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.

Elisabeth Deschaseaux et al.

elisabeth.deschaseaux@gmail.com

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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, this treatment presented an opportunity to investigate the physiological, biochemical and microbial consequences of thermal stress on this relevant phytoplankton in the con-

text of extreme MHWs. We are proposing to provide more details on the choice of temperatures in the manuscript.

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. In response to the Reviewer's concerns, we are proposing to add this information: "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."

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 re-

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garding these potential links will be scaled-back. We are proposing to acknowledge 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."

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 we are offering to acknowledge this point more thoroughly. 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 propose to more clearly point out the reasons for the choice of temperature used in the study and

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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 are proposing to change 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 propose to state 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 are proposing to use 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)".

L131. Julabo, country??

We thank the Reviewer for noticing this omission and will add information has follows: "Temperature and light control was achieved using circulating water heaters (Julabo, USA) and programmable LED lights (Hydra FiftyTwo, AquaIllumination, USA)".

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 will add 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."

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 will be provided as follows: "Detection limit was 50 nM for 500μL headspace injections"

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. This information will be included in the methods as follows: "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." Furthermore, Figure 4 and result section (Biogenic concentrations of DMSP ranged from 424 ± 35 to 1629 ± 170 fmol cell-1) will be 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 in the interpretation of our results.

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 are offering to reorganize this whole section as follows: "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).

DMS and 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 $\pm$ 1.8% mass fraction, National Mea-

surement 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 N2 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 % TiCl3 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 TiCl3 in the same manner as the experimental samples. PBS blanks treated with NaOH and TiCl3 were also run along with the calibration curves. All dimethylated sulfur compounds were normalised to cell density, which best reflects biogenic production."

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 will be added as follows: "All dimethylated sulfur compounds were normalised to cell density, which best reflects biogenic production."

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 will thus modify 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. 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 re-

moving 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 thank the Reviewer for noting this omission and will amend this text accordingly.

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 propose to clarify this detail in the manuscript as follows: "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. This will be changed in the text.

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 this comment. We propose to modify 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 (SimÏŇ, 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)..."

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 propose to make the following change: "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.

Please also note the supplement to this comment:
https://www.biogeosciences-discuss.net/bg-2018-497/bg-2018-497-AC1-supplement.pdf
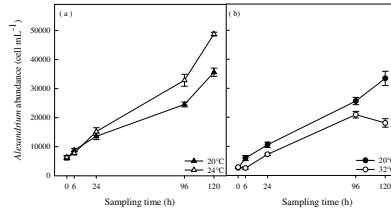
Figure 1

Fig. 1.

Figure 2
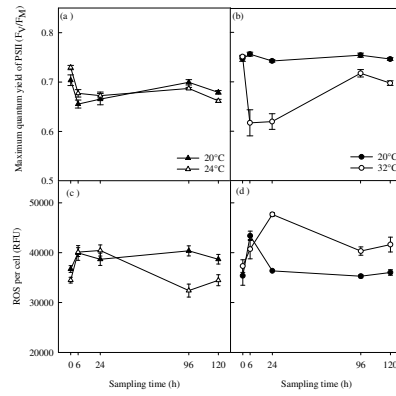
Fig. 2.

Figure 3

$$y = -0.01x + 1120$$
$$R^2 = 0.623$$
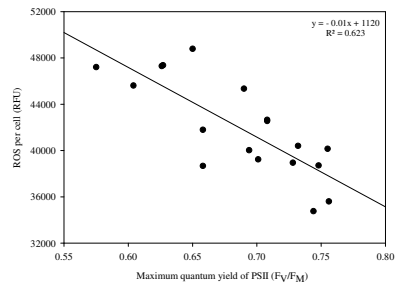
Fig. 3.

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Figure 4

Fig. 4.

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Figure 5

Fig. 5.

Figure 6

Fig. 6.