Understanding carbon regulation in aquatic systems - Bacteriophages as a model [version 1; peer review: 2 approved]

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Abstract
The bacteria and their phages are the most abundant constituents of the aquatic environment, and so represent an ideal model for studying carbon regulation in an aquatic system. The microbe-mediated interconversion of bioavailable organic carbon (OC) into dissolved organic carbon (DOC) by the microbial carbon pump (MCP) has been suggested to have the potential to revolutionize our view of carbon sequestration. It is estimated that DOC is the largest pool of organic matter in the ocean and, though a major component of the global carbon cycle, its source is not yet well understood. A key element of the carbon cycle is the microbial conversion of DOC into inedible forms. The primary aim of this study is to understand the phage conversion from organic to inorganic carbon during phage-host interactions.

Time studies of phage-host interactions under controlled conditions reveal their impact on the total carbon content of the samples and their interconversion of organic and inorganic carbon compared to control samples. A total organic carbon (TOC) analysis showed an increase in inorganic carbon content by 15-25 percent in samples with bacteria and phage compared to samples with bacteria alone. Compared to control samples, the increase in inorganic carbon content was 60-70-fold in samples with bacteria and phage, and 50-55-fold for samples with bacteria alone. This study indicates the potential impact of phages in regulating the carbon cycle of aquatic systems.

Keywords
interconversion, microbial carbon pump, carbon sequestration, refractory carbon, global carbon cycle
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The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

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Introduction
The regulation of carbon in aquatic systems is a major biogeochemical process. The oceans’ surface takes up about 2% more CO₂ gas than they release, a proportion of which dissolves into the water, forming carbonic acid. The increase in CO₂ levels in oceans decreases the pH, resulting in acidification which affects the oceanic ecosystem. Carbon also enters the seas through the food web via photosynthesis, but does not last for long periods and is either released into the atmosphere as CO₂ or sinks to the ocean depths as dead organic matter. However, a significant amount of carbon is present in the water in the form of DOC. The roles that ocean viruses play are very important in shaping microbial population sizes as well as in regenerating carbon and other nutrients. It is estimated that every second, approximately 10²⁵ viral infections occur in the ocean. Therefore, it should not be surprising that viruses are major influential forces behind biogeochemical cycles.

A key element of the carbon cycle is the microbial conversion of dissolved organic carbon into inedible forms. Microbes play a dominant role in “pumping” bioavailable carbon into a pool of relatively inert compounds. The microbial carbon pump (MCP) “may act as one of the conveyor belts that transports and stores carbon in oceans.” The MCP also appears to function in deep waters, where bacteria adapted to the high-pressure environment may be able to degrade refractory DOC. Hiroshi Ogawa et al., showed that marine microbes are able to convert bioavailable DOC to refractory DOC.

The present communication represents time studies of phage-host interactions under controlled conditions, in order to analyze their impact on the total carbon content of the source (nutrient broth) and their interconversion between organic and inorganic forms of carbon with respect to control samples. The control sample is just the nutrient broth without the inoculation of bacterium and their respective phage.

Materials and methods
The experiment was designed to measure the inorganic carbon levels in three conditions: control (nutrient broth only), bacteria alone and bacteria with their specific phage. The bacterium used during our study was E. coli (ATCC, strain 13706) and the bacteriophage used was phi X174 (ATCC, strain 13706 B1). They represent a good model for carbon conversion and interconversion through phage-host interactions and their interaction can be easily determined by the instruments like TOC analyzer.

All three experimental conditions were conducted in 1L of sterilized nutrient broth each as to have a defined composition of the nutrients available for our study (HiMedia Pvt. Ltd.). For the bacteria without phage condition, sterilized nutrient broth media was inoculated with 100 cfu/ml of E. coli (ATCC 13706) previously enriched and incubated at 37°C; for the bacteria with phage condition approximately 1 ml of 1000 pfu/ml of phage were added. All flasks were sealed and incubated at 37°C for 18 hours. For control condition, sterile uninoculated broth was kept at 4°C throughout the experiment.

The initial reading were analyzed by a total organic carbon (TOC) analyzer (Shimadzu, Japan Model: TOC-Vcph) after 18 hours of incubation for all three sets of samples were recorded as “0” hours reading and before inoculation of bacteria and phages (see Table 1 and Table 2). TOC analysis was further carried out after every 2 hours until a stationary state was achieved. The stationary phase for inorganic carbon was defined by no further increase or decrease in the reading of inorganic carbon.

Please refer Figure 1 and Figure 2 for understanding the principle of TOC analysis and different types of carbon compounds. The overall experiment was repeated for 10 times and their averages are represented in the Table 1 and Table 2.

Table 1. TOC analysis results of control and bacterial samples (with and without phage).

<table>
<thead>
<tr>
<th>Experiment No.</th>
<th>Control (ppm)</th>
<th>Sample without phage (ppm)</th>
<th>Sample with phage (ppm)</th>
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<td>TOC TC IC</td>
<td>TOC TC IC</td>
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<td>3091 3196 105.5</td>
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Table 2. TOC analysis results of control and bacterial samples (with and without phage).

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<th>Sample with phage 2 (ppm)</th>
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</table>

Figure 1. Principle of TOC analysis.
Figure 2. Flow chart showing ingredient components of total carbon.

Figure 3. Variation in inorganic carbon content (in ppm) with respect to time (in hours).
Figure 4. Variations in inorganic carbon content (in ppm) with respect to time (in hours).

Results
The average results of the three sets are represented in Table 1 and Table 2, which show that the inorganic carbon content of the samples increased over time (except control) in both sets. The sample set with host-phage inoculation showed a increased reading of inorganic carbon levels compared to bacteria-only. There was an average 15–25 percent increase in inorganic carbon composition of sample set with host-phage inoculation. The result indicates that the phages may have role in regulation of carbon in aquatic systems through carbon sequestration or conversion in different biologically unavailable forms and can elevate inorganic carbon content levels in aquatic environments.

Discussion
The increase in inorganic carbon content may be due to lysis of the host cell releasing its refractory carbon compounds and respiration produced CO$_2$ during utilization of carbon constituent for phage assembly and development. These controlled experiment mimics the continuous viral infections occurring in the different aquatic environments. The consistent rise in the inorganic content is an indicator that, viruses somehow, seems to regulate carbon cycle to a greater extent as observed from the increase in IC level. The analytical results as indicated from the TOC analyzer are sole representation of phage lyses event and are worth analyzing further. If we are able to understand the biochemical mechanism and the byproducts generated during this whole process we may be able to determine the carbon sequestration in a better way. Considerable research activity needs to be initiated involving different environments conditions, parameters, sources, etc to facilitate better understanding of viral life cycle involving carbon cycle as an important area of future research. It can be proposed that carbon conversation during these studies gives us the clear ideas of the possible fate of carbon cycle and the role of phages. Similarly, we can also try to elucidate the role of phages (viruses) influencing other biogeochemical cycles including Nitrogen and Sulphur by using CHNS analyzer for better understanding of this process. It is also known that the infection of microbes also alters host metabolism significantly. Carbon sequestering algae like cyanobacteria are infected by cyanophages, which complicates our understanding further and demanding further in-depth studies. Lysogenic condition established by viruses under nutrient depleted condition or harsh environment can regulate the carbon utilization processes differently. Hence, the effect of viral infection on host metabolism remains unknown.

Future work is essential for understanding the cellular processes especially infected (Lysogenic) host species. It will also prove helpful in deciphering the role of phages in regulating the carbon flow in the aquatic systems like oceans where their concentration outnumbered other species.

Author contributions
All authors have contributed equally to this work. All authors have seen and agreed to the content of the final manuscript.

Grant information
The work was carried out as an in-house activity and was supported by Council of Scientific and Industrial Research (CSIR), Government of India, New Delhi.

I confirm that the funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.
Acknowledgment

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References

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Version 1

Reviewer Report 15 July 2015

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Mayur Bharat Kurade
Department of Natural Resources and Environmental Engineering, Hanyang University, Seoul, South Korea

This paper deals with Bacteriophages as a model for carbon regulation in aquatic systems. The increase in inorganic carbon content was 60-70-fold in samples with bacteria and phage, and 50-55-fold for samples with bacteria alone being reported by the authors. The authors presented their experimental results quite well enough, that can be easily understandable to the readers. This manuscripts meets the necessary standards for this journal. The authors should pay attention on few of the concerns enlisted below. This manuscript should be acceptable after the changes suggested herewith.

1. The figures (3 and 4) are not mentioned in the manuscript text, The discussion part may be elaborated to have much clear idea of the present work and its impact.

2. Please consider to use abbreviations instead of full forms, wherever necessary, e.g. ‘2 h’ instead of ‘2 hours’ in Materials and methods section, and ‘%’ instead of ‘percent’ in “Results” section.

3. Please follow the reference styles with the journal guidelines. e.g. Hiroshi Ogawa et al., ....should be corrected as Ogawa et al., followed by reference number in superscript. Reference number 3 is missing in introduction. References should be arranged in proper sequence. Please check all other references.

4. Authors should elaborate the ‘Results section’, as results of Fig. 1 to 4 are not discussed. I suggest author to overcome short details in this particular section.

Competing Interests: No competing interests were disclosed.

I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Author Response 15 Jul 2015
Swapnil Sanmukh, National Environmental Engineering Research Institute (NEERI), Nagpur, India

Dear Sir,

First of all thank you for approving our manuscript.

Regarding your queries:

- We will change the abbreviations for the units used in the figures and results.
- We will modify the reference style not properly cited for Hiroshi Ogawa et al as Ogawa et al.
- We will elaborate the discussion as well as result section and cite the figures which are not mentioned or not discussed.

We would like to thanks for your time and suggestions for improving the quality of our manuscript.

Competing Interests: No competing interest
CO₂ or sinks to the ocean depths as dead organic matter”. The “seas” should be “sea” and a reference is required.

4. Introduction, Page 2, Line 23: Reference “Hiroshi Ogawa et al.,” should come with the year of publication.

5. Material and methods, this section is written very well and you can understand the experiments conducted by the authors. I have only some queries and suggestions for this section:

   Page 2, Line 8: Kindly provide the manufacturer information for the TOC analyzer in the first usage.

6. Result section is very short (even shorter than abstract) but it’s very informative and well written.

7. Discussion section is also very well written with comparative study of this present work. But I think if you provide 2 or 3 more references then it will be better for this publication.

8. Conclusion; I think authors have been forgotten to provide the conclusion part of this work and it is very important. Authors should be adding this section.

9. Table 1 and 2 are presented very well and it’s looking like whole results are explained by them.

10. Figure 3 and 4 are not cited in the text and it should be cited. These figures are the main results and could be explained in the results and discussion section.

11. Figure 1 and 2 are presented very well and for figure 3 and 4 authors may modify with removing “in hours” by “h” and “in ppm” by “ppm”.

12. Figure 3 and 4: I am unable to understand what differences between both figures are because all the things are same. Should be for table 1 and 2 (ppm 1 and ppm 2 should be come in the figure titles)?

**Competing Interests:** No competing interests were disclosed.

I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

**Author Response 22 Jun 2015**

Swapnil Sanmukh, National Environmental Engineering Research Institute (NEERI), Nagpur, India

Dear Sir,

First of all thank you for approving our manuscript.
Regarding your queries:
- I think most of them are not quite critical but we will cite figure 3 and figure 4 as they are not cited in the article.
- The figures are represented as per journals guidelines.
- We will provide the make of TOC analyser when it is represented firstly in the article.
- We have done the experiments in duplicate so that we can maintain homogeneity and have
We have done the experiments in duplicate so that we can maintain homogeneity and have reproducible results minimizing manual errors. The conclusion part is not included because the article is a research note, not a research article and it is not mandatory for this type of article.

We would like to thanks for your time and suggestions for improving the quality of our manuscript.

Competing Interests: No competing interests are declared.

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