

## ***Interactive comment on “Precipitation of Calcium Carbonate Mineral Induced by Viral Lysis of Cyanobacteria” by Hengchao Xu et al.***

**Hengchao Xu et al.**

xuhengc2007@163.com

Received and published: 5 July 2018

The manuscript by Xu, et al. describes the isolation of a cyanobacterium and cyanophage and the use of this host/bacteriophage system to induce mineral precipitation in artificial medium under laboratory conditions. Using observational data, mineral characterization technologies, microscopic cyanobacterial counts and chemical analyses the authors conclude the presence of cyanophage in a culture of the host cyanobacterium lyses the host and releases cellular constituents into the culture medium. The author propose this release of dissolved and particulate cellular constituents promotes the precipitation of specific polymorphs of calcium carbonate and magnesium hydroxide. General Comments: 1. The manuscript is not well organized, with some sections lacking adequate methodological information. Collectively, these

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issues makes the manuscript a bit difficult to read and interpret. Examples will be specifically described in the Specific Comments section.

Reply: The methodological have been re-organized following the reviewer's suggestion.

2. The use of "calcium carbonate" is used throughout the manuscript but in most instances, this is too general a descriptor within the context of this study. The use of the specific calcium carbonate polymorph names [e.g., amorphous calcium carbonate (ACC), vaterite, aragonite, calcite] will be more appropriate when applicable.

Reply: Thanks for the comments. The specific calcium polymorph names are used at the right place in revised manuscript.

3. The mineral precipitation mechanisms of homogenous and heterogeneous nucleation are not clearly delineated throughout the manuscript and appear to be used interchangeably in some instances. Additionally, mineral nucleation and precipitation are also used interchangeably. This conflation of terms, phrases and concepts makes it more difficult for the reader to read and interpret the manuscript. The experimental design for this study does not allow the detection or characterization of nucleation events, only gross precipitation that can only be indirectly assumed the result of one or both mechanisms of nucleation.

Reply: We highly appreciate the term names clarification and understand that is preferable avoid any confusing terminology in the carbonate nucleation and precipitation. Nucleation of calcium carbonate mineral in the manuscript are deleted from the text.

4. Throughout the manuscript total alkalinity (TA) and alkalinity are used interchangeably. When working with marine carbonate chemistry there is a significant difference between the ways these two alkalinities are calculated. Make it clear to the reader which one is being used or referred to.

Reply: There are three alkalinities (total alkalinity, cytoplasmic alkalinity and carbonate

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alkalinity) used in the manuscript. We agree with the reviewer that we should make them clear to the reader. Total alkalinity used in the manuscript is the measurement of water's ability to neutralize acids. Intracellular alkalinity used in the early version of manuscript refer to the alkalinity released from the cell by lysis of bacteria. We revised it to the "Cytoplasmic bicarbonate" following Lisle and Robbins (2016). Carbonate alkalinity calculates the amount of negatively charged carbonate and bicarbonate atoms in the solution. We talk about the carbonate alkalinity once in the text when we discuss the dissolution of ACC (P7 L28). P6, L7 has been rephrased.

5. There is repeated mention of carbonate chemistry in the text and tables. However, there is no listing of the geochemical data used for or the output from the geochemical modeling analyses. These data sets need to be included.

Reply: The geochemical data are provided in a supplemental table.

6. Saturation indices (SI) are a central component of this manuscript but there is no description of how these were calculated. When working with carbonate chemistry in marine waters most calculations on saturation states are per polymorph, like aragonite and calcite. These calculations are not normally performed within commonly used geochemical modeling programs but rely on CO2SYS or a recently developed application, CO2calc. The calculated SI values from the geochemical modeling software and CO2SYS/CO2calc are not always equivalent.

Reply: Carbonate chemistry was calculated by the program PhreeqC [version 3.3; Watq4f database; United States Geological Survey (USGS), Reston, VA, USA], which has been used in previous cyanobacterial calcification research (e.g. Obst et al., 2009). Compared with CO2calc and CO2SYS, PhreeqC is more convenient to calculate magnesium-related mineral. We agree with reviewer that the saturation states are per polymorph and the calculated SI values from different geochemical modeling software are not always equivalent. Under this circumstances, we give the geochemical modeling analyses with CO2calc and Phreeqc separately as supplemental materials.

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7. The Discussion section is too long and not focused on placing the data and interpretations from this study in context of previously published papers. There is a considerable amount of text dedicated to introducing and developing concepts that are on the periphery of the stated objectives and generated data of this study. This section needs to be edited to remove these passages and re-written to focus the discussion in a focused and concise style.

Reply: We understand the concern pointed out by the reviewer regarding to the discussion section. We take the utmost care to refine the part of discussion. In revised manuscript version, some part of discussion are reduced to a single sentence to expand the importance of viral induced calcification.

Specific Comments: Abstract Pg 1: Ln 10-22. (1) The data do not support the statement that the presence of viruses stabilizes the carbonate minerals detected in this study.

Reply: The precipitate investigated by XRD showing particles of aragonite in viral lysate and brucite in bacterial culture. XRD results combined with chemical parameters change of the non-infected culture revealing that it is unable to calcify to the extent that a stable  $\text{CaCO}_3$  precipitate was formed. As atmospheric  $\text{CO}_2$  dissolved in water, acid-base balance of the system will be changed. Unstable mineral phases can dissolve with acidification. However, with the aid of the viral cycle and the lysis of the host, the dissolution of carbonate seemed not to happen in viral treatment, and a more stable mineral formed. Thus, we conclude that viral lysis of cyanobacteria stabilizes the carbonate minerals detected in this study

(2) There's no evidence in this study of rapid intracellular calcification due to intracellular calcium concentrations.

Reply: We decided to tone down our statement and reformulate the discussion regarding intracellular calcification.

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Introduction Pg 2: Ln 1-2. The precipitation and dissolution of calcium carbonate does later sea water chemistry but do these processes have a more significant influence on the carbonate chemistry than carbon dioxide flux from the atmosphere?

Reply: It has been rephrased. "Formation and dissolution of calcium carbonate is one of the most important process that can change the carbonate chemistry in sea water"

Pg 2: Ln 11-15. This passage describes sedimentary processes and stromatolites. Consideration should be given to removing this passage from the manuscript. This concept is not relevant to the stated theme of this manuscript.

Reply: Thanks for your advice. They are removed.

Pg 2: Ln 16. The precipitation of calcium carbonate, regardless of the mechanism, is always a controlled geochemical or biogeochemical process.

Reply: CaCO<sub>3</sub> biomineralization by cyanobacteria are considered as exclusively extracellular. The sheath structure of some species of cyanobacteria may play a role in the calcification process, but environmental influence are also crucial: the saturation state of the adjacent water, which affects precipitation of calcium carbonate minerals, and the availability of dissolved CO<sub>2</sub>, which affects photosynthesis (reviewed by Riding 2012/Science, VOL 336). Although it is the truth that calcification by coccoliths or bivalves are controlled mineralization and are significant in marine chemistry, cyanobacterial calcification processes introduced in our manuscript are non-controlled.

Pg 2: Ln 23-29. All of the information in this passage is true. However, it's not clear how this information is relevant to the stated objectives and experimental design of this study. Considering should be given to removing this passage from the manuscript.

Reply: Present manuscript focus on the important role of viruses in precipitation of carbonate minerals. This passage reviews the direct effects of viral lysis of microbe in marine system and illustrates why we turn to virus to discuss potential viral effects on CaCO<sub>3</sub> biomineralization. Thus, it is important to the experimental design. We

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value the comments by the reviewer. We reorganize the passage and make it more reasonable and readable.

Pg 2: Ln 33. Which of the calcium carbonate polymorphs was capable of homogenous nucleation in the cited study?

Reply: The thermodynamics calculation proposed by Lisle and Robbins (2016) reveal that the activation energy for nuclei formation thresholds for all three polymorphs is significantly reduced but only vaterite nucleation is energetically favored. It has been described precisely in revised version.

Pg 3: Ln 1-2. The authors state the cited study does not consider the role of magnesium in their calculations. However, they don't tell the reader why it's important in this study. Since the role of magnesium in mineral precipitation is one of the objectives, this would be the place to provide the reader with some background information that will put the data and interpretations in the proper context.

Reply: We appreciate the suggestion from the reviewer. Mg story is included in introduction in the revised version.

Pg 3: Ln 5-14. The information in this passage is not relevant to this study based on the stated objectives and experimental design. Its inclusion is a distraction from the Consideration should be given to removing this passage from the manuscript

Reply: This passage reviews the research on viral particle acting as nucleation sites for different mineral precipitation. We refine the passage to make it more concise.

Pg 3: Ln 17-18. (1) The authors state the understanding of viral influences on the precipitation of carbonate is poorly understood but they in the previous 16 lines of text they list several published studies that do characterize this process.

Reply: Thanks for your reminding. It has been reparsed. "Recent studies of biofilms from hypersaline lakes have shown that hypersaline carbonate minerals can precipitate at the surface of viral particles and have implication to the nano-sized calcium carbon-

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ate structures in various geological settings (Pacton et al., 2014;Lisle and Robbins, 2016;Perri et al., 2017). However, the pathway of precipitation of calcium carbonate onto the surface of virus remains poorly understood”

(2) Here is an example of the confusing use of nucleation and precipitation in the same sentence.

Reply: The change is done accordingly. “When combine with the release of cytoplasmic-associated bicarbonate, which results in the formation of carbonate mineral energetically favored, and available viral capsid for surface-induced precipitation, the comprehending of viral influence on the precipitation of carbonate is extremely limit.”

Pg 3: Ln 30. (1) A virus infected cyanobacterium is not a cyanophage. (2) Interpreting Figure 2 as showing a cyanophage and its host bacterium is a bit of a reach. These images could be almost anything.

Reply: “Cyanophages, which infect this ecologically important group of cyanobacteria were isolate from the surface seawater from Sanya Bay.” TEM image of cyanophage by negative staining is included in the revised version of manuscript.

Pg 3: Ln 31-32. There needs to be, at a minimum, an abbreviated description of the cited method used to isolate, purify and identify the cyanobacterial specie. It should not be incumbent on the reader to run the most basic description of a method. Pg 4: Ln 1-2. (1) As noted in the previous comment, at a minimum, an abbreviated description of the cited method for the cyanophage isolation needs to be provided. (2) There is a reference to metagenomics analysis, including a supplemental data file. The method of sample collection, processing, sequencing and sequence analysis (including the bioinformatics) is not mentioned or described anywhere in this manuscript. This is a significant deficiency in this version of the manuscript.

Reply: Thanks for your suggestion. Although isolation and identification of cyanobac-

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teria and cyanophage are out of scope of the main goal of the manuscript, they are used for simulation experiment. Detailed methods for isolation and identification are provided as supplemental file in revised version.

Pg 4: Ln 3-17. (1) This section is very poorly organized and developed with respect to the different methods mentioned. There is no reasonable way a person could replicate this research effort or interpret the data from these methods using this section for guidance. (2) In this reviewer's opinion there are seven distinct methods: culture growth conditions; cyanobacteria counts; ion chromatography; salinity, total alkalinity and DIC measurements; geochemical modeling of carbonate chemistry; metagenomics analysis. Consider giving each of these their own sub-section within Experimental Setup and develop each section so the reader will understand how the methods were performed. (3) List the incubation or experimental times for each experiment type. (4) What is meant by a "pre-culture"? (5) A "one treatment" is mentioned. Are there other treatments? If so, describe those treatments and their respective differences. (6) List the volumes for each experimental container, the volumes of the sub-samples and the times at which the sub-samples were collected. (7) For the cyanobacterial growth cultures provide the light wavelength and dose.

Reply: We agree with the reviewer that each of the method should be given a sub-section in revised version of manuscript. Changes are done accordingly.

Pg 4: Ln 15-17. There is reference to geochemical modeling using a specific program. However, there is no mention or listing of the data on which the geochemical analyses were performed or the outputs from those analyses (e.g., activities of carbonate species). Both of these data sets need to be included in this manuscript. Additionally, the PHREEQC code used for these analyses needs to be included, most likely as part of the Supplemental Data files.

Reply: Data used for geochemical calculating are provided in revised version. The PHREEQC code used in the early version of the manuscript, as referred, is Wateq4f

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database. In order to make it more directly, we list it in the supplemental data files together with data calculated by CO2calc.

Pg 4: Ln 19. Here and in several later passages, there are references to “phases” of the cyanobacterial cultures. Based on the references it’s assumed these phases are similar to those commonly measured during the growth of bacteria in the laboratory (i.e., lag, exponential, stationary). However, the methods used to determine these growth phases and the data from those experiments are not provided. This is another method that should be included in the Experimental Setup section.

Reply: The change is done accordingly. They growth curve is determined by the counting of autofluorescence during the culture of cyanobacteria.

Results Pg 5: Ln 4-5. How many cyanophage were inoculated into the cyanobacterium culture? Were the cyanophage titered? If so, the titer data need to be included. Without these data you cannot know the ratio of host cells-to-cyanophage (i.e., MOI), which has a significant influence on infection rates.

Reply: Cyanophage were enumerated from the culture by epifluorescence microscopy with SYBR Green I staining (Patel et al., 2007).

Pg 5: Ln 5-7. (1) Based on the brief description of the counting method and Figure 1b, it appears the cyanobacterial host abundances are based solely on the autofluorescence of the photosynthesizing microorganism. How can you be sure that nonfluorescent bacteria are not present in these cultures? Without knowing this, the possibility that some or all of the observed responses are due to a non-host bacterium or bacteria cannot be ruled out.

Reply: Cyanobacteria have been isolated and purified using standard microbiological techniques. During all the treatment, operations are asepsis strictly. Even if there is non-host and non-fluorescent bacterium, the culture media inoculation of the cyanophage will be turbid, which is caused by the growth of mixed bacteria. But Fig.3

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b and d show the lysate are clear (Fig 3b and d). Even though it is not stained, non-fluorescent bacterium, if there is, may be seen from the optical microscope images. But this is conflicts with the optical microscope result. Under these conditions, we believe they are pure culture.

(2) From this passage, it appears the cyanobacterial host abundances in the two culture types were measured using fluorescent microscopy but the cyanophage abundances were not determined in the co-culture. How can you conclude the cyanophage were responsible for any of the observations if there is no indication of how many were added to the host culture at time zero and their abundances at the different time points are not known? These cyanophage abundance counts from the different sub-samples should show increases as the host abundances decrease. Without the cyanophage abundance data you can't support the reductions in host abundances as being solely due to cyanophage induced lysis

Reply: In the earlier version of manuscript, we made the conclusion that cyanophage were responsible for the host mortality after comparing the growth curve of culture with and without inoculation of cyanophage. But even if it is, we agree with the reviewer that the abundance of virus particles are needed.

Pg 5: Ln 13-23. (1) This section needs to be revised into a more organized and concise presentation of the geochemical data. It's very difficult to interpret in its current format.

Reply: Most of this section has been rewritten in revised version of manuscript.

(2) Throughout this section there are repeated references to different subsamples and geochemical data associated with those sub-samples. This suggests sub-samples were collected at specific time points during the incubations and those sub-samples were then analyzed for the presence and concentrations of analytes required for the geochemical modeling of changes in the carbonate chemistry. The times of the sub-samples are not provided, the analytical data from those sub-samples is not presented and the outputs from the geochemical modeling analyses are not listed. Collectively,

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this information and data have to be included for the proper interpretation of the documented observations. For example, reference is made to seemingly unrealistic changes in total alkalinity, DIC and calcium and magnesium removal over the different growth phases of the host culture. The geochemical modeling data need to be presented to support these observations.

Reply: We understand the concern pointed out by the reviewer regarding to the data acquisition and data view. We have included in the supplemental material a table of raw data and a comparison between different geochemical model outputs.

(3) Was pCO<sub>2</sub> measured during these experiments? If so, this method needs to be included and described.

Reply: We do not measure the pCO<sub>2</sub> during the experiments. Alternatively, we calculate pCO<sub>2</sub> with total alkalinity and DIC with the known temperature, salinity and pressure. This is included in the supplemental material in revised version.

Discussion Pg 6: Ln 9. Its not clear what this balanced equation is representing? Its not at all clear from the text how formaldehyde (CH<sub>2</sub>O) is formed from bicarbonate and water or what the significance of CH<sub>2</sub>O is to the study described by this manuscript. Was the intention to let CH<sub>2</sub>O be a general reference to a carbohydrate? Or the dissociation of bicarbonate to carbon dioxide?

Reply: It refers to the photosynthetic bicarbonate uptake uptake and its conversion within the cell to carbohydrate.

Pg 6: Ln 14-17, Ln 21-26; Pg 7: Ln 9-10, Ln 16-17, Ln 19-21, Ln 25-29. All of these passages require the reader to have access to the geochemical modeling input data and output results for each sample before an independent interpretation and evaluation of the observations can be made.

Reply: Data used for geochemical calculating are provided in a supplemental data files in revised version.

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Pg 6: Ln 1 and Ln 32. These are the first time brucite is mentioned, other than the abstract. As this mineral seems to a significant product of the processes described in this manuscript, consideration should be given to bring this mineral and its relative importance into the manuscript in the Introduction and the Materials and Methods sections, including the geochemical modeling sub-section.

Reply: The change has been done accordingly.

Pg 6: Ln 22-23. Here and within other passages later in the manuscript, there is reference to the saturation index (SI) as being the metric for determining the saturation, supersaturation and under-saturation state of the culture medium. Though true, the SI alone will not tell you if a mineral will precipitate or dissolve. For example, if this were unconditionally true then average seawater, which has an SI between 2-3, one or more of the calcium carbonate polymorphs would be precipitating out of solution all the time. Instead, average seawater is metastable with no precipitation, indicating there are more geochemical factors other than SI that dictate if a precipitation even will proceed or not. Here is another example of where the geochemical modeling data would assist in the discussion of the results from this study.

Reply: We agree with the reviewer that more information and comments about the geochemical factors can be helpful. Data used for geochemical calculating are provided in a supplemental data files in revised version. The precipitation and dissolution of calcium and magnesium is mainly derived from the ion concentration in the medium. When we know the ion concentration change, we calculate the saturated state of the medium and show the calculated results supporting the precipitation or dissolution.

Pg 8: Ln 1-16 and Ln 24-31; Pg 9: Ln 1-17. These passages do not add anything supportive to the manuscript, based on the stated objectives, experimental design and presented data. Consider deleting these from the manuscript.

Reply: We decided to tone down our statement and reformulate the discussion regarding intracellular calcification. The part 4.3 in the early version of manuscript is reduced

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following the suggestion from the reviewer.

Pg 8: Ln 20-23, Ln 28-33. The precipitation of one of the calcium carbonate polymorphs in tropical marine waters, or whittings, has been shown via peer reviewed publications to be driven by biological processes and not the simple physical re-suspension of established carbonate sediments.

Reply: Thanks for reminding. The discussion about whiting is deleted following the reviewer's suggestion.

Conclusion Pg 9: Ln 19-20. Without the geochemical data and model outputs a detailed view of carbonate chemistry changes have not been provided. Also, without cyanophage abundance counts the cyanophage infection and lysis of the host cells cannot be definitively stated.

Reply: These two data sets are included in the revised version of manuscript.

References Pg 10-13. There are 65 references listed. Based on there being issues with citations being incomplete, having punctuation errors, odd symbols inserted (which may have been a conversion issue) and inconsistency with the DOI information format, there are 36 references that need to be reviewed and corrected if needed. There were too many to list individually.

Reply: Change was done accordingly

Table 1. (1) This are several modified f/2 medium formulations, but the recipe listed in this table is not included in those published formulation. If there is a citation for this formulation of f/2 please include it. Also if the mineral and vitamin solutions were purchased then this needs to be noted as well. If these solutions were made in the laboratory, then there are several ingredients that are missing (e.g., EDTA). (2) The final pH (which should be between 7.8-8.2) of the media needs to be included as well.

Reply: F/2 mediums reported in literature are prepared by stocks adding to filtered seawater. Only NaNO<sub>3</sub>, NaH<sub>2</sub>PO<sub>4</sub>, Na<sub>2</sub>SiO<sub>3</sub>, trace mental stock and vitamin stock

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are defined. Here, the artificial seawater base modified from Harrison et al (2010). We apologize for the mistaking of repeating  $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$  in the table and missing  $\text{Na}_2\text{EDTA} \cdot 2\text{H}_2\text{O}$ . The final pH of the media was adjusted to 8.

Guillard R.R.L. and Ryther J.H. (1962) Studies of marine planktonic diatoms. I. *Cyclotella nana* Hustedt and *Detonula confervacea* Cleve. *Can. J. Microbiol.* 8: 229-239.  
Guillard R.R.L. (1975) Culture of phytoplankton for feeding marine invertebrates. in "Culture of Marine Invertebrate Animals." (eds: Smith W.L. and Chanley M.H.) Plenum Press, New York, USA. pp 26-60. Harrison P J, Waters R E, Taylor F J R. A BROAD SPECTRUM ARTIFICIAL SEA WATER MEDIUM FOR COASTAL AND OPEN OCEAN PHYTOPLANKTON. *Journal of Phycology*, 2010, 16(1):28-35.

Table 2: (1) The pH values for all of the samples are relatively high compared to the initial pH of f/2. Were these measured or the products of the geochemical modeling? (2) The higher pH values are unrealistically high for open marine waters but, interestingly, the SI values are all less than those for the two dominant polymorphs (aragonite and calcite) in typical marine water. At these pH values, higher SI values, relative to typical marine water, would be predicted. This is another example of where having the geochemical modeling data is critical for the proper interpretation and assessment of the research data.

Reply: The pH values were calculated from the PhreeqC. Photosynthetic bicarbonate uptake and its conversion within the cell to  $\text{CO}_2$  by carbonic anhydrase lead to increased pH in the immediate vicinity of the cell (Reviewed by Ridding 2011). In present study, when lytic rates run over the bacterial replication, photosynthesis ceases making the atmospheric  $\text{CO}_2$  dissolved in the water and changing the acid-base balance of the system. In present study, SI value calc and  $\Omega$  were determined following:  $\text{SI} = \log \Omega = \log[\text{IAP}/\text{Ksp Mineral}]$  wherein,  $\text{IAP} = [\text{Ca}^{2+}][\text{CO}_3^{2-}]$  Thus, SI values in table 2 are higher than typical marine water. We calculate the  $\Omega$  of aragonite and calcite in  $\text{CO}_2$  Calc in revised Supplemental table and the results also support our conclusion.

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Figures 1 and 2. These images do not contribute additional information to the reader. Consider deleting these from the manuscript.

Reply: We agree with the reviewer and give the descriptions of cyanobacteria and cyanophage in a supplemental material in revised version of manuscript.

Figure 5. Panel 5c is not cited in the manuscript.

Reply: The citation is done accordingly.

Figure 8. The simple shape of an object is not enough to proclaim it being a microbial cell, virus or encrusted microbial structure. Unless more definitive proof can be provided that will support the declaration that this images are what's written in the legend, consideration should be given to deleting this figure from the manuscript.

Reply: We agree with the reviewer that morphological evidence is not enough to discriminate the microbial and mineral. We attempted to take EDS or EDS-mapping of the encrusted structure. Unfortunately, SEM under EDS model could not acquire the morphology of particle with hundreds nanometers. Since we have decided to tone down our statement regarding intracellular calcification, electron microscopic photographs are revised following the reviewers suggestion.

Figure 10. This is a nice graphic but it's not explained well in the manuscript and there is a lot of information which is not even mentioned in the manuscript. This figure would be a nice summary graphic but the information in the image will have to be presented in the manuscript.

Reply: Fig. 10 are revised following the suggestion of reviewer on the mechanism of viral induced calcification. We try our best to explained it well in revised version of manuscript.

Supplementary Figure. The information in this figure stands alone because nothing about or within this figure is mentioned or described in the manuscript. In the manuscript's current format, this figure does not support anything presented in the

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Results or Discussion sections. For this reason, consideration should be given to removing this figure from the manuscript.

Reply: We understand the concern pointed out by the reviewer regarding to the supplementary Figure. Since the method of isolation and characterization of cyanophage are needed in the revised manuscript, we interpret this figure together with the characterization of cyanophage in supplementary material.

Please also note the supplement to this comment:

<https://www.biogeosciences-discuss.net/bg-2018-194/bg-2018-194-AC2-supplement.zip>

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Interactive comment on Biogeosciences Discuss., <https://doi.org/10.5194/bg-2018-194>, 2018.

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