Report from Anonymous Referee #1

Manuscript BG-2023-51 manuscript: "Properties of exopolymeric substances (EPS) produced during cyanobacterial growth: potential role in whiting events."

1. Anonymous Referee #1

The authors conducted a culture-based study and used a complementary arsenal of carefully done measurements to verify their results and observations and infer how the precipitation of CaCO3 in pelagic cyanobacterial blooms may occur (during whiting events). FTIR, pH drift assays, EPS compositional analyses, etc. They were able to show that the precipitation of CaCO3 (calcite, and to a lesser extent vaterite) coincided with the magnitude of EPS production and the available functional groups on the EPS occurring in early stationary phase cultures. Larger precips were formed during the exponential phase, and smaller, more abundant precips were formed during the stationary phase.

The larger precipitates early on (Exponential phase cultures) and smaller precipitates observed in later stationary phase is somewhat puzzling. But interpretations were made that help to explain these outcomes, especially when considering natural bloom systems.

The "pH of cultures was around 10, and remained steady". Given that cultures were grown under 12/12 light/dark cycles, was pH measured in darkness? It should be clarified if pH was measured during light conditions or dark, or both. Please clarify?

Author's response: *Synechococcus* cultures were grown under a light/dark cycle of 12 hours each. However, pH measurements were exclusively carries out during the light cycle. The pH values were measured approximately 3-4 hours after the completion of the dark cycle. This explains the consistently high pH values depicted in Figure 1B. We have now incorporated this information into the main text (Line 113).

Figure 1. What may have caused the dip in both pH (B), and numbers of cells (A) during stationary phase (near day 40) in Experiment 1? Any suggestions?

Author's response: Indeed, in Experiment 1, we observed an atypical phenomenon where the cultures appeared to undergo a collapse around day 40. Both pH and cell density values decreased but started to increase again within approximately 4 days. One possible explanation for this unusual pattern could be a disruption or change in nutrient availability. This could include phosphorous (PO_4^{3-}) deficiencies or a transition to a different nitrogen (N) source (e.g., from NH_4^+ to NO_3^-). Another probable factor is the depletion or insufficiency of CO_2 during this specific phase of cultivation. The shift in the carbon source from CO_2 to HCO_3^- might trigger a metabolic response in the cells, leading to their re-adaptation, which in turn could influence cell growth and account for the observed pattern in Experiment 1. It is important to note that this explanation is merely a supposition based on information gathered from the

literature (e.g. Rückert et al., 2004) and that further measurements would be necessary to confirm this hypothesis. Nevertheless, when comparing pH values and cell numbers between Experiment 1 and Experiment 2, no significant differences were found (p-value > 0.05, as shown in Figure AA and AB).

Anova: Single Factor											
SUMMARY											
Groups	*Count*	*Sum*	*Average*	*Variance*							
Column 1	6	58,48292	9,747153	0,923947							
Column 2	6	58,845	9,8075	0,735155							
ANOVA											
Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*					
Between Groups	0,010925	1	0,010925	0,01317	0,910906	4,964603					
Within Groups	8,29551	10	0,829551								
Total	8,306435	11									

	pH	
	Column 1	Column 2
Time (day	Experiment 1	Experiment 2
0	8,24	8,58
3	8,94	8,87
14	10,28	10,10
28	10,45	10,28
41	9,89	10,50
56	10,70	10,52

Figure AA. Anova Single-factor statistical test comparing pH values obtained from growth experiments 1 and 2.

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Total	8,306435	11									

	cell density (cells.L-1)	
	Column 1	Column 2
Time (day	Experiment 1	Experiment 2
0	9,52E+10	7,12E+10
3	2,58E+11	2,03E+11
14	1,74E+12	5,65E+11
28	2,10E+12	1,48E+12
41	1,81E+12	1,24E+12
56	2,69E+12	1,44E+12

Figure AB. Anova Single-factor statistical test comparing cell numbers obtained from growth experiments 1 and 2.

Using FTIR, highest protein levels (line 256, 257) were indicated, and later using colorimetric assays of protein (line 271) it is stated that highest protein occurred in EPS also during early stationary phase (also shown in Table 3) – good verification!

Author's response: Thank you.

The FTIR results for EPS are especially informative and helpful. The authors should consider summarizing these in a separate Table for easier reference by the reader.

Author's response: We conducted an FTIR analysis of the EPS extracted at various *Synechococcus* growth stages and included the corresponding results in the supplementary materials document as Table 1S.

Although this is a laboratory-based study, it sheds light on a longer standing issue of how whiting events occur during blooms in natural systems. The authors are to be commended on the nice, careful work examining this whiting-related process. The ms was well written, and only minor changes are suggested.

Author's response: We thank the reviewer for the positive feedback.

Report from Referee #2

Manuscript BG-2023-51 manuscript: "Properties of exopolymeric substances (EPS) produced during cyanobacterial growth: potential role in whiting events."

2. **Referee #2: Dr. Tobias-Hunefeld, Sven**
sven.hunefeldt@igb-berlin.de

Summary:

The authors investigated the role of EPS in carbonate precipitation during a Synechococcus bloom experiment. EPS characteristics were assessed with FT-IR spectroscopy, SDS-PAGE and forced carbonate mineral precipitation experiments. The authors separated the bloom into exponential, and early and late exponential stages. Stationary phases contained higher abundances of negatively charged groups than during the exponential phase, leading to smaller (< 50 um) but more abundant carbonate minerals in the stationary phases, whereas the exponential phase contained larger (> 50 um) but less abundant carbonate percipitates.

I believe this manuscript gives a small but important contribution to the field. I especially applaud the many different approaches taken to measure the EPS and associated factors. My comments mainly center around cutting down on the amount of references, only using the key ones, and ensuring that the different sections (introduction/results/discussion) don't intersect with each other. Other comments include ways to convey data reliability and acknowledging limitations.

Major comments:

The abstract should make clearer what techniques were used for measurements, at least the ones that the major findings were drawn upon.

Author's response: Thank you for this remark. We have followed your suggestion and this information is now added to the abstract (Lines 21-23).

The graphical abstract is well made and explains the stated findings well. However, I am unsure of what the 'Negatively charged groups' are, is it the connecting lines of the different phases?

Author's response: The negatively charged groups are represented by the "connecting lines" and resemble a honeycomb-like structure.

In that case I would remove the Exponential/Early stationary/Late stationary illustrations and explanations from the Legend.

Author's response: We acknowledge the reviewer's observation regarding the repetition of the terms "Exponential/Early stationary/Late stationary phase." In response, we have taken their suggestion and eliminated them from the legend and decided to keep it solely as subtitles in the Graphical abstract.

There are many references throughout the introduction and discussion, to the degree that the text in the intro is 50% references and 50% informative text. I would focus more on relevant and landmark references and use these key references to make your point. Rather than having 3-5 references for every statement. For example: L83-84: There are 3 references that say that cyanobacteria are known EPS producers, and then another 2 that state this is especially true during blooms. It would be best to have only those last two as they are the most relevant to your point.

Author's response: After thorough examination, we carefully reviewed the entire manuscript once again. As a result, we have made the decision to retain only the three most relevant references for each statement. We acknowledge the reviewer's point that certain subsections contained an excessive number of citations. However, it is important to emphasize that is a lab-based study in which we present a [theoretical] model that we have extrapolated to the natural system. To effectively integrate our findings with the natural system scenario, it is imperative to access wide range of disciplines and explore relevant literature.

Methods seem to match the stated goals of the study. Many measurements however were done in duplicate, with no justification as to why. Such as protein, sugar and glycosaminoglycan quantifications. Unless measurements were extremely similar, triplicates would convey a greater degree of measurement precision. I would recommend either measuring an additional sample (if available), stating the reason for why duplicates were chosen for this, or acknowledging this as a study limitation.

Author's response: Protein measurements were conducted in quadruplicate, sugar measurements in triplicate and GAGs (glycosaminoglycans) measurements in duplicate. The reported values in the study represent the mean of these measurements taken from two replicates of EPS samples. Therefore, the reported averages were obtained from a total of eight protein measurements, six sugar measurements and four GAG measurements. We have now included this information in the main text at lines 265-266.

As there were two experimental runs that were temporally displaced, I would have liked to see a brief comparison of their measurements to show that these two test runs are not significantly different.

Author's response: Comparison of pH and cell number measurements of Experiment 1 and Experiment 2 showed that they are not significantly different (p-value > 0.05, see Figure AA and Figure AB). This information is now included in the main text (Lines 192, 198).

Anova: Single Factor

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Figure AA. Anova Single-factor statistical test comparing pH values obtained from growth experiments 1 and 2.

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56	2,69E+12	1,44E+12

Figure AB. Anova Single-factor statistical test comparing cell numbers obtained from growth experiments 1 and 2.

Additionally, is there an explanation why experiment 1 had a 7 day and experiment 2 had a 14 day exponential phase?

Author's response: Although the inoculum was consistent between Experiment I and II (we calculated the same final optical density), the initial cell count on day 0 was ~ 1.3 times higher in Experiment I ($9.52 \times 10^{10}\ cells.L^{-1}$) than in Experiment II ($7.12 \times 10^{10}\ cells.L^{-1}$). This difference in cell density could potentially account for the faster cell growth and shorter exponential phase observed in Experiment I.

With the difference in exponential phase, why were the chosen sample days the same for both experiments? Specificallly: D0, D14, D8, D56.

Author's response: In our previous publication (Martinho de Brito et al., 2022), we demonstrated that the exponential growth phase of *Synechococcus* spp. lasts for a minimum of 18 days. Based on this finding, we selected day 14 as the first sampling point in the current

study, representing the mid of the exponential phase. The choice of the second sampling point was based on the observed stability in cell numbers and pH values in Experiment 1, suggesting the transition from the exponential phase to the stationary phase. Thus, we selected day 28 for the second sampling point. Initially, we anticipated sampling during the death phase for the final time point. However, as stated in the main text, we did not observe a distinct death phase during the 56-day experiment. Consequently, we collected the last sample during the late stationary phase. For Experiment II, we established the same sampling time to correlate the obtained data with EPS production. Although the number of cells may not be the same in both experiments, the growth stages of the cultures overlap significantly (p-value = 0.91).

By picking only crystals above 10 µm, could this be introducing some bias? Or was the 10 µm limit chosen due to methodological limitations? If yes, should be stated. Otherwise, the 10 µm size cut off should be justified.

Author's response: Based on the observations made during the precipitation experiment, we identified two primary size classes. To analyze these size classes, we used an image analysis software and selected two crystal size classes: crystals with a size < 50 µm and crystals with a size > 50 µm. It is important to note that the < 50 µm size class encompasses all crystals ranging from 0 µm to < 50 µm, which includes crystals smaller and larger than 10 µm. Therefore, crystals above 10 µm were counted and their counts are included in the reported results. Please refer to the "Crystal count and size distribution" (Subsection 2.6.2) in the Materials and Methods section for a more detailed explanation of this counting process.

Please add confidence intervals/standard deviations to Figure 5.

Author's response: Each one of the curves shown in Figure 5 represents pH values that were measured at a frequency of every 2 seconds, resulting in a minimum of 400 measurements per curve. Due to the large number of measurements and to maintain the clarity of the graph, we opted not to include the standard deviation (SD) for each curve. Including the SD in the graph would render it unreadable and overcrowded. However, it is important to note that the general trend is preserved. We have included a supplementary Figure (Figure 2S) that displays the replication of the *in vitro* inhibition of calcium carbonate precipitation, demonstrating the consistent pattern observed in the main figure.

I would also like to see the statistics represented on all relevant figures. Between negative controls and measured values, as well as between time points.

Author's response: After a careful examination of all the tables and figures of the manuscript, we confirmed that only Table 1 was lacking the addition of standard deviations (SD). We have now corrected this error and added the necessary SD values to Table 1. Please refer to Table 1 (Lines 213-215) in the manuscript for this updated information.

Please increase the readability of the introduction and discussion, lots of results were included and some points are made and justified either in the next discussion section or 20 lines below. Based on this there were some other points to make:

As EPS characteristics are phytoplankton/organism specific it should be noted that these findings need to be expanded on with additional studies and other phytoplankton.

Author's response: We acknowledge the reviewer's valuable input regarding this significant aspect. As a result, we have included the relevant information in the Discussion section of the manuscript (Line 476).

Tables and Figures that were already presented in the results section should not be mentioned again in the discussion.

Author's response: We have implemented changes throughout the entire text to address this concern. Specifically, we have removed several references to tables and figures from the discussion section, as suggested by the reviewer.

The same is true for the discussion section, do not reiterate results in this section, but rather focus only on the implications and what this means for the field.

Author's response: We have made revisions and modifications to the Discussion section as required.

Ensure that conclusion drawn from the data are found only in the discussion, and not in the results. Such as L276, L278-279.

Author's response: We have removed these sentences from the Results section and that information is now included in the Discussion section.

Please ensure that you do not discuss or provide justification for the chosen methods in your results, but rather in the introduction, methods section, or discussion (as relevant).

Author's response: We have changed the text accordingly.

Please make clear how discussion L381-399 relates to whiting events. The text is very information dense, however how it relates to phytoplankton associated whiting events is not clearly stated.

Author's response: We agree that there is significant repetition within this paragraph. As some of this information was already presented in the Introduction section, we have condensed the paragraph into three lines for brevity and clarity. Please refer to Lines 451-454 for the summarized version.

I believe it would help to have L400-417 before L381-399 or remove L381-399 from the manuscript.

Author's response: After careful consideration, we have decided to retain the original version of Lines 381-399. We believe that this section presents the narrative in a logical and comprehensive manner. Our aim was to establish a connection between cell growth/metabolic activity (high photosynthetic activity and high pH levels and alkalinity, as well as CO_2 limitation) with EPS production, exploring its impact on carbonate precipitation. The same structured approach was consistently applied throughout the discussion of all *Synechococcus* growth stages, as outlined in Sections 4.1, 4.2, and 4.3.

As EPS characteristics are phytoplankton/organism specific it should be noted that these findings need to be expanded on with additional studies and other phytoplankton.

Author's response: We acknowledge the reviewer's valuable input regarding this significant aspect. As a result, we have included the relevant information in the Discussion section of the manuscript (Line 476).

Minor comments (please also see attached pdf with comments):

The limit of detection was stated in µg.L^{-1} in text, however Table 1 values are in µM. The same units should be used throughout the manuscript.

Author's response: The limit of detection was modified and is now expressed in µM (Line 209).

I would also be interested in day 0 values throughout the text, as it was stated that they were measured but are not shown in the manuscript.

Author's response: We agree that this was confusing as stated and we have rectified it by amending the caption of Table 1 (Line 212). The measurements were done in the medium before the inoculation (t0). Therefore, in Table 1, t0 corresponds to the column entitled "Initial concentrations in the medium".

Only Figure 2 is required. Table 2 should then be relegated to the supplementary.

Author's response: We prefer to keep Table 2 and Figure 2 in the main text as they complement each other. The calculations of cell-specific EPS production described in Figure 2 were derived from the data presented in Table 2, which includes information on cell yield and EPS production at the three different growth stages. By including both Table 2 and Figure 2, we aim to enhance the clarity and comprehension of the study's findings.

FT-IR mentions a lot of different spectra, it would be best to only focus on the ones from the discussion or those relevant to the findings.

Author's response: We strongly believe that providing a comprehensive description of the Infra-Red spectra is highly useful for a deeper comprehension of the EPS, including its composition and structure. To our knowledge, there is still limited availability of this type of information for exopolymeric substances. Therefore, we emphasize the importance of highlighting and describing all the peaks in the spectra. Additionally, we would like to emphasize that the other reviewer of this paper suggested moving Table 1S "Attribution of main infrared absorption bands of EPS samples" from the supplementary materials to the main text due to its relevance. The reviewer found this information highly pertinent, further reinforcing the significance of including it in the main text.

Can Figure 4 be redone with the inclusion of a negative control?

Author's response: Usually the use of Alcian Blue (AB) does not require a negative control since it is not expected to stain non-polyanionic substances such as proteins and sugars. This is a commonly accepted practice, and several studies examining polyanionic matrices on gels have been published without including a negative control (Marie et al., 2007; Gaspard et al., 2008). There are instances where non-polyanionic matrices exhibit no staining at all with AB,

as demonstrated by Pavat et al. (2012). Moreover, in our gel, the lane corresponding to the molecular weight (MW) ladder can be considered as an appropriate negative control.