with lower ~~cellular~~ DMS and DMSO concentrations than in the 20°C control, although this was only evident 24h after the start of the experiment. Since algal stress responses often result in increased cellular sulfur concentrations in dinoflagellates (McLenon and DiTullio, 2012;Berdalet et al., 2011), it is perhaps not surprising that DMS and DMSO concentrations ~~decreased~~were lower under what appear to have been more optimal growth temperature conditions.

In contrast to the ~~decreases in~~lower DMS and DMSO concentrations observed at 24°C compared to the 20°C control, exposure to 32°C resulted in spikes in DMS and DMSO 6h after the start of the experiment, which accompanied decreased algal growth and impaired photosystem II. ~~The~~ Although sporadic, the increases in DMS and DMSO observed in the 32°C treatment may have resulted from enhanced intracellular DMSP cleavage by phytoplankton (Del Valle et al., 2011) or enhanced DMSP exudation from phytoplankton cells during cell lysis (Simó, 2001), resulting in an increasing pool of dissolved DMSP made readily available to both bacteria and phytoplankton DMSP-lyases (Riedel et al., 2015;Alcolombri et al., 2015;Todd et al., 2009;Todd et al., 2007). ~~Indeed, although DMSP-lyases can be present both extracellularly and intracellularly in marine bacteria (Yoch et al., 1997), algal DMSP-lyases seem to be exclusively located extra-cellularly (Stefels and Dijkhuizen, 1996), indicating that DMSP-cleavage to DMS is mainly possible when DMSP exudes from phytoplankton cells during lysis (Simó, 2001).~~ However, it is notable that lower DMSP concentrations in the 32°C treatment than in the control only occurred on day 4, whereas the spike in DMS and DMSO were evident at the outset of the experiment (6h). Since this decrease in DMSP at 96h was not coupled with an increase in DMS, this could alternatively be indicative of a decrease in methionine synthase activity (McLenon and DiTullio, 2012) or assimilation of DMSP-sulfur by bacterioplankton for *de novo* protein synthesis (Kiene et al., 2000), with this demethylation pathway often accounting for more than 80% of DMSP turnover in marine surface waters. The spike in DMSO measured 6h after the increase in temperature to 32°C most likely indicated rapid DMS oxidation by ROS under thermal stress (Sunda et al., 2002;Niki et al., 2000). At

that time however, we found no evidence for ROS build up in *A. minutum* cultures, possibly because ROS concentrations were kept in check by sufficient DMS synthesis and active DMS-mediated ROS scavenging (Lesser, 2006;Sunda et al., 2002). In contrast, 24h after the start of the experiment, increased ROS coincided with an abrupt decline in DMS and DMSO, perhaps suggestive of serial oxidation via active ROS scavenging of both DMS to DMSO and DMSO to methane sulfinic acid (MSNA) (Sunda et al., 2002~~). ~~), although it is always difficult to confidently link DMS(O) and ROS dynamics unless using tracing techniques.

The only previous study that has examined sulfur responses to stress exposure in *A. minutum* examined the effect of physical turbulence by shaking *A. minutum* cultures for up to four days (Berdalet et al., 2011). While the authors of that study also observed slower cell growth as a response to stress exposure, in contrast to our study, cellular DMSP concentrations increased by 20%. Here, a drop in DMSP concentration was observed at 96h between the control and temperature treatment. Therefore, even though DMSP concentrations were quantified with a similar approach as in Berdalet et al. (2011) (no filtration of the samples with assuming that particulate DMSP concentrations overrule dissolved DMSP and DMS concentrations), it seems that heat stress and turbulence triggered a dissimilar sulfur response to stress in *A. minutum*.

Overall, a 12°C increase in temperature led to lower photosynthetic efficiency, increased oxidative stress and slower cell growth in the red-tide mediating dinoflagellate *A. minutum*. This physiological stress response was coupled with a differential biogenic sulfur cycling as shown by spikes in DMS and DMSO as well as lower DMSP concentrations, most likely translating ROS scavenging and DMSP uptake by bacterioplankton, respectively. Because the turnover of DMS, DMSP and DMSO in biological systems can occur very quickly (Simo et al 2000), DMS and DMSO concentrations can change rapidly, which sometimes makes it difficult to clearly establish cause-effect relationships between physiological stress and the biogenic sulfur response.

4.3. A shift in A. minutum associated-bacteria composition triggered by thermal stress

In light of DMSP and related biogenic sulfur compounds constituting an important source of carbon and sulfur to phytoplankton-associated bacteria (Kiene et al., 2000), it follows that any shift in biogenic sulfur concentrations could influence the microbiome composition of *A. minutum*. ~~Indeed~~However, it is undeniable that a shift in the microbial community could also

be driven by a range of physiological and biochemical parameters that were not measured in this study. Nevertheless, the most pronounced temporal shift in the composition of the bacterial community associated with *A. minutum* occurred in the 32°C treatment. This shift was primarily characterized by a statistically significant increase in the relative abundance of OTUs classified as members of the *Oceanicaulis, Phycisphaeraceae* and *Balneola* and a significant decrease in OTUs classified as members of the *Maribacter, Marinoscillum* and *Seohaeicola*.

To predict any potential role of these key OTUs in biogenic sulfur cycling processes, we screened the genomes of members of these groups using BLAST for four genes commonly involved in DMSP ~~metabolism~~catabolism: *dmdA*, CP000031.2 (Howard et al., 2006); *dddP*, KP639186 (Todd et al., 2009); *tmm*, JN797862 (Chen et al., 2011); and *dsyB*, KT989543 (Kageyama et al., 2018). A BLAST query of the sequences in the NCBI nucleotide collection (nr/nt) database revealed that previously sequenced members of the genera *Maribacter* (taxid:252356, 357 sequences), *Oceanicaulis* (taxid:153232, 36 sequences), *Marinoscillum* (taxid:643701, 23 sequences), *Seohaeicola* (taxid:481178, 18 sequences) and *Balneola* (taxid:455358, 44 sequences) did not possess any homologs of these sulfur cycling genes. While no homologs were found in the genus *SM1A02*, perhaps because very little genomic information is available for this genus~~. However,~~ a close phylogenetic relative to *SM1A02* (99% query cover, 80% identical, E-value = 0.0), and also a member of the *Phycisphaeraceae* family (*P. mikurensis* 10266; genbank accession numbers AP012338.1), possessed significant homologues to all four query genes involved in DMSP metabolism: *dmdA* (92% identical, E-value < 0.001), *dddP* (87% identical, E-value = 0.003), *tmm* (82% identical, E-value = 0.002) and *dsyB* (92% identical, E-value < 0.001). It is thus possible that the spike in DMS and DMSO concentrations in the early stage of the 32°C heat treatment was a consequence of (or contributed to) the preferential recruitment of *Phycisphaeraceae SM1A02*.

Some members of the *Rhodobacter* family such as several members of the *Roseobacter* genus and *Rhodobacter sphaeroides* are known to possess homologues of either or both *dmdA* and *ddd* genes, which are responsible for DMSP demethylation and DMSP-to-DMS cleavage, respectively (Howard et al., 2006;Curson et al., 2008). However, none of the available reference genomes ~~form~~for *Seohaeicola*, a member of the *Rhodobacteracea*, possessed any homologs of targeted biogenic sulfur cycling. Similarly, members of the *Maribacter*, which was the main contributor to the difference in microbiome structure between the control and thermal stress treatment, are known not to possess DMSP/DMS transformation pathways

(Kessler et al., 2018). Hence, the decline of ~~these~~this taxa in the heat stress treatments, where an upshift in biogenic sulfur availability occurred, is perhaps not surprising. However, this change in microbial abundance could have also been triggered by a range of other parameters that were not measured in this study.

Ultimately, the ~~quick conversion of DMSP to~~rapid changes in DMS ~~(Wolfe et al., 2002)~~ and ~~oxidation of DMS to~~ DMSO ~~(Sunda et al., 2002) was~~concentrations were potentially caused by (or led to) a shift in microbiome composition towards the preferential growth of sulfur-consuming bacteria (e.g. *Phycisphaeraceae* SM1A02) at the expense of other types of bacteria (e.g. *Seohaeicola*). Alternatively, the observed shifts in microbiome structure may have occurred independently to the biogenic sulfur cycling processes and was instead related to other metabolic shifts in the heat-stressed *A. minitum*. Notably, the temporal shift in bacterial composition under thermal stress was associated with increased cellular ROS at the end of the experiment, indicating a potential link to oxidative stress. ~~However, in light of the phylogenetic patterns discussed above, this correlation could also reflect a secondary correlation driven by a sulfur-related cascade response, whereby an increase in ROS could have led to an up-regulation of DMSP synthesis (McLenon and DiTullio, 2012;Sunda et al., 2002) and DMSP exudation from *A. minutum* cells (Simó, 2001).~~

5. Conclusion

Abrupt and intense increases in seawater temperatures associated with MHWs are predicted to become more frequent and intense (Oliver et al., 2018) and have the potential to influence the structure of coastal microbial assemblages and the nature of the important biogeochemical processes that they mediate. Here, we hypothesized that ~~an~~a very acute increase in temperature, mimicking ~~a~~extreme coastal ~~MHW~~MHWs, would trigger both a physiological and biochemical stress response in the DMSP-producing dinoflagellate *A. minutum*. This response was indeed observed following a 12°C-increase in temperature, with evidence for impaired photosynthetic efficiency, oxidative stress, spikes in DMS and DMSO concentrations, a drop in DMSP concentration and a shift in the composition of the *A. minutum* microbiome. These patterns are indicative of a profound shift in the physiological state and biochemical function of ~~an~~this

ecologically relevant dinoflagellate ~~under MHW conditions~~ in the context of MHWs and suggest that ~~MHWs have~~extreme thermal stress has the potential to not only influence the composition and interactions of coastal microbial food-webs, but re-shape sulfur budgets in coastal waters.

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Author contribution:

ED, KP and JS devised the experimental design. ED and JOB conducted the thermal stress experiments, including sampling and sample analysis. NS and JOB processed sequencing data while ED processed the physiological and sulfur data. ED wrote the manuscript with significant contributions from all co-authors.

Competing interests:

The authors declare that they have no conflict of interest.

Figure captions

Figure 1 – Algal cell abundance in *A. minutum* cultures in experiment 1 (20°C and 24°C) (A) and experiment 2 (20°C and 32°C) (B); average ± SE, $n = 4$.

Figure 2 – Photosynthetic efficiency (A, B) and reactive oxygen species (ROS) (C, D) in *A. minutum* cultures in experiment 1 (20°C and 24°C) (A, C) and experiment 2 (20°C and 32°C) (B, D); average ± SE, $n = 4$.

Figure 3 – Correlation between the photosynthetic efficiency and reactive oxygen species (ROS) in *Alexandrium minutum* under the 32°C thermal stress treatment; $n = 18$.

Figure 4 – DMSP (A, B), DMS (C, D) and DMSO (E, F) concentrations in *A. minutum* cultures in experiment 1 (20°C and 24°C) (A, C, E) and experiment 2 (20°C and 32°C) (B, D, F); average ± SE, $n = 4$.

Figure 5 – Bacterial cell abundance in *A. minutum* cultures in experiment 1 (20°C and 24°C) (A) and experiment 2 (20°C and 32°C) (B); average ± SE, $n = 4$.

Figure 6 – Multi-dimensional scaling (MDS) of the three phylogenetic groups defined by 16s sequencing of the bacteria population associated with *A. minutum* cultures grown under control conditions (20°C) and acute thermal stress (32°C) at T_0 and T_{120} (**A**) and MDS excluding the T_0 control (**B**).Vectors represent the factors that most likely drove the shift in bacterial composition between groups. The taxonomic groups that significantly contributed to the difference in bacterial composition between T_0 and T_{120} at 32°C **(1)**, between T_0 and T_{120} at 20°C **(2)** and between 32°C and 20°C at T_{120} **(3)** appear in bold next to the heatmap (**C**), with scaling being based on relative abundance.

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Table 1. Output of repeated measures analysis of variance (rmANOVA) for algal ($CELLS_A$) and bacterial ($CELLS_B$) cell abundance, photosynthetic efficiency (F_V/F_M), oxidative stress (ROS), dimethylsulfoniopropionate (DMSP), dimethylsulfide (DMS) and dimethylsulfoxide (DMSO) concentrations as a function of temperature (24°C or 32°C) and time. Numbers in bold indicate significant data based on the level of significance $p < 0.05$. df1 = numerator df; df2= denominator df.

		24°C – mild thermal stress			32°C – mild thermal stress		
Parameters		temperature	time	temperature × time	temperature	time	temperature × time
$CELLS_A$	*F*	4.04	**335**	**4.16**	**27.47**	**237.62**	**8.28**
	df1	1	**4**	**4**	**1**	**2.04**	**2.04**
	df2	6	**24**	**24**	**6**	**12.26**	**12.26**
	p	0.91	**< 0.001**	**0.01**	**< 0.001**	**< 0.001**	**0.005**
$CELLS_B$	*F*	2.13	**52.2**	1.35	**32.56**	**199.8**	**22.26**
	df1	1	**1.29**	1.29	**1**	**4**	**4**
	df2	6	**7.74**	7.74	**6**	**24**	**24**
	p	0.2	**< 0.001**	0.3	**0.001**	**< 0.001**	**< 0.001**
F_V/F_M	*F*	**0.42**	**33.43**	**6.90**	**48.79**	**12.58**	**13.11**
	df1	**1**	**4**	**4**	**1**	**1.19**	**1.19**
	df2	**6**	**24**	**24**	**5**	**5.93**	**5.93**
	p	**0.54**	**< 0.001**	**0.001**	**0.001**	**0.01**	**0.01**
ROS	*F*	**37.26**	**6.30**	**5.88**	**33.23**	**8.85**	**8.41**
	df1	**1**	**4**	**4**	**1**	**2.32**	**2.32**
	df2	**6**	**24**	**24**	**6**	**13.9**	**13.9**
	p	**0.001**	**0.001**	**0.002**	**0.001**	**0.003**	**0.003**
DMSP	*F*	0.79	**31.16**	0.95	3.03	**15.18**	**3.17**
	df1	1	**1.56**	1.56	1	**4**	**4**
	df2	6	**9.35**	9.35	6	**24**	**24**
	p	0.41	**<0.001**	0.4	0.13	**< 0.001**	**0.03**
DMS	*F*	**51.5**	**38.73**	2.01	5.08	**30.77**	**5.23**
	df1	**1**	**2.14**	2.14	1	**4**	**4**
	df2	**6**	**12.87**	12.87	6	**24**	**24**
	p	**< 0.001**	**< 0.001**	0.17	0.07	**< 0.001**	**0.004**
DMSO	*F*	**36.56**	**26.64**	**7.21**	4.68	**14.74**	**7.14**
	df1	**1**	**4**	**4**	1	**4**	**4**
	df2	**6**	**24**	**24**	6	**24**	**24**
	p	**0.001**	**< 0.001**	**0.001**	0.07	**< 0.001**	**0.001**

Figure 1

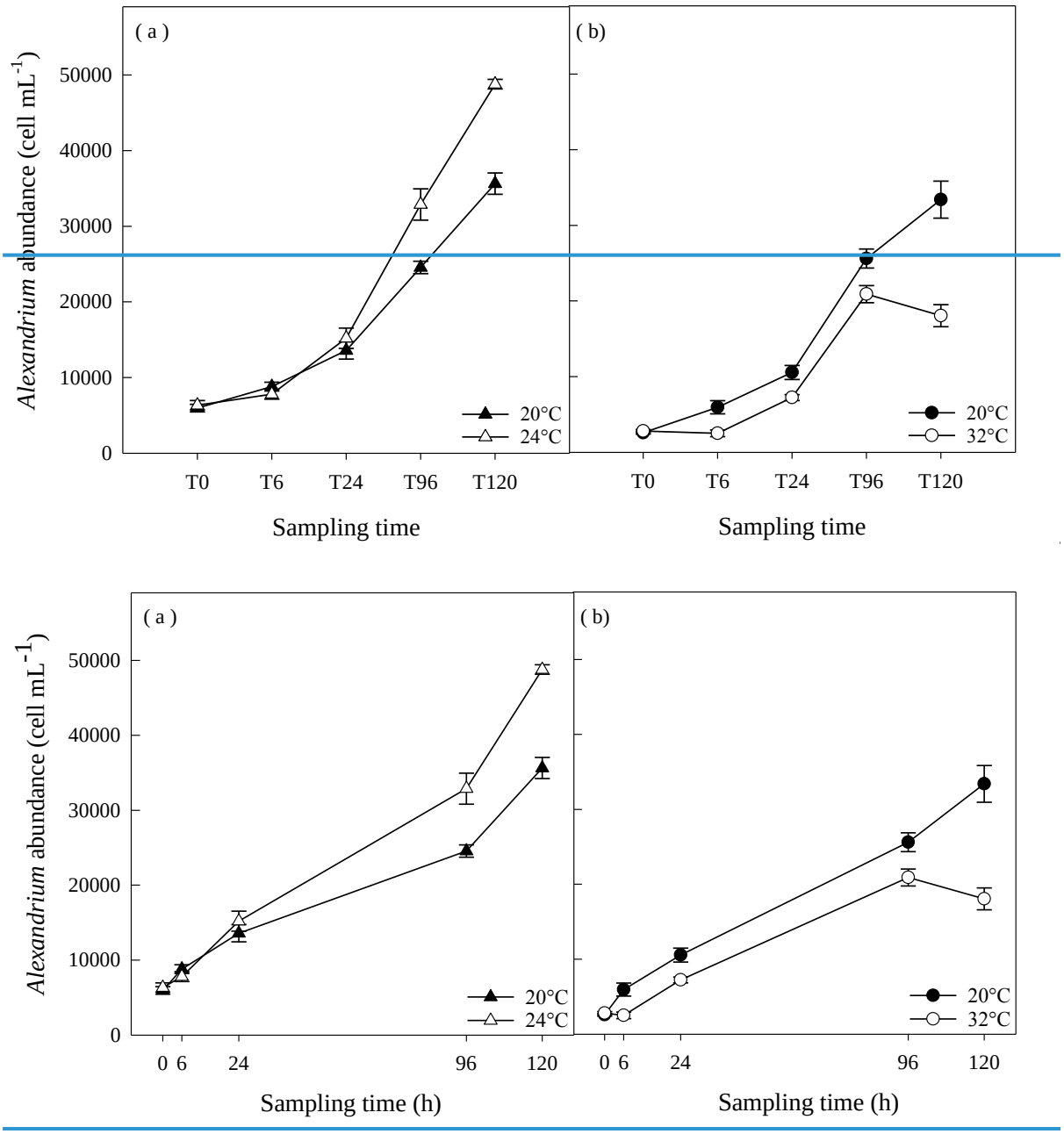
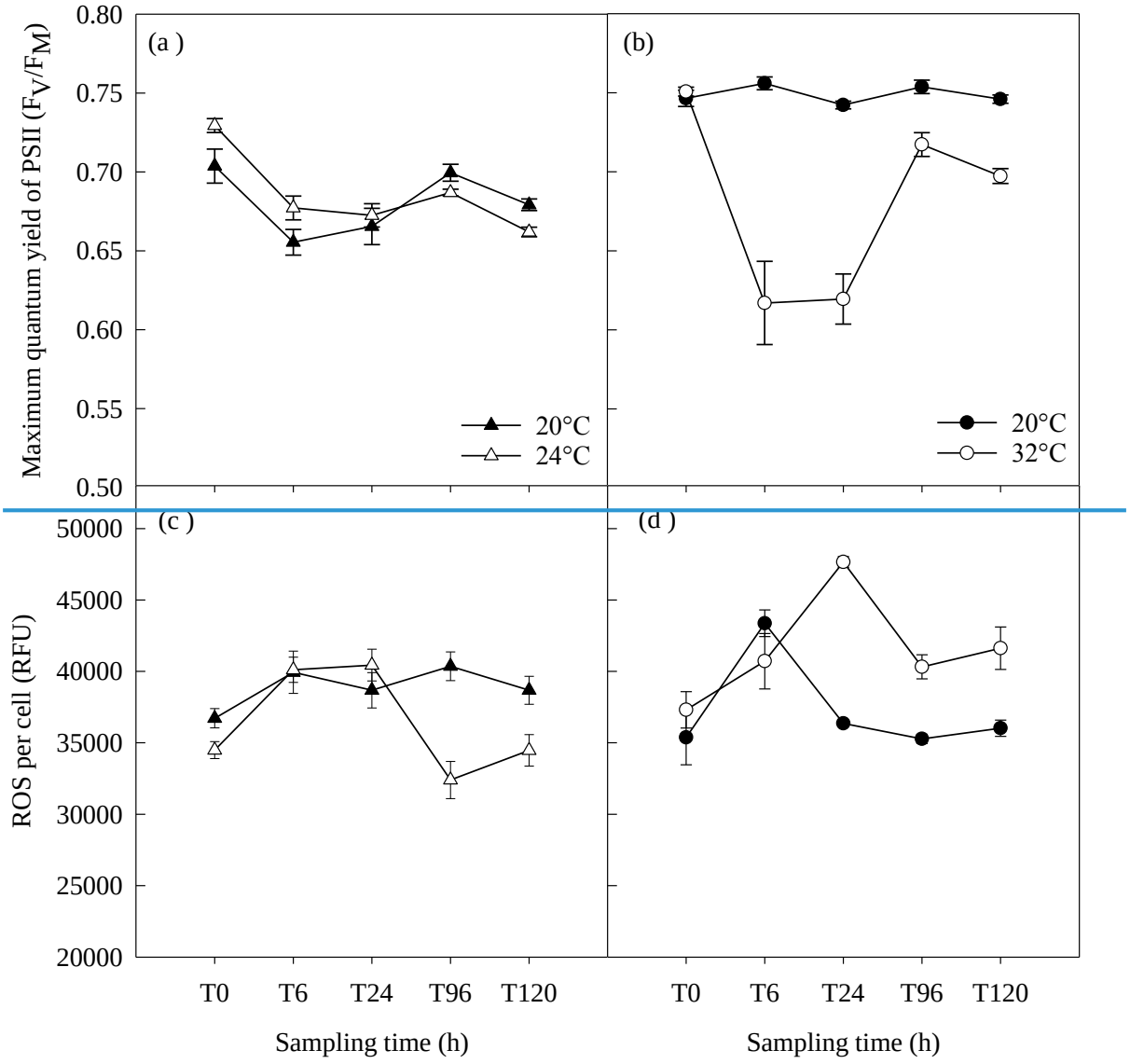

(a)
(b)
Alexandrium abundance (cell mL-1)
50000
40000
30000
20000
10000
0
T0
T6
T24
T96
T120
Sampling time
20°C
24°C
32°C
0 6
24
96
120
Sampling time (h)

Figure 2


(a)
(b)
(c)
(d)
Maximum quantum yield of PSII (F_V/F_M)
ROS per cell (RFU)
0.80
0.75
0.70
0.65
0.60
0.55
0.50
50000
45000
40000
35000
30000
25000
20000
20°C
24°C
20°C
32°C
T0
T6
T24
T96
T120
Sampling time (h)
Sampling time (h)

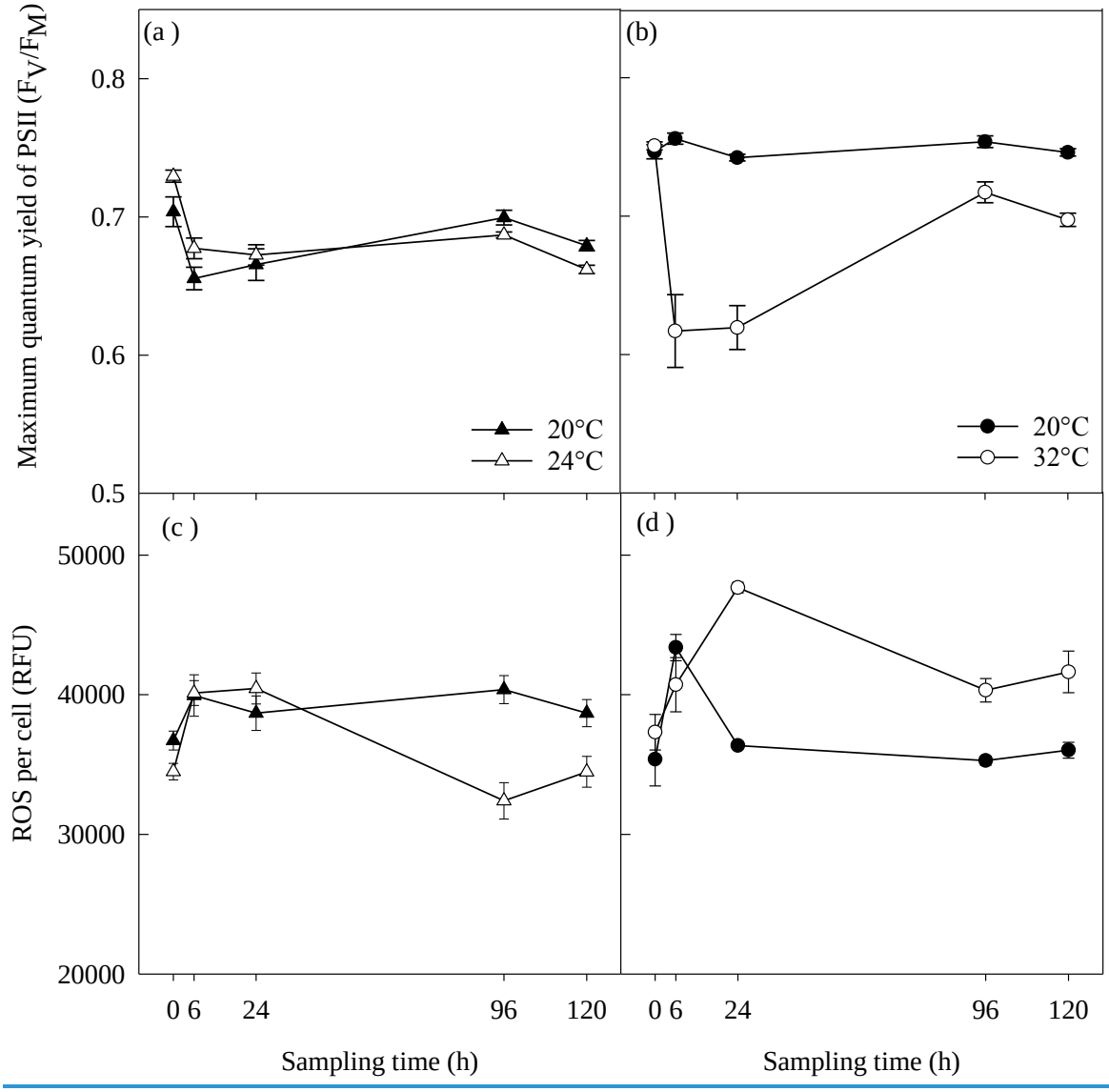
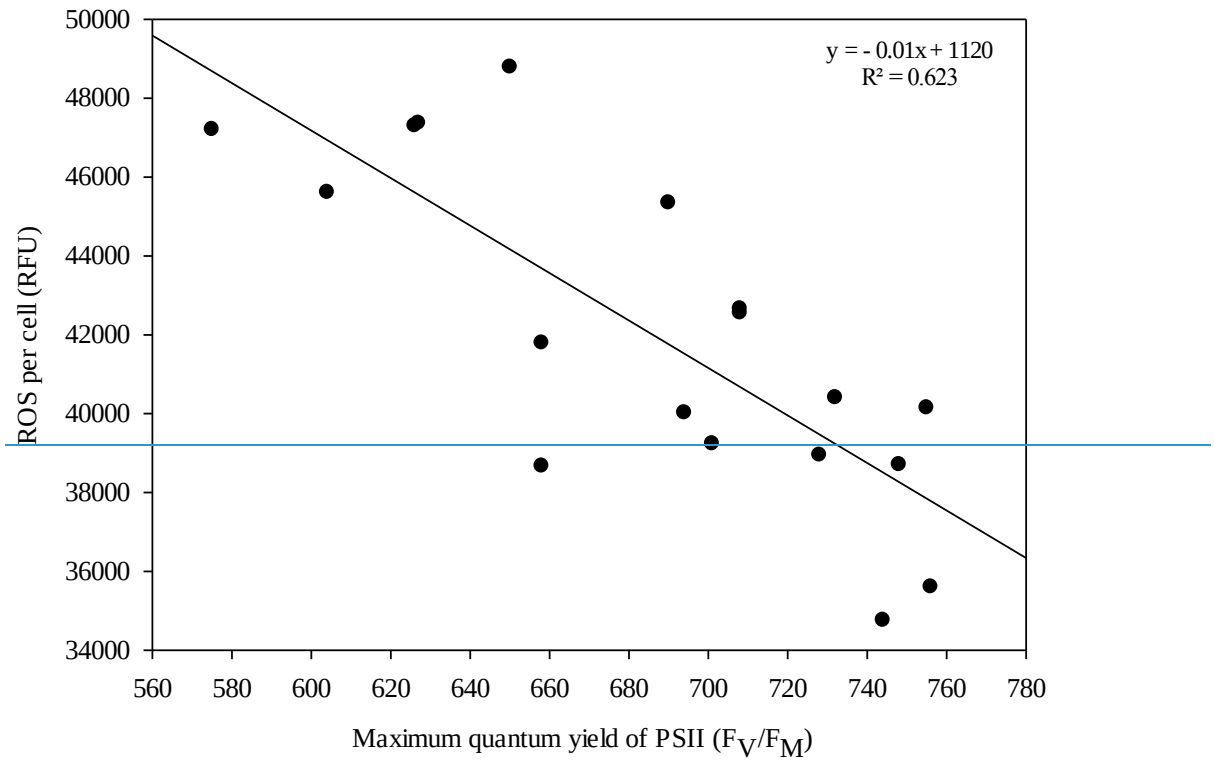

(a)
(b)
(c)
(d)
Maximum quantum yield of PSII (F_V/F_M)
ROS per cell (RFU)
0.8
0.7
0.6
0.5
50000
40000
30000
20000
20°C
24°C
20°C
32°C
0 6
24
96
120
Sampling time (h)
Sampling time (h)

Figure 3


y = - 0.01x + 1120
R² = 0.623
ROS per cell (RFU)
50000
48000
46000
44000
42000
40000
38000
36000
34000
560
580
600
620
640
660
680
700
720
740
760
780
Maximum quantum yield of PSII (FV/FM)

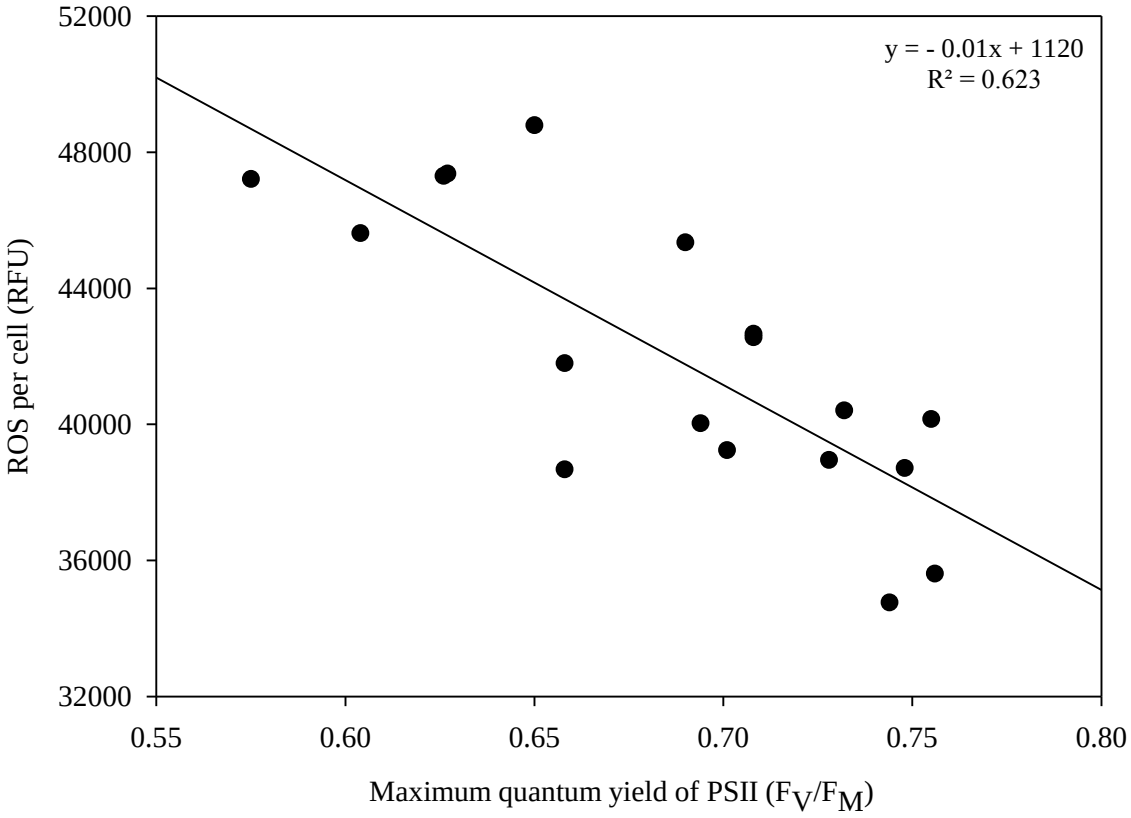
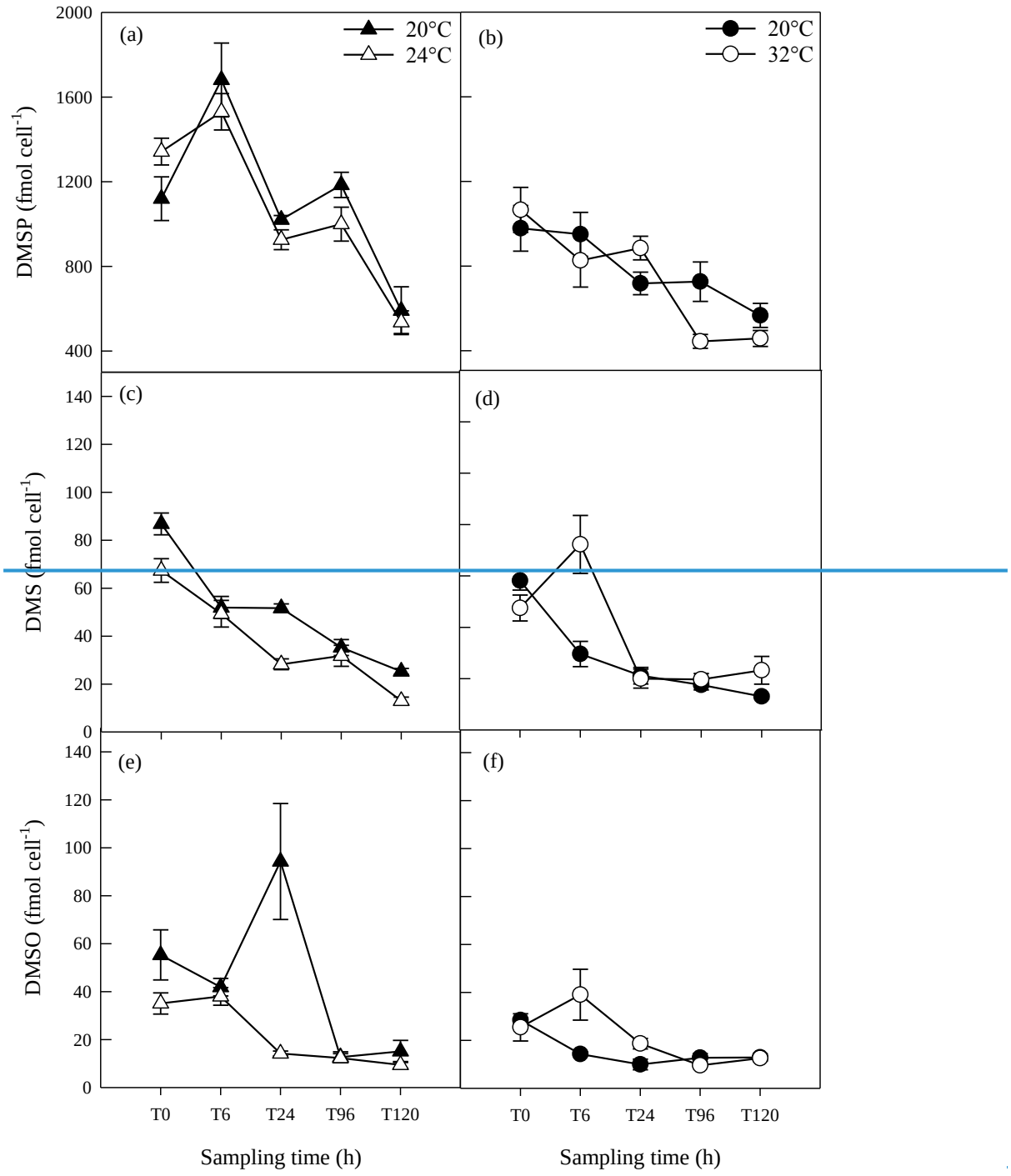

y = - 0.01x + 1120
R² = 0.623
52000
48000
44000
40000
36000
32000
0.55
0.60
0.65
0.70
0.75
0.80
ROS per cell (RFU)
Maximum quantum yield of PSII (F_V/F_M)

Figure 4


(a)
20°C
24°C
(b)
20°C
32°C
DMSP (fmol cell-1)
2000
1600
1200
800
400
(c)
(d)
DMS (fmol cell-1)
140
120
100
80
60
40
20
0
(e)
(f)
DMSO (fmol cell-1)
T0
T6
T24
T96
T120
Sampling time (h)
Sampling time (h)

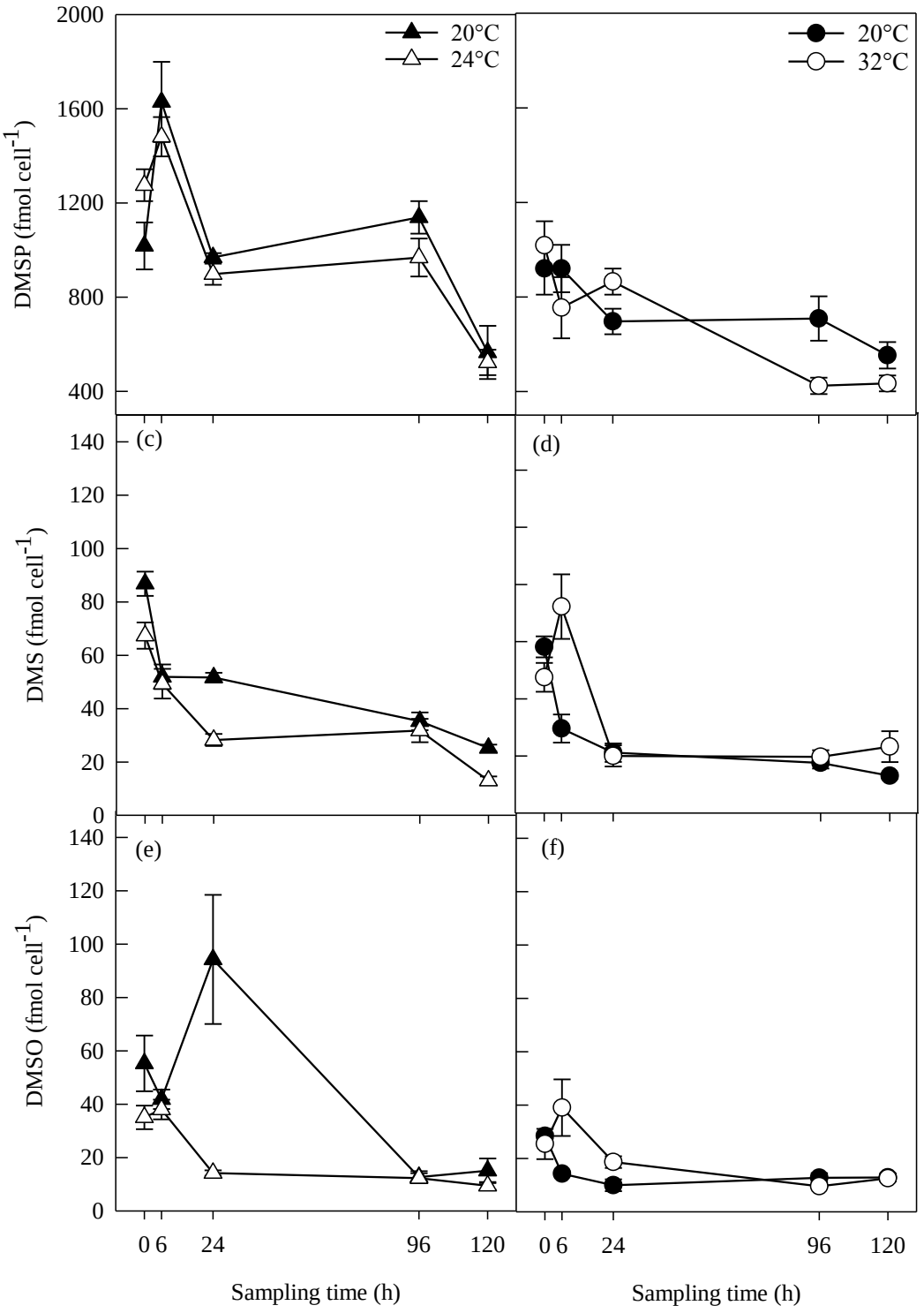

20°C
24°C
20°C
32°C
DMSP (fmol cell-1)
DMS (fmol cell-1)
DMSO (fmol cell-1)
(c)
(d)
(e)
(f)
2000
1600
1200
800
400
140
120
100
80
60
40
20
0
0 6 24 96 120
Sampling time (h)
Sampling time (h)

Figure 5

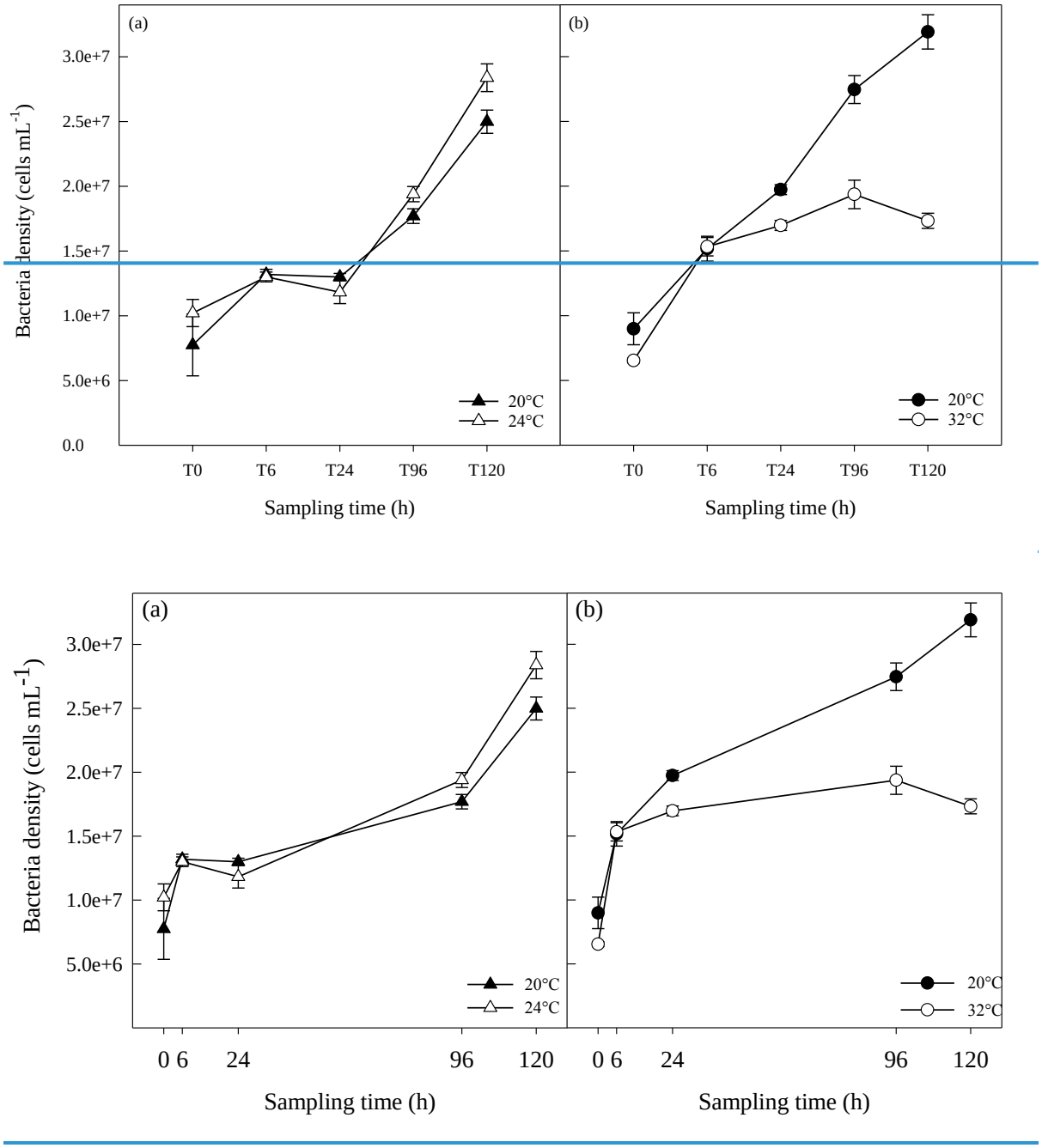
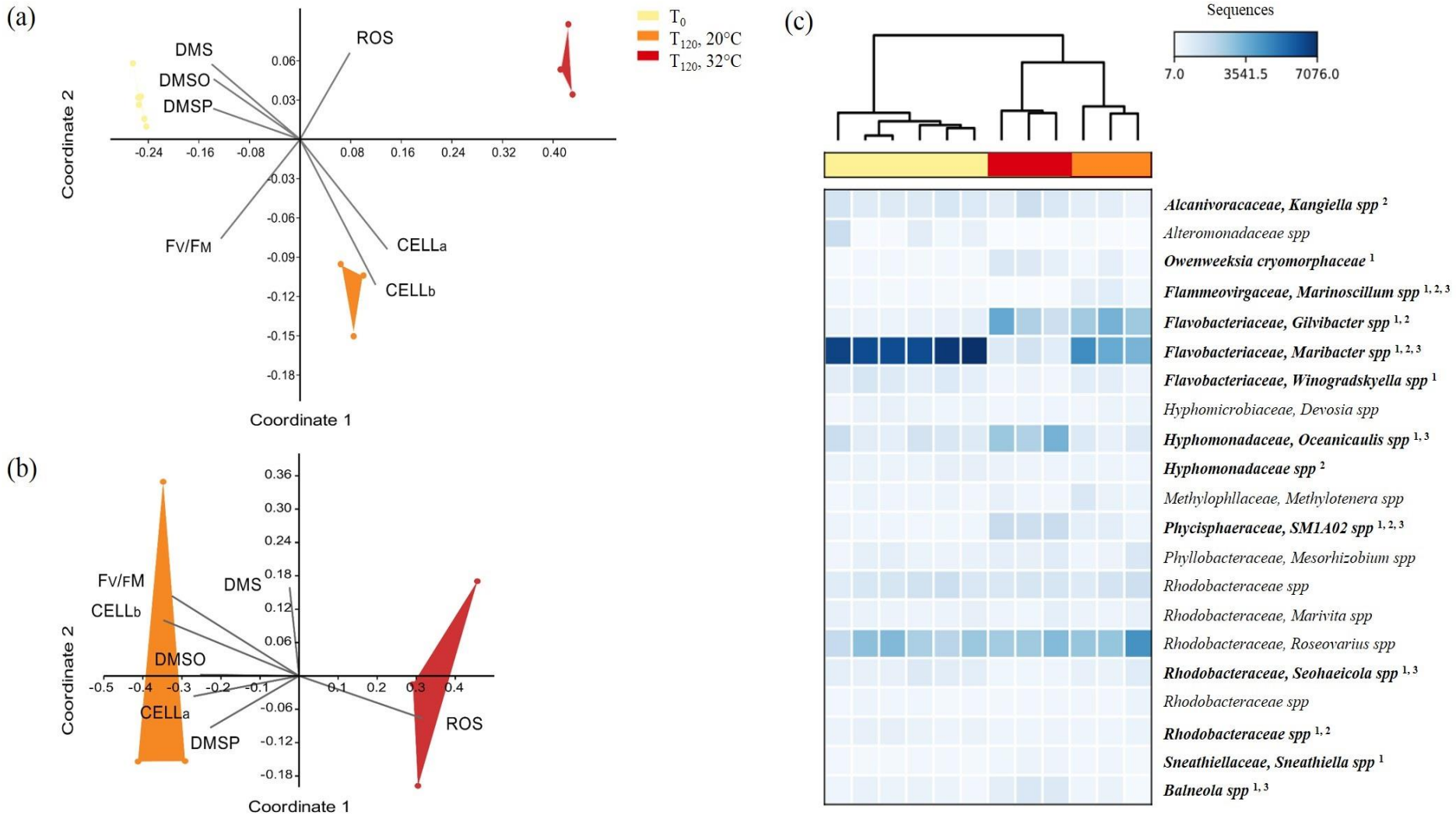

(a)
(b)
Bacteria density (cells mL-1)
3.0e+7
2.5e+7
2.0e+7
1.5e+7
1.0e+7
5.0e+6
0.0
20°C
24°C
32°C
T0
T6
T24
T96
T120
Sampling time (h)
0 6
24
96
120

Figure 6


(a)
DMS
DMSO
DMSP
ROS
Fv/Fm
CELLa
CELLb
Coordinate 1
Coordinate 2
T0
T120, 20°C
T120, 32°C
(b)
Fv/Fm
CELLb
DMS
DMSO
CELLa
DMSP
ROS
Coordinate 1
Coordinate 2
(c)
Sequences
7.0
3541.5
7076.0
Alcanivoracaceae, Kangiella spp 2
Alteromonadaceae spp
Owenweeksia cryomorphaceae 1
Flammeovirgaceae, Marinoscillum spp 1, 2, 3
Flavobacteriaceae, Gilvibacter spp 1, 2
Flavobacteriaceae, Maribacter spp 1, 2, 3
Flavobacteriaceae, Winogradskyella spp 1
Hyphomicrobiaceae, Devosia spp
Hyphomonadaceae, Oceanicaulis spp 1, 3
Hyphomonadaceae spp 2
Methylophllaceae, Methylotenera spp
Phycisphaeraceae, SM1A02 spp 1, 2, 3
Phyllobacteraceae, Mesorhizobium spp
Rhodobacteraceae spp
Rhodobacteraceae, Marivita spp
Rhodobacteraceae, Roseovarius spp
Rhodobacteraceae, Seohaeicola spp 1, 3
Rhodobacteraceae spp
Rhodobacteraceae spp 1, 2
Sneathiellaceae, Sneathiella spp 1
Balneola spp 1, 3