Rebuttal (May 30, 2023)

Report from Referee #2

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

1. **Referee #2: Dr. Tobias-Hunefeld, Sven**
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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									
SUMMARY									
Groups	*Count*	*Sum*	*Average*	*Variance*				pH	
Column 1	6	58,48292	9,747153	0,923947				Column 1	Column 2
Column 2	6	58,845	9,8075	0,735155			Time (day	Experiment 1	Experiment 2
							0	8,24	8,58
							3	8,94	8,87
ANOVA							14	10,28	10,10
Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*	28	10,45	10,28
Between Groups	0,010925	1	0,010925	0,01317	0,910906	4,964603	41	9,89	10,50
Within Groups	8,29551	10	0,829551				56	10,70	10,52
Total	8,306435	11							

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

Anova: Single Factor

SUMMARY						cell density (cells.L-1)	
Groups	Count	Sum	Average	Variance		Column 1	Column 2
Column 1	6	58,48292	9,747153	0,923947	Time (day	Experiment 1	Experiment 2
Column 2	6	58,845	9,8075	0,735155	0	9,52E+10	7,12E+10
					3	2,58E+11	2,03E+11
					14	1,74E+12	5,65E+11
ANOVA					28	2,10E+12	1,48E+12
Source of Variation	SS	df	MS	F	41	1,81E+12	1,24E+12
Between Groups	0,010925	1	0,010925	0,01317	56	2,69E+12	1,44E+12

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				

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 (9.52×10^{10} $cells.L^{-1}$) than in Experiment II (7.12×10^{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? Specifically: 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.

Properties of exopolymeric substances (EPS) produced during cyanobacterial growth: potential role in whiting events

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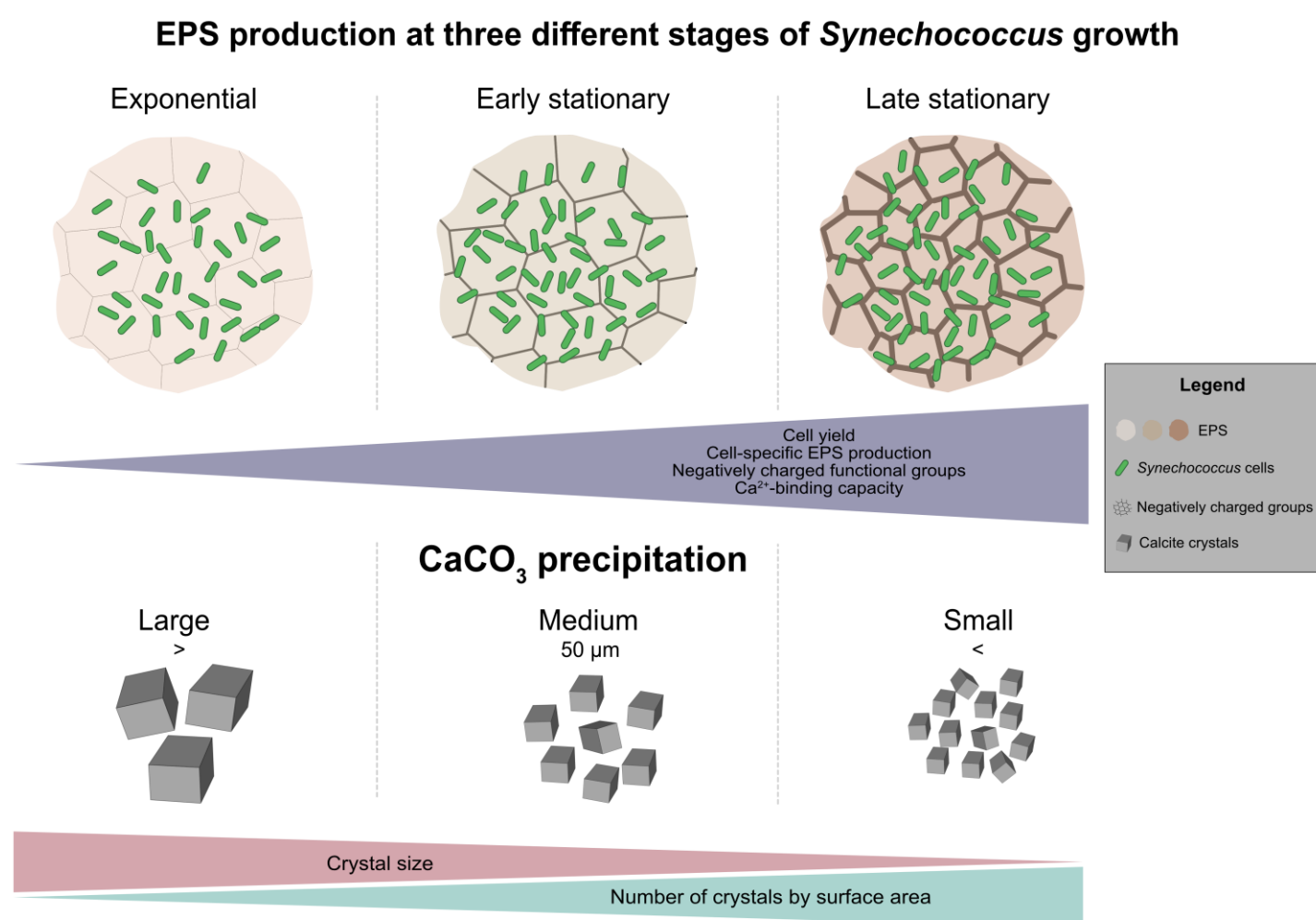
Abstract

Extracellular polymeric substances (EPS) are an important organic carbon reservoir in many pelagic and benthic environments. The production of EPS is intimately associated with the growth of phyto- and picoplankton. EPS plays a critical role in carbonate precipitation through the binding of cations and by acting as a nucleation site for minerals. Large-scale episodes of fine-grained calcium carbonate precipitation in the water column (whiting events) have been linked to cyanobacterial blooms, including of *Synechococcus* spp.. The mechanisms that trigger these precipitation events are still debated. We pose that the cyanobacterial EPS, produced during exponential and stationary growth phases plays a critical role in the formation of whitings. The aim of this study was to investigate the production of EPS during a two-month cyanobacterial growth, mimicking a bloom. The production and characteristics of EPS were examined in different growth stages of *Synechococcus* spp. using various techniques such as FT-IR spectroscopy, colorimetric and SDS-PAGE assays. We further evaluated the potential role of EPS in carbonate precipitation through *in vitro* forced precipitation experiments. EPS produced during the early and late stationary phase contained a larger amount of negatively charged groups than present in EPS produced during the exponential phase. Consequently, a higher Ca^{2+} binding affinity of the stationary phase-EPS led to the formation of a larger amount of smaller carbonate minerals ($<50\,\mu m$) compared to crystals formed in exponential phase-EPS, which were less abundant and larger ($>50\,\mu m$). These findings were used to establish a conceptual model for picoplankton bloom-mediated $CaCO_3$ precipitation that can explain the role of EPS in whitings (see graphical abstract).

Graphical abstract

1. Introduction

1.1 Significance of this study

Massive carbonate precipitation episodes in the water column, also referred to as 'whiting events' are a well-known phenomenon of modern freshwater (Schultze-Lam et al., 1997; Hodell et al., 1998; Stanton et al., 2021) and marine environment (Shinn et al., 1989; Robbins and Blackwelder, 1992; Larson and Mylroie, 2014). Whitings are caused by large-scale precipitation of micron-sized calcium carbonate particles (visible from space) and represent a major sink in the carbon cycle. The particles associated with whitings can make up a major sedimentary constituent of the modern-day and ancient carbonate rock records (Pomar and Hallock, 2008). Whiting events can be triggered by a combination of biological and physicochemical processes. Among the biological mechanisms that have been studied in this context, picocyanobacterial proliferations have often been invoked in the initiation of whitings (Hodell et al., 1998; Thompson, 2000; Obst et al., 2009). Photosynthesis increases pH levels and alkalinity during cyanobacterial blooms, ultimately causing the saturation state of calcium carbonate to rise, thereby leading to its potential precipitation. The role of *Synechococcus* spp. bloom-forming cyanobacteria in $CaCO_3$ precipitation has been demonstrated in laboratory experiments (Yates and Robbins, 1998; Dittrich et al., 2003; Obst et al., 2009; Bundeleva et al., 2014; Martinho de Brito et al., 2022) and observed in field investigations (Wells and Iling, 1964; Thompson et al., 1990; Dittrich and Obst., 2004). Change in temperature, salinity, CO_2 pressure as well as turbulence are some of the physicochemical factors

44   that can lead to the formation of supersaturated solutions and subsequent precipitation of $CaCO_3$ thus initiating the whiting. Even though several possible biogenic

45   and abiotic mechanisms have been identified, the formation of whitings is still poorly understood.

1.2 Overview of phytoplankton blooms

47   Phytoplankton blooms, including those of picoplankton, are dense accumulations of cells resulting in a visible discoloration of the surface water layers (Reynolds and

48   Walsby, 1975; Huisman et al., 2018). Their occurrence has been recorded worldwide in marine and freshwater bodies (Paerl et al., 2001; Paerl and Huisman, 2008;

49   Ploug, 2008). Light intensity, water temperature, nutrient availability, weather conditions and hydrodynamics are key factors that determine the onset and persistence

50   of a bloom. Blooms are typically seasonal, frequently observed during late spring or summer, and can be dominated by picoplankton (Huisman et al., 2018). Some

51   phytoplankton organisms, notably cyanobacteria, may produce toxins and form large-scale harmful algal blooms (Paerl et al., 2001). The intensity and frequency of

52   cyanobacterial blooms have been increasing due to anthropogenic eutrophication (Heisler et al., 2008; O'Neil et al., 2012), a trend expected to exacerbate due to

53   climate change (Lürling et al., 2018). Cyanobacteria comprise a diverse group of photoautotrophic organisms that play a pivotal role in global primary production and

54   are key players in the biogeochemical cycles of carbon, nitrogen and oxygen (Callieri and Stockner, 2000; Raven et al., 2017). The unicellular cyanobacterium

55   *Synechococcus* is one of the most abundant photosynthetic microorganisms on Earth (Whitton and Potts, 2012), which contribute substantially to the picoplankton

56   community in marine (Murphy and Haugen, 1985; Coello-Camba and Agustí, 2021) and freshwater environments (Weisse, 1993) that can form dense blooms

57   (Schultze-Lam et al., 1992; Phlips et al., 1999; Dittrich and Obst, 2004).

1.3 Phytoplankton blooms and $CaCO_3$ precipitation

59   During the occurrence of dense phytoplankton blooms, high rates of photosynthetic activity lead to a rapid depletion of CO_2 in the surface waters, increasing alkalinity.

60   Depending on the buffering capacity of the water, this could result in pH values ≥ 9 to as high as 11 (Ibelings and Maberly, 1998; Zepernick et al., 2021). Consequently,

61   the inorganic carbonate equilibrium shifts towards carbonate (CO_3^{2-}). Some cyanobacteria possess a carbon concentrating mechanism (CCM) that converts HCO_3^- to

62   CO_2 through the action of carbonic anhydrase enzymes (Price et al., 1998; Badger et al., 2002) and produce hydroxide ions (Kupriyanova and Pronina, 2011). The

63   activity of extracellular carbonic anhydrase (eCA) may contribute to the create an alkaline microenvironment in the extracellular polymeric substances (EPS)

64   surrounding the cyanobacterial cells (Price et al., 2002; Dupraz et al., 2009). When OH^- ions are released during photosynthesis it causes the pH to rise, which favors

65   carbonate mineral precipitation, assuming there are enough calcium ions available (Kamennaya et al., 2012). Consequently, during blooms, carbonate minerals can

66   form on EPS or precipitated in the microenvironment surrounding cyanobacterial cells.

1.4 The role of EPS

68   Cyanobacteria are known producers of EPS, especially during blooms (Pannard et al., 2016; Liu et al., 2018). EPS serve as a boundary between cells and their

69   immediate environment (Whitton and Potts, 2012) and may act as a template for $CaCO_3$ nucleation (Dupraz and Visscher, 2005; Dupraz et al., 2009; Kamennaya et

70   al., 2012). EPS are high molecular weight organic molecules composed of polysaccharides, proteins, nucleic acids and lipids (Pereira et al., 2009; Marvasi et al., 2010;

71   Decho and Gutierrez, 2017). This complex mixture of molecules may contain specific monomer components, such as uronic or sialic acids (monosaccharides), aspartic

72   or glutamic acids (amino acids) or functions (sulfate, phosphate), which carry negative charges in physiological conditions and can therefore bind cations, such as

73   Ca^{2+}, and promote the nucleation of $CaCO_3$ crystals (Trichet and Defarge, 1995; Dupraz et al., 2009; Walker et al., 2019). Conversely, polyanionic EPS in solution

74   can inhibit crystal growth by poisoning the faces of growing nuclei by an adsorption mechanism, according to a classical and accepted view prevailing for other

75   macromolecules of similar charge properties: synthetic peptides (Wheeler et al., 1991), skeletal proteins (Wheeler et al., 1981; Addadi and Weiner, 1985), coccolith-

76   associated polysaccharides (Borman et al., 1982) or natural organic matter dissolved in seawater (Mitterer and Cunningham, 1985). The production and composition

77   of EPS differ among different species of microorganisms and their type of metabolism and depend on environment in which they live, stressors (e.g., nutrient

78   availability, pH, temperature, light, salinity) and the stage of their growth (Pereira et al., 2009; Pannard et al., 2016; Martinho de Brito et al., 2022). The deprotonation

79   of functional groups at elevated pH enhances the binding capacity of cations such as Ca^{2+} and controls crystal nucleation and growth by reducing the interfacial energy

80   barrier between the crystal and the EPS substrate (Dupraz et al., 2009; Dittrich and Sibler, 2010). EPS play a two-fold role in carbonate formation by initially inhibiting

81   (through Ca^{2+} binding) and subsequently promoting carbonate precipitation by releasing calcium ions during EPS alteration and degradation (Dupraz and Visscher,

82   2005. Furthermore, through specific functional group composition and structural architecture, EPS may also exert control over the mineralogy, morphology and/or

83   abundance of the minerals that are formed (Trichet and Defarge, 1995; Dupraz et al., 2009).

1.5 The goal of this study

85   We have previously reported that the pH of *Synechococcus* cultures increased when grown in a non-buffered medium (Martinho de Brito et al., 2022). In these growth

86   conditions, the production of EPS was enhanced compared to growth in a buffered medium. Furthermore, the EPS from cells grown in non-buffered conditions

87   contained more negatively-charged functional groups that impacted the properties of the carbonate minerals that precipitated. The current study further investigates

88   the properties of EPS produced during different growth phases of *Synechococcus* spp. Over an extended incubation time (mimicking a prolonged natural bloom). We

89   aim to better understand the role of cyanobacterial blooms in carbonate precipitation through EPS production and develop a conceptual model of picoplankton-

90   mediated organomineralization to explain the biological origin of whiting events.

2. Materials and Methods

2.1 *Synechococcus* PCC7942 strain and culture growth conditions

93   *Synechococcus* PCC7942 was obtained from the Centre de Ressources Biologiques de l'Institut Pasteur (Paris). Cultures were grown in a one-third-strength, non-

94   buffered liquid BG-11 medium (Allen, 1968; Rippka et al., 1979). The medium consists of (per liter): 1.5 g of $NaNO_3$; 0.04 g of $K_2HPO_4 2H_2O$; 0.075 g of $MgSO_4 7H_2O$;

95   0.036 g of $CaCl_2 2H_2O$; 6 mg of citric acid combined with 6 mg of ferric citrate; 0.001 g of $Na_2EDTA 2H_2O$ and 0.02 g of Na_2CO_3. Trace metal solutions contained

96   (per liter) 2.86 mg of H_3BO_3; 1.81 mg of $MnCl_2 4H_2O$; 0.222 mg of $ZnSO_4 7H_2O$; 0.39 mg of $Na_2MoO_4 2H_2O$; 0.079 mg of $CuSO_4 5H_2O$ and 0.0494 mg of

97 $Co(NO_3)_2 6H_2O$. Cultures were incubated at room temperature ($21°C\pm2$), in a light/dark cycle of 12h/12h under 36.8 $\mu E\ m^{-2}\ s^{-1}$ of photon irradiance while shaken at

98 200 rpm in a Cimarec i Multipoint Stirrer, 6 Position, 2000 rpm, 3L per Multipoint, 100-240 VAC rotary shaker.

99 **2.2 Experimental design of *Synechococcus*-bloom formation**

100 Two independent growth experiments were performed in 1L glass serum bottles containing 800 mL of $1/3$ BG-11 medium adjusted to pH 7.5, sealed with silicone caps

101 to allow gas exchange. Cells used for the inoculum (pH = 9.2) were pre-cultured in a full-strength BG-11. Immediately after inoculation (30 mL/bottle), the pH

102 increased to approximately 8.2.

103 **2.2.1 Experiment I**

104 In the first growth experiment, six bottles were inoculated with *Synechococcus* PCC7942. Cell growth and EPS production were examined. Optical density (OD_{750nm}),

105 pH and cell counts were monitored weekly (2-3 times by week). EPS was extracted on days 14, 28 and 56 of cultivation (two bottles were harvested at each sampling

106 time).

107 **2.2.2 Experiment II**

108 The second growth experiment was performed in quadruplicate. Chlorophyll *a* (Chl*a*), extracellular carbonic anhydrase activity (eCA), nutrients (NO_3^- and PO_4^{3-}) and

109 calcium concentration were analysed at 0, 14, 28 and 56 days of cultivation. pH values, OD and cell counts were also assessed at longer intervals (once per week) than

110 in Experiment I.

111 **2.3 Growth assessment**

112 **2.3.1 pH values, optical density (OD) and cell counts**

113 The pH value was measured about 3-4 h after the light cycle started with a CRISON GLP 21 pH meter (Crison Instruments SA, Alella, Spain). Cell growth was

114 monitored through cell counts and OD_{750} measurements. Cell counts were performed using a counting chamber (Neubauer, Mariangela, Germany) by randomly

115 selecting five fields of view and counting approximately 100-200 cells. The OD at 750 nm of a 1-ml sample of the culture was measured in a Bio-Rad SmartSpec Plus

116 Spectrophotometer (Bio-Rad, Hercules, CA, USA).

117 **2.3.2 Chlorophyll-a extraction**

118 Chl*a* was extracted from 2 ml culture aliquots using a methanol extraction method (Stal et al., 1984). Following the extraction in the dark at 4 °C, samples were

119 centrifuged. The Chl*a* absorbance was measured in the supernatant at 665 nm using a Bio-Rad SmartSpec Plus Spectrophotometer (Bio-Rad, Hercules, CA, USA).

120 **2.3.3 Extracellular carbonic anhydrase activity**

121 The extracellular carbonic anhydrase (eCA) activity was measured using a BioVision Carbonic Anhydrase Activity Assay Kit Kit (BioVision, Ref. K472-100, Abcam,

122 Waltham, MA, USA) according to the manufacturer's specifications. Aliquots of ~ 5 ml were analysed immediately after the collection. To avoid cell lysis and

123 intracellular CA contamination, samples were not centrifuged. The cells were separated from the supernatant by using a 1 mL syringe and a 0.20 µm NALGENE®

124 syringe filter. The absorbance was measured in a Bio-Rad Model 680 Microplate Reader at 405 nm.

125 **2.3.4 Nitrogen, phosphorus and calcium measurements**

126 Phosphate, nitrate and calcium concentrations were determined in the growth medium at 0, 14, 28 and 56 days of cultivation. Cells were removed by centrifugation

127 and filtration through a 0.20 µm Millipore filter under a mild vacuum. The samples were stored at 4°C in the dark until measured by ion chromatography. Analyses

128 were realized within the PEA²t technical platform of the Chrono-Environment Laboratory UMR6249 (Université de Franche-Comté, Besançon, France) and the Ca^{2+}

129 concentration was determined by ICP-AES (dual axial and radial view iCAP Pro XP model with fast loop, Thermofisher Scientific, Courtaboeuf, France) available at

130 the University of Franche-Comté, Besançon, France.

131 **2.4 EPS extraction and purification**

132 EPS were extracted from the *Synechococcus* cultures as previously described by Martinho de Brito et al. (2022). EPS were harvested after 14, 28 and 56 days of

133 cultivation. Cyanobacterial cells were inspected by microscopy to ensure that no cell lysis had occurred during the extraction process. The pure EPS fractions were

134 obtained by ultrafiltration (>10 kDa = retentate) for volume reduction and the weight of the material was determined following by dialysis (using a 1 kDa Membrane)

135 lyophilization on a high-precision analytical balance (Quintix 35-1S, Sartorius, Gottingen, Germany).

136 **2.5 EPS characterization**

137 **2.5.1 Fourier Transform-Infrared Spectroscopy**

138 FT-IR spectra were obtained from freeze-dried EPS on an FT-IR Bruker Alpha spectrometer (Bruker Optics SARL, Marne la Vallée, France) fitted with an Attenuated

139 Total Reflectance (ATR) ALPHA-P device equipped with a mono-reflection diamond crystal. A total of 24 scans were performed on each sample at a spectral

140 resolution of 4 cm^{-1} in the 4000–375 cm^{-1} wavenumber range. The qualitative assignment of absorption bands was performed by comparison with spectra available in

141 the literature (Coates, 2000).

142 **2.5.2 Protein, sugar and glycosaminoglycan [quantification]**

143 The total protein content of EPS was determined using the Bicinchoninic acid assay (Pierce® BCA Protein Assay Kit) and bovine serum albumin as the standard. The

144 total sugar content was determined by a modified phenol-sulfuric acid method (Dubois et al., 1956) and xanthan and dextran were used as standards (Sigma-Aldrich,

145 St. Louis, MO, USA). The total glycosaminoglycan (GAGs) content was quantified using the Blyscan Assay according to the manufacturer's protocol (Blyscan Kit

146 B1000, Biocolor Ltd., Antrim, UK) with chondroitin sulphate as the standard. All assays were carried out in duplicated EPS samples.

2.5.3 Visualization of polyanionic macromolecules on Alcian Blue stained gels

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) followed by Alcian Blue staining (Wall and Gyi, 1988) were used to separate and to stain negatively charged macromolecules (10- > 170 kDa), respectively. Alcian Blue is a dye that specifically binds to glycoconjugates with an acidic character (*e.g.,* containing carboxylated or sulfated functional groups). Samples were analysed on one-dimensional precast grafdient protein gels (TGX Gel 4-15%, 90 mm x 70 mm) on a Mini-Protean 3 cell (Bio-Rad, Hercules, CA, USA), according to the method previously described by Martinho de Brito et al. (Martinho de Brito et al., 2022). Prior to migration, samples were heat-denatured in standard 2x Laemmli sample buffer (5 min., 99°C, ref. 1610737, Bio-Rad). A pre-stained protein ladder (Euromedex, #06P-0111; MW: 10 kDa to > 170 kDa) was used as a reference.

2.5.4 Inhibitory effect of EPS using pH-drift assay

The capacity of negatively charged functional groups in EPS to inhibit the *in vitro* precipitation of calcium carbonate was tested with the pH-drift assay (Wheeler et al., 1981; Marin et al., 2000; Kawaguchi and Decho, 2002). This assay was performed as previously described by Martinho de Brito et al. (2022). Briefly, the pH was recorded by a pH meter (Laboratory Research Grade Benchtop pH/mV Meter with 0.001 pH Resolution-HI5221) connected to a PC via a USB cable. Data were recorded by the HANNA HI92000 software. The pH was measured every two seconds for ~15 min. The shape of the curve (after reaching its maximum, about one minute after T0) reflects directly the inhibitory capacity of the tested EPS: a fast decrease in pH (decreasing exponential) indicates ongoing precipitation i.e. the absence of inhibition. A delayed decrease in pH, resulting in a plateau around pH 8, indicates an inhibitory effect, proportional to the length of the plateau. Between each experiment, the electrode was refreshed with dilute acid and blank tests (without EPS) were performed.

2.6 Interaction of EPS with the *in vitro* precipitation of $CaCO_3$

The potential of the EPS matrix to interact with the precipitation of calcium carbonate was tested via the diffusion method in the presence of a closed ammonia-CO_2 saturated atmosphere (Albeck et al., 1993). 200 µL of the mixture containing pre-filtered (0.22 µm) $CaCl_2$ solution (10 mM) and EPS at increasing concentrations (3, 18, and 36 µg.mL^{-1}) were incubated in duplicate in 16-well plates (Lab-Tek, Nunc/Thermo Scientific, Rochester, NY, USA). The EPS concentrations were selected to match the EPS yields at the extraction times (14, 28 and 56 days of cultivation). The plastic covers of the well plates were perforated to allow the reaction between $CaCl_2$ solutions containing EPS and ammonium bicarbonate. The well plates were placed in a desiccator that was incubated at 4°C in the dark for 72 hrs. At the completion of the incubation period, the pH value was measured in each well, the overlying solutions were carefully removed to dryness and $CaCO_3$ crystals analysed. Blank experiments were performed without any EPS. The experiment was carried out in duplicates.

2.6.1 Morphology and mineralogy of the crystals

The 16-well plates containing crystals were used in two manners: first, the morphology of the $CaCO_3$ crystals was checked with a tabletop scanning electron microscope (Hitachi TM 1000, Ibariki, Japan) in back-scattered electron mode. To this end, the glass plate base was unsealed from its plastic well part and directly observed without carbon or gold sputtering. Secondly, the polymorph of the calcium carbonate minerals was determined by FT-IR spectroscopy using an FT-IR Bruker Alpha (Bruker Optics, SARL, Champs-sur-Marne, France). Mineral phases were determined by comparison of the spectra with the reference spectra available in the RRUFF Project database (https://rruff.info, accessed on January1st, 2022).

2.6.2 Crystal counts and size distribution

$CaCO_3$ crystals were counted directly in the 16-well plates using an inverted microscope (Nachet, Paris, France) equipped with Mosaic 2.2.1 image analysis software. Images were processed to obtain crystal sizes (average width and length of size classes < 50 µm and > 50 µm) and the total count of crystals in each well. A total of ten fields of view (10 squares) accounting for 15.5 mm^2 were analysed. The results are reported as the mean ± standard deviation.

2.7 Statistical analysis

All the data concerning *Synechococcus* growth and EPS production are representative of two independent experiments with two technical replicates (four replicates for EPS extracted at 56 days of culture). The results are reported as the mean ± standard error of the mean. Statistical significance was assessed by performing single-factor ANOVA tests; p-values < 0.05 were statistically different.

3. Results

3.1 Trends in *Synechococcus* PCC7942 growth experiments and pH evolution

Cell density and pH values increased over the *Synechococcus* cultivation period (Figure 1A and 1B). The growth of *Synechococcus* cells showed a typical pattern including a brief lag phase (~6-7 days) followed by a 7-day (experiment I) and 14-day (experiment II) exponential phase and finally a stationary phase. The stationary phase (early stationary phase) was reached after 14 and 21 days of growth in experiment I and II, respectively, and lasted until day 56 of cultivation in both experiments (late stationary phase) (Figure 1A). Growth experiments I and II started with a similar cell density of approximately 10^{10} cells.L^{-1} and demonstrated reproducible growth patterns (p-value = 0.91). At the time of inoculation, cell density was 9.5×10^{10} in experiment I and 7.1×10^{10} cells.L^{-1} in experiment II (Figure 1A). *Synechococcus* grew exponentially until reaching a maximum of 1.7×10^{12} in experiment I at 14-day of growth and 1.5×10^{12} cells.L^{-1} after 21 days of growth in experiment II. At the end of the exponential growth phase, the cell numbers levelled off and achieved a stable growth stage (stationary phase). Typical evolutions of pH values in culture media during the *Synechococcus* growth experiments are presented in Figure 1B. As a general trend, pH is linked to the photosynthetic activity of cyanobacteria. The pH levels rose rapidly during the exponential phase in both experiments, reaching around 10, and stayed steady during the stationary phase. While experiment I experienced significant pH fluctuations during the latter part of the stationary phase, overall, the pH evolution trends for both experiments are comparable (p-value = 0.91; Figure 1B). The p-values for pH and cell numbers showed that the two independent growth experiments are not significantly different.

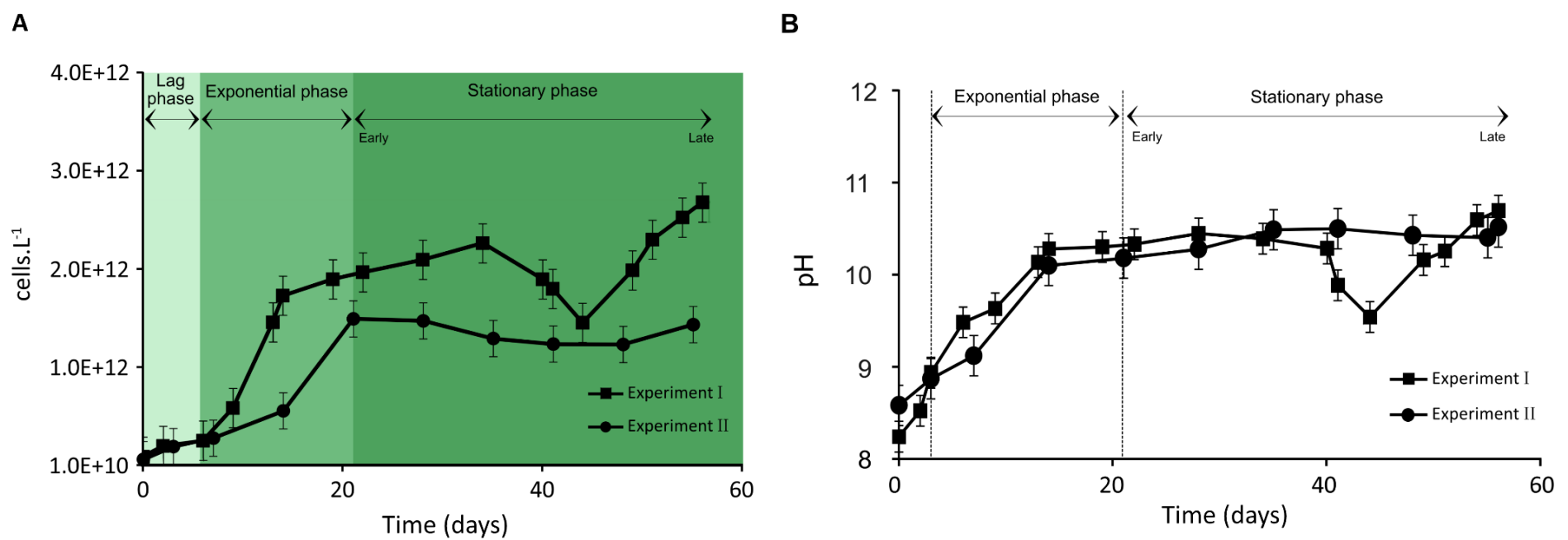



Figure 1. Evolution of biomass of *Synechococcus* PCC 7942 culture **(A)** and pH evolution **(B)** during exponential, early and late stationary phases. The vertical dotted lines (B) represent the stage transition between lag, exponential and stationary phases. Each value is the mean ± SD of all replicate values.

3.2 Extracellular carbonic anhydrase

The activity of extracellular carbonic anhydrase (eCA) in solution changed slightly over the growth experiment (Figure 1S). The highest eCA activity (~1600) was detected after 14 days of culture, during the exponential phase. The lowest activity was measured after 56 days of growth, in the late stationary phase.

3.3 Nutrient concentrations during growth

High nitrate concentrations supported exponential growth and high cell density (Table 1). The results show that aa major decrease in nitrate and phosphate concentrations occurred during the exponential growth phase and remained slowed down progressively over the stationary phase. At the end of the stationary phase, the phosphate concentration had decreased to approximately 30% of its initial level. On the other hand, the nitrate concentration was still high, with approximately 67% of its initial concentration remaining. Ammonium concentration was below the limit of detection (2.2 µM). Calcium concentrations decreased gradually and accounted for the total calcium concentration of 81% in the late stationary phase. Other medium constituents should be present in excess and were thus not measured.

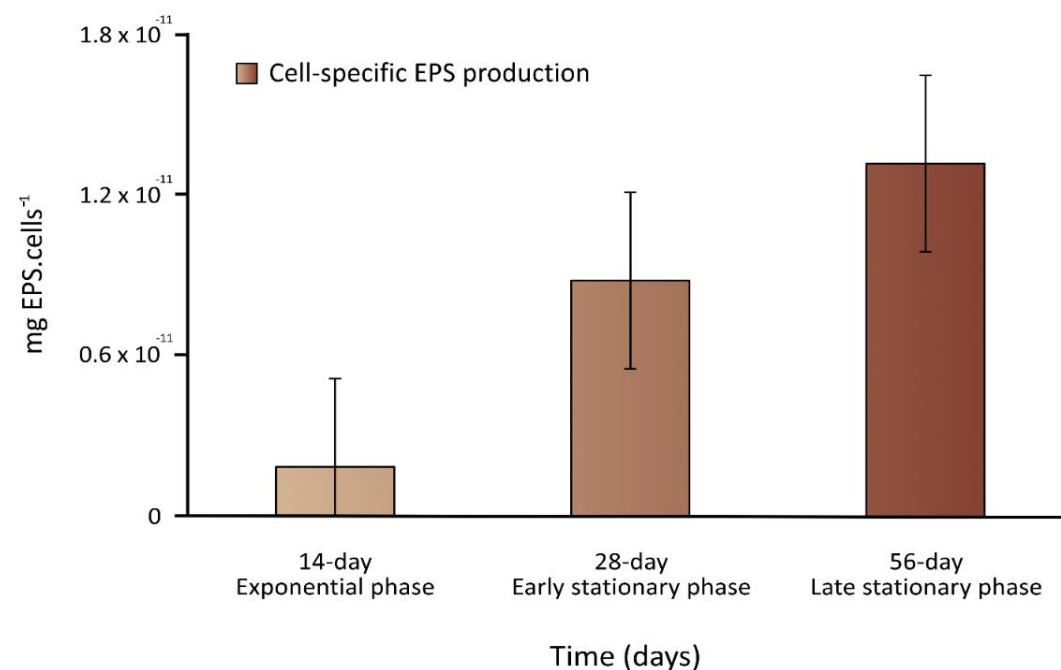
Table 1. Concentrations of NO_3^-, PO_4^{3-} and Ca^{2+} (µM) in the culture medium before inoculation (initial concentrations in the medium) and during exponential, early and late stationary of *Synechococcus* growth phases are given as mean concentrations of four replicates (n=4).

Major anions and cations (µM)	Initial concentrations in the medium	*Synechococcus* growth phases		
		Exponential	**Early stationary**	**Late stationary**
NO_3^-	7082±58.7	5731±328.9	5544±57.9	4716±250.1
PO_4^{3-}	68±0.6	39±4.7	41±2.2	21±6.7
Ca^{2+}	102±0.5	91±2.1	88±1.7	83±4.8

3.3 Abundance of EPS

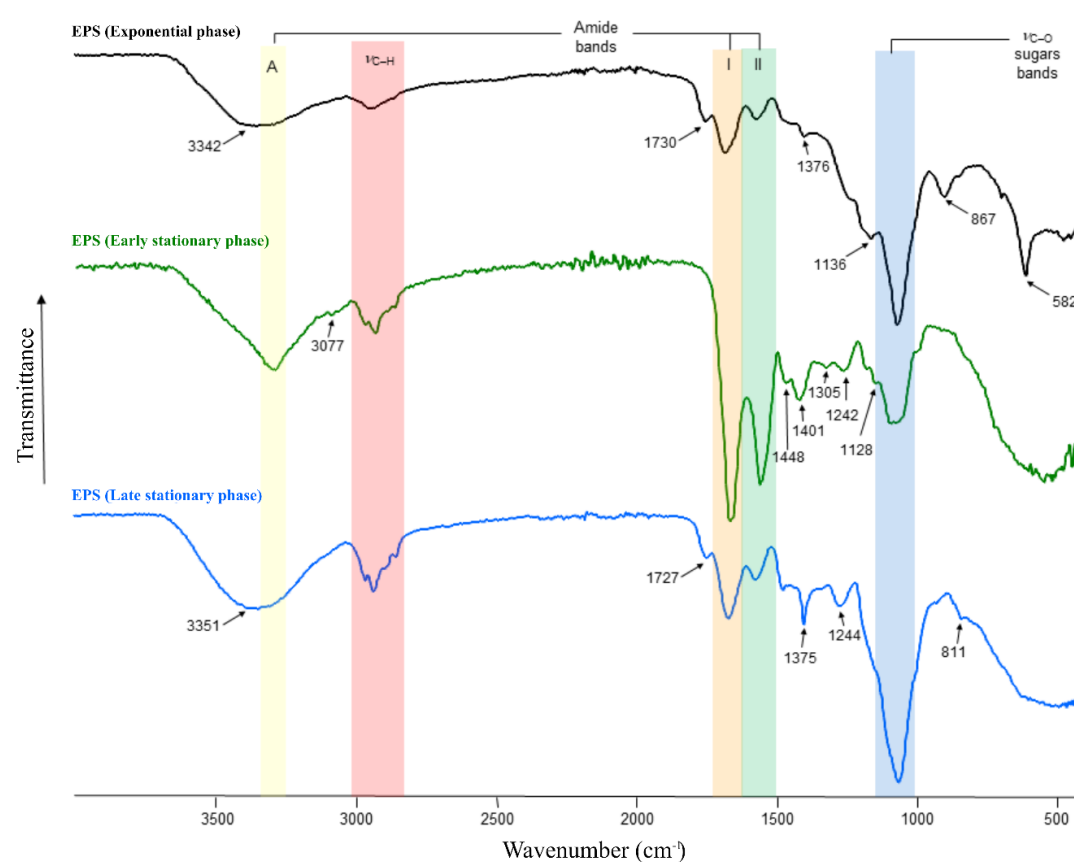
The recovery yields of the EPS produced (mean ± SD) resulting from the applied extraction method are listed in Table 2. The EPS yields varied from 2.9±0.5 to 18.6±2.1 mg.L^{-1} during exponential and early stationary phases and reached the highest yield of 35.4±4.2 mg.L^{-1} at 56 days of culture, in the late stationary phase (Table 2). When the values were normalized per cell yield, results showed that the EPS concentration increased significantly between the exponential and late stationary phases (p-value < 0.05) (Figure 2). *Synechococcus* continuously produced EPS during the 56-day experiment. In the first 14 days of growth, cells grew exponentially and EPS production was deficient. Between exponential and early stationary phases, EPS production increased by a factor of five to seven, reaching a maximum in the late stationary phase, after the 56-day growth experiment.

Table 2. Cell yield, total EPS production and cell-specific EPS production in *Synechococcus* PCC7942 cultures during exponential, early and late stationary growth phases. Data represent the means of two independent experiments.

	Time of harvest (growth phase)		
	Exponential	**Early stationary**	**Late stationary**
Cell yield (cells.L^{-1})	(161.6±21.6)10^{10}	(211.2±6.0)10^{10}	(268.8±14.4)10^{10}
EPS yield (mg.L^{-1})	2.9±0.5	18.6±2.1	35.4±4.2
Cell-specific EPS production (mg. cells^{-1})	(1.9±0.6)10^{-12}	(8.8±0.8)10^{-12}	(13.1±0.9)10^{-12}

227
228
Figure 2. Cell-specific EPS production during the exponential, early and late stationary phases. MEAN±SD replicates from (n=2).

3.4 Chemical properties of EPS

3.4.1 FT-IR spectroscopy of EPS

FT-IR spectroscopy was used to check the overall EPS properties and composition. The IR spectra of EPS harvested during the exponential, early and late stationary phases of the growth experiment are depicted in Figure 3. The three spectra show strong similarities, exhibiting characteristic absorption bands for polysaccharides and protein moieties (highlighted in Figure 3 by vertical-coloured areas). However, differences in sample composition were also revealed by the presence of additional absorptions indicated by arrows in Figure 3. Interestingly, the spectrum of the exponential phase EPS exhibits a strong band, isolated at 582 cm^{-1}, which according to the literature on EPS could be assigned to a C–X stretch of alkyl halides (Kavita et al., 2011). Bands at 811-868 cm^{-1}, most likely representing the glycosidic linkage between sugar monomers, were only present in EPS extracts in the early and late stationary phases. Bands at 1039 – 1128 cm^{-1} (C–O and C–O–C stretching vibrations) could be assigned to polysaccharides and polysaccharide-like structures (Wang et al., 2012) and were observed in all EPS samples (Figure 3, blue area). In contrast, the small shoulders observed in the early and late stationary phase EPS, at ~1242 and 1244 cm^{-1} correspond to sulfate groups (nS=O stretching vibrations). Low-intensity bands observed in the range of 1370-1450 cm^{-1} are assigned to CH$_3$ and CH$_2$ deformations (bends) of proteins (Kansiz et al., 1999). These absorption bands were more evident in EPS obtained during the early stationary phase. The bands present in the range of 1660 and 1540 cm^{-1} are attributed to C=O and C–N stretching vibrations and are characteristic of Amide I and II functions (Figure 3, orange and green areas, respectively), which are typically associated with proteins (Coates, 2000). Spectra of the early stationary phase EPS showed higher peaks of protein than those observed in EPS from exponential and late stationary phases. The medium bands at 1730 and 1727 cm^{-1}, present in samples extracted from exponential and late stationary phases, can be attributed to C=O stretching vibrations resulting from lipids and fatty acids (Kansiz et al., 1999). Absorptions in the range of 2960–2850 cm^{-1} corresponding to C–H stretching vibrations of aliphatic hydrocarbons and possibly indicative of long-chain polymers (*e.g.,* sugars or proteins), were observable in all EPS extracts. The amide A band (3345 cm^{-1}), characteristic of the N–H vibration of peptide groups in proteins, is present in all spectra (Figure 3, yellow area), but is particularly visible on the early stationary phase EPS spectrum. In the samples at 14 and 56 days of growth, this band is included in shoulders due to the presence of OH absorptions centred at 3342 and 3351 cm^{-1}, respectively. The list of band assignments is summarized in supplementary material (Table 1S).

250
Figure 3. FT-IR spectra of EPS produced during the exponential (black line), early (green line) and late (blue line) stationary phases. Amide A absorbs in the range of 3342-3351 cm^{-1} (yellow area), amides I-II at 1542–1650 cm^{-1} (orange and green areas), sulfate groups at ~1242-1244 cm^{-1}, polysaccharides at ~1040–1070 cm^{-1} (blue area), and the β-glycosidic linkages are visible as a shoulder at ~867cm^{-1}.

3.4.2 Protein, sugar and glycosaminoglycan (GAGs) contents

The EPS produced during the exponential growth phase revealed the lowest concentration of protein (79±9 µg. mg⁻¹ EPS) (Table 3). The highest protein concentration was measured in EPS produced during the early stationary phase (253±42 µg.mg⁻¹ EPS), whereas during the late stationary phase EPS, the protein concentration decreased by ~ two-fold. When accounting for the cell yield at times of EPS extraction, cells produced EPS with *ca* 11-15 times more protein in the stationary phase than in the exponential phase. The sugar content in the EPS harvested during the three different growth stages did not vary significantly. The EPS produced during the exponential phase contained a slightly higher sugar content (584±9 µg of xanthan and 504±78 µg of dextran equivalents. mg⁻¹ EPS) than that measured in EPS produced during the early and late stationary phases (1.8 times and 1.3 times lower, respectively). Our results show that, over the cultivation time, cells enhanced the production of larger amounts of glycosaminoglycans (GAGs) which can be associated with amino sugars and glycoproteins. The highest fraction of sulfated groups (GAGs) to total EPS (217±143 µg GAGs.mg mg⁻¹ EPS) was found in the late stationary phase EPS.

Table 3. Protein, sugar and glycosaminoglycan content of the harvested EPS at times 14, 28 and 56 days of *Synechococcus* PCC7942 culture. Values represent the average of four, three and two measurements of protein, sugar and GAGs, respectively, in two EPS replicated samples (n=2).

	Time of EPS harvesting (days/growth phase)		
Components of EPS yield	**14 days** **Exponential**	**28 days** **Early stationary**	**56 days** **Late stationary**
Protein (µg·mg⁻¹ EPS)	79±9	253±42	128±13
Cell-specific protein production (µg protein.cell⁻¹)	$(1.5±0.6) \times 10^{-10}$	$(2.2±0.1)\times 10^{-9}$	$(1.7±0.0)\times10^{-9}$
Sugar (µg xanthan equivalents·mg⁻¹ EPS)	584±95	326±26	434±11
Cell-specific sugar production (µg xanthan equivalent.cell⁻¹)	$(1.0±0.2)\times10^{-9}$	$(2.8±0.1)\times10^{-9}$	$(5.7±0.2)\times10^{-9}$
Sugar (µg dextran equivalents·mg⁻¹ EPS)	504±78	292±22	381±90
Cell-specific sugar production (µg dextran equivalent.cell⁻¹)	$(8.9±1.4)\times10^{-10}$	$(2.6±0.1)\times10^{-9}$	$(5.0±0.2)\times10^{-9}$
Glycosaminoglycans (µg GAGs·mg⁻¹ EPS)	4±0	31±13	217±143
Cell-specific GAGs production (µg GAGs.cell⁻¹)	$(5.5±5.5)\times10^{-12}$	$(2.6±0.8)\times10^{-10}$	$(3.0±2.0)\times10^{-9}$
GAGs/Sugar (xanthan) ratio	0.01±00	0.09±00	0.51±0.3
GAGs/Sugar (xanthan) ratio	0.01±00	0.10±00	0.58±0.4

3.4.3 SDS-PAGE

The results of gel electrophoresis after the migration of exponential, early and late stationary phase EPS samples are illustrated in Figure 4. Replicates showed similar band patterns that are distributed between 10 and > 170 kDa. A sharp greenish band in the migration front is strongly stained in late stationary phase EPS (Figure 4) and may correspond to chlorophyll. A less pronounced smear is visible in extracts obtained from the early stationary phase (Lanes 4 and 5). Bands of < 10 kDa were not detected in the EPS produced during the exponential phase (Lanes 2 and 3). A marked smear pattern is evidenced in all EPS extracted between 10-26 kDa: one prominent band was individualized at 17 kDa. A discrete blue smear (> 17-43 or 55 kDa) is evidenced in exponential phase EPS samples (Lanes 2 and 3) and is less obvious in EPS samples from the early and late stationary phase (lanes 4-5 and 6-7, respectively). No specific bands were individualized in the > 17-43 kDa molecular mass range, for the three growth phases. A band at about 45-47 kDa was strongly stained in exponential phase only. An area between 43 and 170 kDa was noted in all EPS extracts, accounting for 5-6 individualized bands that may correspond to the consecutive addition of an identical 'module', because the progression is logarithmic: is clearly seen in the early and late stationary phase lanes (lanes 4-7). The individualized bands were densely stained in EPS from the late stationary phase, including a smear at ~43-55 or 72 kDa (Lanes 6 and 7) and a prominent band at > 170 kDa (Lanes 6 and 7).

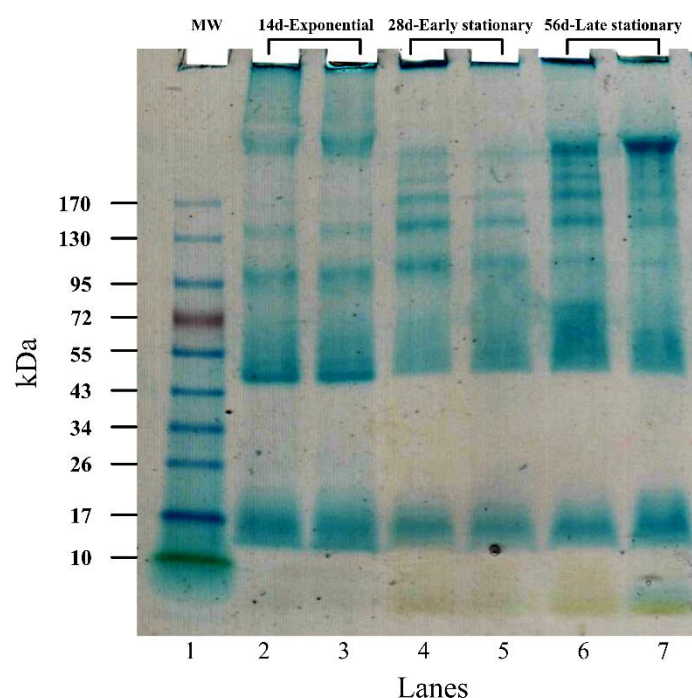



Figure 4. SDS-PAGE of EPS harvested during exponential (lanes 2-3), early (lanes 4-5) and late (lanes 6-7) stationary phases. Alcian blue staining was applied. The molecular ladder (MW) reference is shown in lane 1.

3.4.4 pH-drift assay

Recordings of the pH-drift assay are shown in Figure 5. The pH-drift assay determined the inhibitory effect of the EPS matrix (e.g., negatively charged functional groups) on the rate of $CaCO_3$ precipitation. Negatively charged groups of EPS can bind calcium ions from the solution and inhibit the nucleation of carbonates. When

CaCO₃ minerals start to nucleate, the pH of the solution decreases. Results show that the inhibitory effect was concentration-dependent and clear differences were visible between EPS extracted in the exponential (Figure 5A), early (Figure 5B) and late (Figure 5C) growth phases. EPS matrices from the stationary phase of culture growth (Figures 5B and 5C) exhibited a stronger inhibitory effect on CaCO₃ precipitation than the EPS extracted during the exponential phase (Figure 5A). Complete inhibition was only reached in EPS from early and late stationary phases when 50 µg of EPS.mL⁻¹ was tested. In this case, a drop in pH was not observed and nucleation of crystals did not occur (Figure 5B and 5C), which means that the inhibition was total. Conversely, the exponential phase EPS exhibited less inhibition of CaCO₃ precipitation (Figure 5A). The shorter plateau shows that the mineral-binding capacity of the matrix delayed CaCO₃ precipitation but that consequently the pH dropped and visible precipitates formed, showing a less powerful inhibitory effect of the EPS compared to stationary phases EPS matrices.

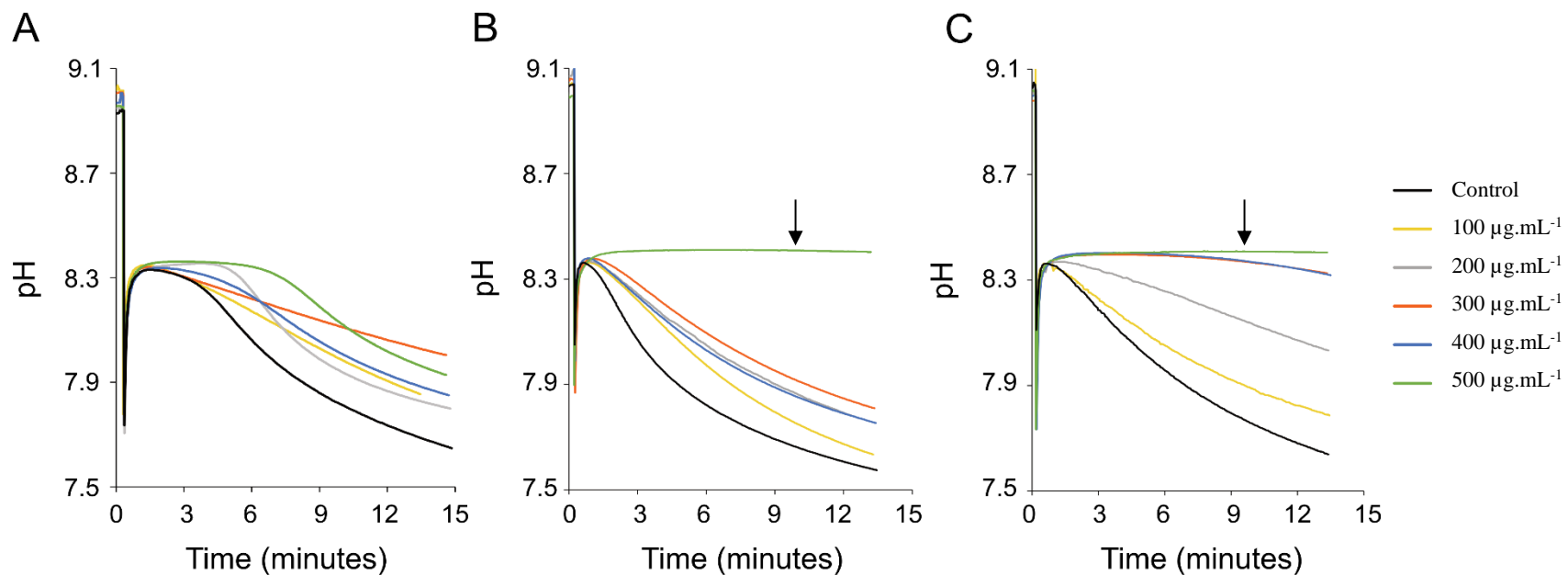



Figure 5. *In vitro* inhibition of calcium carbonate precipitation by using EPS extracted during exponential **(A)**, early **(B)** and late **(C)** stationary phases. Each panel shows the effect of six different EPS concentrations (0, 100, 200, 300, 400 and 500 µg EPS. mL⁻¹) on CaCO₃ precipitation, using the pH-drift assay method. The drop in pH indicates nucleation of CaCO₃ (= precipitation) and a plateau indicates inhibition of precipitation. A larger plateau indicates a higher Ca-binding capacity of the matrix and thus stronger inhibition. Complete inhibition was observed when 50 µg of EPS solution from early and late stationary phases were used (*e.g.,* see arrows). The results in each panel represent single experiments. Replication showed identical results (see Supplementary Figure 2S).

3.5 Calcium carbonate crystallization in the presence of EPS

Forced CaCO₃ experiments were performed using a control solution (without EPS) and EPS solutions, at same pH, with concentrations of 3, 18 and 36 µg.mL⁻¹. Each concentration corresponds to the EPS yield at different growth stages: exponential phase (= 3 µgEPS.mL⁻¹), early (18 µgEPS.mL⁻¹) and late (36 µgEPS.mL⁻¹) stationary phases. The crystals formed in the various EPS solutions showed different morphological (Figure 6) and mineralogical (Figure 3S) features as well as distinct crystal sizes and distributions compared to those formed in control solution (Figure 7).

3.5.1 Mineral morphology

A preliminary light microscopic analysis was carried out in order to identify the most significant samples to analyse by SEM (Figure 6). The morphology of crystals precipitated in the negative controls was very homogeneous and predominantly composed of calcite rhombohedrons that sometimes formed polycrystalline aggregates of size > 50µm (Figure 6A). All control solutions tested for the various EPS harvested during exponential and stationary phases showed similar crystal characteristics. In the EPS solutions, CaCO₃ crystals showed both rhombohedral and spheroidal morphologies (Figure 6B-D). The morphology of crystals appears to change with increasing EPS concentrations. Spherical minerals formation was observed in the exponential phase-EPS solution (Figure 6B) and were less frequent in the EPS solution from early stationary phase (Figure6C). In the late stationary phase-EPS solution, rhombohedrons represented the prevalent crystal morphology while spherical minerals were absent (Figure 6D).

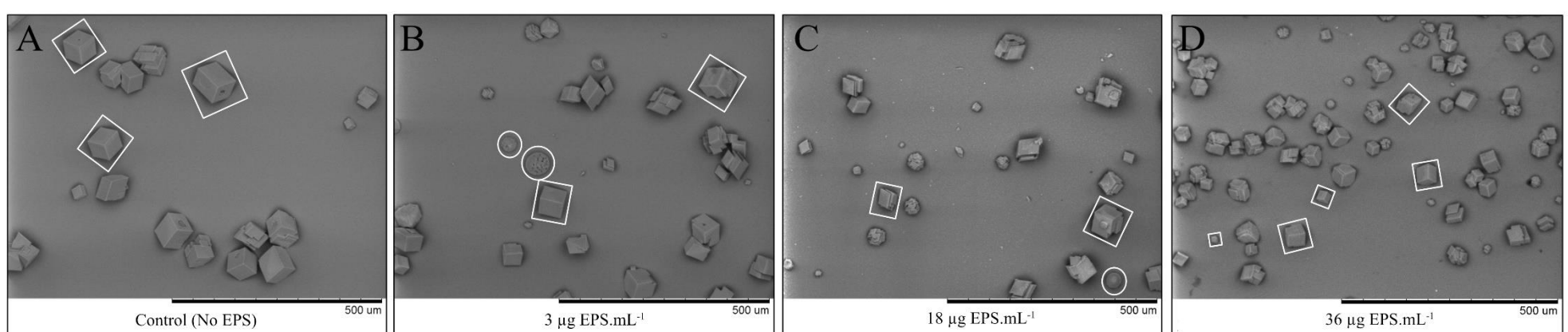



Figure 6. *In vitro* forced CaCO₃ precipitation assay in **(A)** the absence of the EPS (control solution) and in the presence of EPS extracted during the **(B)** exponential, **(C)** early and **(D)** late stationary phases under increasing EPS concentrations of 3, 18 and 36 µg.mL⁻¹, respectively. The images show two different CaCO₃ morphologies: rhombohedral (white squares) and spheroidal (white circles), in some cases shown as polycrystalline crystals. The scale bar (black) at the bottom right of the images is 500 µm.

317 **3.5.2 Crystal mineralogy**

318 The crystals' mineralogy was assessed by FT-IR microscopy performed on selected individual crystals of > 10 µm (Figure 3S). The results revealed that calcite was

319 the only $CaCO_3$ polymorph formed in the control solution. Calcite and vaterite formed in all EPS solutions tested. The FT-IR spectra revealed that all rhombohedrons

320 and polycrystalline aggregates with "sharp edges" represent calcite polymorphs. In contrast, spheroidal crystals revealed a vaterite signature (Figure 3S).

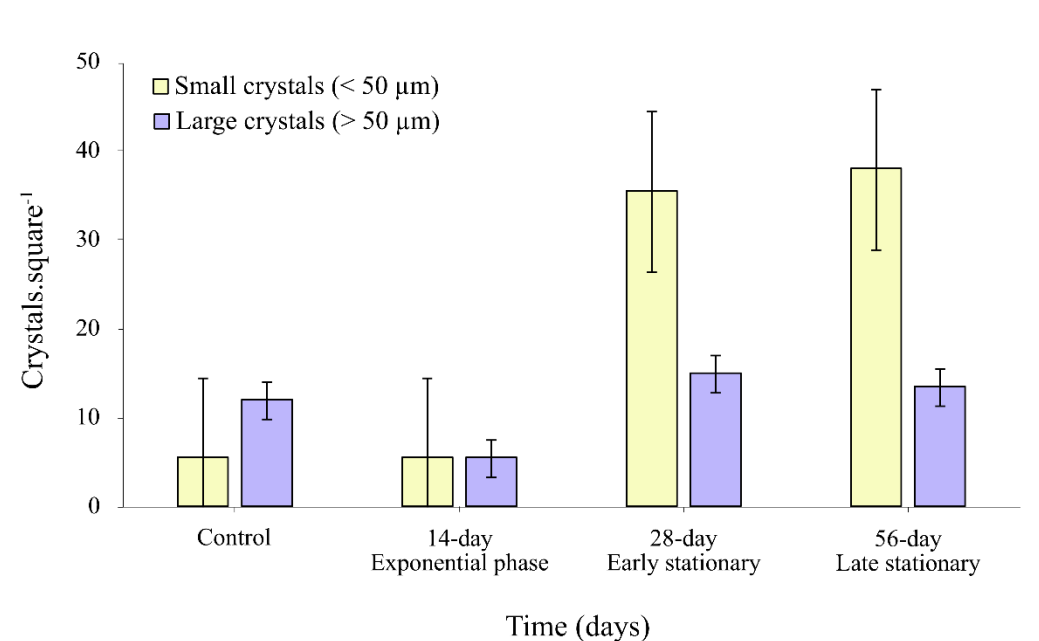
321 **3.5.3 Crystal size and distribution**

322 The results from image analysis showed that a larger quantity of crystals precipitated in the stationary phase (early and late) EPS solutions (Figure 7) and that major

323 differences were also observed in crystal size distribution (Figure 7). A comparison of the class of small crystal sizes (< 50 µm) with the large crystal size class

324 (>50µm) showed a clear trend of an increasing total number of small crystals in the stationary phase EPS solutions compared to those formed in the EPS solutions

325 from the exponential phase. The size reduction of the crystals at 18 and 36 mg/L (Figure 7, early and late stationary phases) suggests a partial inhibitory effect of the

326 EPS on the formation of calcium carbonate.

327
328 **Figure 7.** Total numbers of small (< 50 µm) and large (> 50 µm) crystal size classes of precipitated $CaCO_3$ in EPS solutions obtained from exponential and stationary phases, in EPS

329 concentrations of 3, 18 and 36 µg.mL^{-1}, respectively.

330 **4. Discussion**

331 Our study demonstrates that the amount and properties of EPS change significantly (p-value < 0.05) at the three different stages of *Synechococcus* growth in an

332 artificial bloom experiment. Cells continuously produce EPS that increases in concentration and become more negatively charged in the stationary phase. We sampled

333 this EPS over the exponential, early and late stationary phases and studied its role in carbonate mineral precipitation. Based on this, a conceptual model was developed

334 to correlate the findings of this investigation with the potential EPS production of the naturally occurring picoplankton blooms and its possible involvement in whiting

335 events. Though natural blooms experience a variety of factors that are not represented in the experiments described in this paper, the first part of the discussion is

336 focussing only on the experimental data, whereas the interaction of these basic processes with other biotic and abiotic factors acting in the environment is discussed

337 afterwards.

338 **4.1 Exponential growth phase**

339 Macronutrients, such as nitrogen (N) and phosphorus (P) promote the initiation of cyanobacterial blooms (Reynolds and Walsby, 1975; Paerl, 2008; Xu et al., 2015).

340 In our growth experiment, the beginning of the exponential phase (and the persistence of bloom) (Figure 1A) was positively correlated with the high initial nutrient

341 concentration in the medium (Table 1). Environmental factors such as water temperature, light intensity, hydrodynamics and availability of dissolved inorganic carbon

342 (DIC) are also important determinants of cyanobacteria bloom development (Clark and Flynn, 2000; Dokulil and Teubner, 2000; Havens, 2008). Blooms can

343 dramatically alter the supply of inorganic carbon for photosynthesis, which causes the pH to increase (Ibelings and Maberly, 1998). In the early exponential phase of

344 our batch cultures, the high photosynthetic activity of cyanobacteria cultures resulted in fast pH increase thereby reducing the total inorganic carbon of the grown

345 medium. Light and CO_2 are the sources of energy and carbon for cyanobacteria, and are of critical importance for their growth (Takahashi et al., 2004). At pH 9

346 (Figure 1B), the concentration of CO_2 predicted is close to zero (< 1µM) and the HCO_3^- concentration is 475 µM (PhreeqC data). A similar scenario was observed in

347 natural blooms occurrence: the population of cyanobacteria draws down the partial pressure of CO_2 (pCO_2) in the photic zone, increasing the surface water pH up to

348 9-10 (Ibelings and Maberly, 1998; Verspagen et al., 2014) and CO_2 concentration can become completely depleted or reach values close to zero (Maberly, 1996).

349 Under extreme conditions, the concentration of HCO_3^- can also become markedly reduced (Talling, 1976; Maberly, 1996). When the rate of photosynthesis is greater

350 than the combined rate of resupply of CO_2 from the atmosphere and DIC in the hypolimnion, deviation from the air equilibrium occurs, favouring $CaCO_3$ precipitation.

351 The pH of most aquatic systems ranges from 7.5-8.1 and keeps inorganic carbon primarily in the form of bicarbonate (O'Neil et al., 2012). In poorly buffered systems,

352 such as highly productive lakes, the pH and speciation of DIC experience large fluctuations which vary widely on a scale from daily (diel) to episodic, to seasonal

353 (Maberly, 1996) with diel variations as high as two pH units and 60 µmol DIC.L^{-1} (Maberly, 1996). Because CO_2 favors the C_3 photosynthesis (C_3 cycle operation of

354 Calvin-Beson cycle), the high pH of ~ 10 in our growth medium could be associated with carbon limitation (Ibelings and Maberly, 1998; Verspagen et al., 2014).

355

356 To alleviate CO_2 limitation, cyanobacteria have developed an efficient CO_2-concentrating mechanism (CCM) (Aizawa and Miyachi, 1986; Badger and Price, 1992;

357 Badger et al., 2002; Burnap et al., 2015) and can use bicarbonate as an inorganic carbon source for photosynthesis (Price et al., 1998; Giordano et al., 2005; Sandrini

358 et al., 2016). By activating CCM, cyanobacteria concentrate CO_2 by a factor of up to a thousand (Badger and Andrews, 1982; Badger et al., 2002; Price, 2011). CO_2-

359 deficient conditions experienced during the exponential phase of our growth experiment, coupled with the continuous cellular demand for inorganic carbon to support

360 photosynthetic carbon fixation likely led the cells to activate CCM. The predicted concentrations of CO_2 and HCO_3^- in the growth medium (PhreeqC data) in the early

361 and late exponential phase infer that *Synechococcus* cells actively transported across the membrane and accumulated DIC into the cell, where the HCO_3^- pool was

362 utilized to generate elevated CO_2 levels around Rubisco (Badger et al., 2002; Price et al., 2008). The CCM of cyanobacteria accomplishes very high carbon

363 concentrating factors ($C_{extrenal} : C_{internal}$) at deficient specificity factors of RuBisCo (Tortell, 2000; Tortell et al., 2000). CCM involves bicarbonate transporters in the

364 cell membrane, intracellular (iCA) and extracellular (eCA) carbonic anhydrase enzymes and concentrated RuBisCO activity located in carboxysomes (Badger et al.,

365 2006; Price et al., 2008; Rae et al., 2013). CA converts HCO_3^- to CO_2 (Badger and Price, 1994), which increases the external pH in close proximity to the cells. In our

366 study, eCA activity was ~ 1.6-2.0 times higher during the exponential growth phase and reduced gradually through the stationary phase (Supplementary Figure 1S).

367 The strongly stained band only present in the exponential phase-EPS at around 45-47 kDa (Figure 4, lanes 2, 3) may be indicative of eCA, as reported by Kupriyanova

368 et al., 2018, but this requires further investigation. Another plausible explanation for the 45-47 kDa band could be the presence of chlorophyll f synthase, which

369 typically migrates at around 46 kDa (Shen et al., 2019). Similarly, Yang et al. (2023) measured the CA anhydrase in solution over a 30-day growth experiment with

370 *Synechococcus* PCC 7942 and reported an increase over the lag phase and large fluctuations over the exponential phase. During the stationary phase, CA did not vary

371 greatly but a minor decrease was recorded in the late stationary phase (Yang et al., 2023). In our study, the higher eCA activity recorded could explain the strongly

372 stained ~45-47-kDa band that was only identified in our SDS-PAGE gels of EPS produced during the exponential phase (Figure 4, lanes 2-3). The molecular weight

373 (MW) of this band is similar to a 42-43 kDa eCA previously identified by Kupriyanova et al. (2018) and discussed by Martinho de Brito et al. (2022). As explained

374 in the Results section 3.4.3, we cannot exclude that the band is chlorophyll f synthase, which seems to show up around 46 kDa. A more substantiated demonstration

375 of the identity of the SDS-PAGE band will require other approaches (beyond the scope of the present study), such as micro-sequencing of the purified 43 kDa band

376 or the use of a CA-specific antibody.

377

378 Active uptake of HCO_3^- and accumulation of Ci species requires the input of metabolic energy e.g., ATP (BCT1 HCO_3^- transporter), NADPH or reduced ferredoxin

379 (CO_2 uptake) or coupling to an electrochemical Na^+ gradient (SbtA or BicA HCO_3^- transport) (Badger et al., 2002; Price et al., 2008). This energetic cost may therefore

380 reflect on the growth rates achieved. *Synechococcus* PCC 7942 grows at > 80% of its maximum growth rate when provided with HCO_3^- as its main inorganic carbon

381 source (Miller et al., 1984). During the exponential phase, the carbon production from photosynthesis is mainly allocated for biomass production, not for EPS synthesis.

382 During this phase (Figure 2 and Table 2), the small amount of EPS produced comprises a higher proportion of sugars and lower amounts of protein and GAG compared

383 to EPS produced during the stationary phase (Figure 3 and Table 3). Our study indicates that rather than proteins, sugars are the major component in all EPS extracts.

384 This finding is supported by the data obtained from FTIR analysis (Figure 3). The smaller amount of negatively charged groups of the EPS during the exponential

385 phase (Figure 4, lanes 2-3) compared to those of EPS from the early and late stationary phases (Figure 4, lanes 4-7) resulted in weak to moderate inhibitory capacity

386 (Figure 5A). The main phenomenon observed in the pH-drift assay (Figure 5) is the initial Ca binding to negatively charged groups in EPS prior to carbonate addition,

387 which initiates $CaCO_3$ precipitation. This results in a decrease of pH. The pH drift assay showed that EPS from exponential phase (Figure 5C) having a larger plateau,

388 and thus a lower a calcium binding capacity then the EPS from the stationary phase. This observation was further corroborated by the forced precipitation experiments,

389 which showed that EPS from the exponential phase induced small amount of mostly large-sized carbonate crystals (>50 µm), very similarly to the negative control

390 experiment (Figure 7) (Martinho de Brito et al., 2022). The high concentration of Ca^{2+} in the medium (83 µM) compared to the initial $[Ca^{2+}]$ at the beginning of the

391 experiment (103 µM), indicates that a small amount of calcium ions was bound to negatively charged functional groups of EPS (Table 1, see $[Ca^{2+}]$).

392 In our batch experiment, cells continue to grow exponentially for ~20 days of cultivation. At this point, cultures reached the maximum cell density (Figure 1A) and

393 pH values ranged between 10-11 (Figure 1B). Based on our calculations, under these alkaline conditions, CO_2 was completely depleted (1.7×10^{-3} µM) in the growth

394 medium, whereas HCO_3^- was extremely low (~ 79 µM). Thus, the dominant inorganic carbon speciation was CO_3^{2-} (421 µM). Because cells cannot take up CO_3^{2-} and

395 HCO_3^- concentration seems to be insufficient to cover the carbon demands of cyanobacterial growth, we assume that this may have been the cause of cell numbers

396 starting to level off (Figure 1A, early stationary phase). Consequently, cultures entered a stationary state due to a lack of inorganic carbon availability required to

397 increase cell population (Miller et al., 1984; Mayo et al., 1989; Verspagen et al., 2014). The excess of nutrients measured in the medium in the late exponential phase

398 (Table 1) suggested that the specific growth rate was not limited by nutrient availability but by a rather low level of CO_2 carbon content.

399

400

401 **4.2 Early stationary phase**

402 Insufficient CO_2 availability is considered to be the external stress factor constraining the growth rate of cyanobacteria (Maberly, 1996; Hein, 1997; Ibelings and

403 Maberly, 1998) and low $[HCO_3^-]$ could sustain a constant population density for at least ~ 40 days (See Figure 1A, stationary phase). Our results suggest that at this

404 point, carbon fixation was allocated to EPS synthesis, not to biomass production (Miller et al., 1984). Increased EPS production is usually associated with external

405 stress factors (Rossi and De Philippis, 2015), including high pH conditions (Martinho de Brito et al., 2022). Moreover, metabolic stress may also alter the composition

406 of EPS (Babele et al., 2019; Martinho de Brito et al., 2022). In the present study, the negative functional group abundance increased, resulting in a higher acidity of

407 EPS (Figure 4, lanes 4-5) due to an increase in protein and sulfated glycan (GAG) (Table 3). In the pH conditions of the early stationary phase, all the functional

408 groups of the EPS matrix are deprotonated and are able to bind calcium ions (Figure 5B) (Dupraz and Visscher, 2005; Braissant et al., 2007; Dittrich and Sibler, 2010)

409 and bind calcium more efficiently nanometric nuclei in formation (if their formation is thermodynamically favoured). We suggest that the increased calcium-binding

410 capacity of the EPS probably accounts for lower the Ca^{2+} concentration measured in the medium (Table 2, see $[Ca^{2+}]$). In our *in vitro* forced precipitation assay, we

411 measure the second effect, the inhibitory one (mineral-binding effect), which results in the production of small-sized calcium carbonate crystals (< 50 µm), in

412 comparison to what happens in the exponential phase (Figure 7).

413

4.3 Late stationary phase

As mentioned above, we assume that the continuous increase in EPS production over the late stationary phase, including an overall augmentation of negatively charged functional groups (Figure 4, lanes 6-7), including GAG content (Table 2), might be a specific response to a stress scenario As expected, the present study shows that the greater amount of negatively charged functional groups of EPS from the late stationary phase (Figure 4, lanes 6-7) resulted in a higher Ca-binding capacity than exponential and early stationary phase-EPS (Figure 5C). Our forced precipitation experiments showed that minerals produced in the late stationary-EPS solutions are smaller and more abundant than those formed in EPS solutions from the early stationary phase (Figure 7). Under natural conditions, when the Ca^{2+} supply is continuous, the crystals may or may not continue to grow, depending on the physical space within the EPS matrix (Dupraz et al., 2009). Based on the high concentration of nitrate (4720 µM) measured in the late stationary phase (Table 1), we assume that the abundance of this nutrient supported the persistence of the stationary phase, i.e., similar to a prolonged bloom in natural conditions. The death phase was not observed in our 56-day-long experiment. Given that our cultures were continuously stirred, we can assume that light was not limiting cyanobacterial growth. Furthermore, in natural blooms, the increase in population density may affect cells at greater depth through self-shading by decreasing the light available for photosynthesis (Townsend et al., 1994). Yet, cyanobacteria (including *Synechococcus*) are known to be well-adapted to low-light conditions (Campbell and Carpenter, 1986; Palenik, 2001; Callieri et al., 2011). Additionally, the presence of sulfated constituents on late stationary phase-EPS contributes to a higher negative charge of the matrix and higher Ca-binding potential (Decho and Kawaguchi, 2003; Skoog et al., 2022), compared to EPS extracted in the exponential phase which contained significantly lower GAG (Table 2). The present study shows that the greater amount of negatively charged functional groups of EPS from the late stationary phase (Figure 4, lanes 6-7) resulted in a higher Ca-binding capacity than exponential and early stationary phase-EPS (Figure 5C). Our forced precipitation experiments showed that minerals produced in the late stationary-EPS solutions are smaller and more abundant than those formed in EPS solutions from the early stationary phase (Figure 7), suggesting an increased inhibitory ability of the late stationary-EPS.

4.4 Natural bloom and formation of whitings – Conceptual model

Our observations made during exponential and stationary phases can be applied to generate a conceptual model of EPS properties during a *Synechococcus* bloom event (Figure 8A-C). The onset of a bloom starts with an increase in cell numbers, with high values in spring-summer (exceeding 10^5-10^7 cells.mL^{-1}) and lower values in winter months ($< 10^5$ cells.mL^{-1}) in both marine (Agawin et al., 1998; Phlips et al., 1999) and freshwater (Maeda et al., 1992; Tai and Palenik, 2009) environments. This resembles the exponential growth phase in our study (Figure 1, exponential phase). We predict that during the initial phase of a natural bloom, there is little EPS production: cells grow relatively quickly and the carbon fixed during photosynthesis is predominantly allocated to biomass production (Figure 8A). The fast growth is followed by a phase during which cell numbers level off, typically due to stress conditions, which is represented by the early stationary phase in our study. Under certain conditions, blooms can be sustained for weeks and possibly longer (Anderson et al., 2002; Havens, 2008; Zhao et al., 2013), similar to what we observed in our growth experiments (Figure 1A, early stationary phase). The maintenance of a bloom requires continuous input of nutrients, which is also the case in our experiment (Table 1) or in the case of natural systems, a turnover from lysing cells recycled by other microbes. During this phase, we did not observe a significant increase in cell density but the production of EPS continued at a disproportionately high rate (Figure 8B-C). Our findings are in agreement with the lab studies using diatom cultures which show that EPS production is low during exponential growth and increases in the stationary phase (Myklestad and Haug, 1972; Myklestad et al., 1989; Bhosle et al., 1995). These authors reported that nutrient-deficient conditions enhanced the production of EPS over the growth phases. If carbon fixation continues and some critically required nutrient is lacking from the growth medium, most likely the phototrophic organisms produce carbohydrate reservoirs (Ciebiada et al., 2020). These include storage polymers like glycogen and the production of other carbohydrate-rich compounds, including EPS (De Philippis et al., 1996, 2001; Decho and Gutierrez, 2017). The decline of blooms in natural environments is typically associated with nutrient, low or high light intensity, grazing or viral infection. Under these stressful conditions, an increase in EPS production by the phyto/picoplankton community may be expected.

Synechococcus spp. blooms can cause whiting events (Thompson, 2000), characterized by the presence of large amounts of $CaCO_3$ minerals in surface water. Various mechanisms have been proposed for this phenomenon, including chemical and physical processes (Shinn and Steinen, 1989: Larson and Mylroie, 2014) as well as biologically mediated precipitation (Thompson and Ferris, 1990; Robbins and Blackwelder, 1992; Stanton et al., 2021). However, no consensus has been reached on the precise cause of these events. Carefully transporting the results from forced precipitation experiments to a whiting event, we suggest that early in the bloom (Figure 8A), relatively large $CaCO_3$ crystals form, provided sufficient Ca^{2+} is available (Figure 8D). As the bloom continues to grow, progressively the larger quantity of negatively charged functional groups in the EPS provides more cation-binding sites and thus inhibits calcium carbonate precipitation largely. Depending on the three-dimensional structure of the EPS and surface properties (Wang et al., 2012), nucleation may yield smaller $CaCO_3$ crystals (Figure 8). If this occurs, then the production of a more negatively charged matrix (largely contributed by the enrichment in sulfated polysaccharides) may offer some selective advantage to the cyanobacteria population, by inhibiting and/or delaying mineral precipitation and by reducing crystal size formed around the cells. This might result in slow sinking rates, extending the residence time of the cyanobacterial community in the photic zone. If the bloom occurrence is short (*e.g.,* similar to 14-28 days in our growth experiment), minerals making up the whiting will be relatively larger. Consequently, the aggregates of cyanobacteria, EPS and $CaCO_3$ minerals may sink faster because mineral precipitation in EPS increases the cyanobacterial-specific density several-fold. The *Synechococcus* specific density (ρ) is 1.040 g·cm^{-3} (Reynolds, 1987), near-neutrally buoyant, whereas $\rho_{calcite}$ is 2.710 g·cm^{-3} (Lange, 1999). The production of larger amounts of more negatively charged EPS may act as a protection mechanism against carbonate formation in the vicinity of the cell wall (Martinez et al., 2010; Bundeleva et al., 2012), thus allowing the organisms to reside longer in the photic zone. Interestingly, the production of EPS that contained sulfated groups among bacteria seems to be exclusive to cyanobacteria (Pereira et al., 2009; Maeda et al., 2021). Maeda et al. (2021) reported that the cyanobacterium *Synechocystis* 6803 produced large amounts of GAG compounds during an experimental bloom formation. The authors suggested that these constituents can be advantageous for the development of surface bloom as it may increase the buoyancy, permitting cells to migrate upward rapidly when the water column is stable (Walsby et al., 1995). Thus, GAG production may be considered as an alternative for organisms that lack gas vesicles to remain longer in the photic zone (Maeda et al., 2021). The negative charge of EPS produced containing high sulfated content also protects the community against viral infection (Matsunaga et al., 1996). Therefore, the production of GAG by pelagic cyanobacteria contributes to stress tolerance and viral infectivity, helping in the persistence of bloom. In our growth experiments, a decline in cell numbers was not observed, which would represent the end of the bloom. In the natural environment, nutrient depletion, grazing or viral lysis/infection are the most likely causes of terminating a bloom (Gons et al., 2002). The cell lysis releases organic matter, which

supports the growth of heterotrophic bacteria (Kjelleberg et al., 1987; Hagström et al., 1988; Kieft et al., 2021). Photosynthetically derived organic carbon is one of the major carbon and energy sources for heterotrophic bacteria (Allgaier et al., 2008). These heterotrophs can degrade EPS and liberate bound Ca^{2+} (Visscher et al. 1998; Ionescu et al., 2015; Diaz et al., 2017). In addition, microbial respiration will produce HCO_3^-/CO_2, increasing the saturation index of $CaCO_3$, and may enhance the whiting (Figure 8). Although our model is [somewhat] largely theoretical at this stage and explore the role of one picoplankton species, its merit is to focus on an overlooked actor of whiting events, the EPS. Furthermore, it provides a conceptual framework to work with, for designing novel experiments and measurements both in natural systems and at the lab bench, to validate the molecular mechanisms involved in microbial bloom associated $CaCO_3$ formation in marine and lacustrine models.

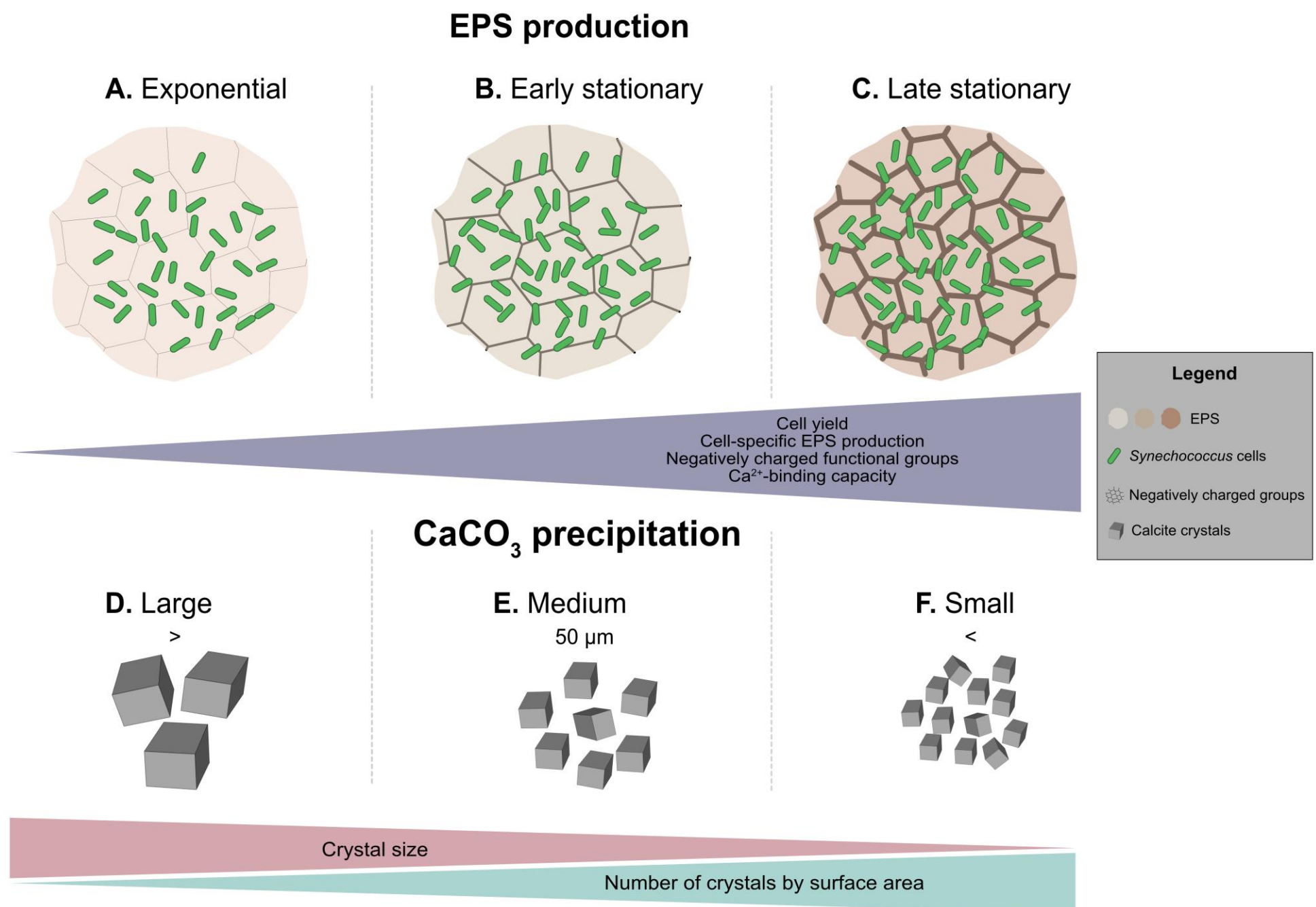



Figure 8. Conceptual diagram of proposed EPS-supported carbonate precipitation mechanism explaining the origin of whiting events.

Data availability

All raw data can be provided by the corresponding authors upon request.

Author contributions

M.M.d.B., I.B. and P.T.V. designed the study in a project directed by P.T.V., I.B. and E.V.; M.M.d.B., I.B., P.T.V., F.M., A.W. and L.P. developed the methodology; M.M.d.B. and I.B. carried out the laboratory measurements; M.M.d.B., P.T.V. and I.B. analysed the data; M.M.d.B. wrote the manuscript draft with significant contributions of P.T.V. and I.B. M.M.d.B., P.T.V., I.B., E.V., F.M., A.W and L.P. reviewed and edited the manuscript. All authors have read and agreed to the published version of the manuscript.

Competing interests

The authors declare that they have no conflict of interest.

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