Reviewer comments:

Review of "Insignificant effects of elevated CO_2 on bacterioplankton community in a eutrophic coastal mesocosm experiment"

Referee#1

The manuscript addresses the research question if bacterial communities in eutrophic coastal areas will be affected by elevated CO_2 concentration. The topic is highly relevant given the possible effects of changes in oceanic carbon chemistry on bacterioplankton communities and subsequent biogeochemical nutrient cycles. The authors state that they found "insignificant effects of elevated CO_2 on bacterioplankton communities)", however their methodology and experimental setup is poor and insufficient to test the hypothesis.

The major criticisms of the manuscript is that the bacterial community composition (BCC) resulted from contamination of tubing and material used, as well as non-axenic phytoplankton cultures and hardly represents a natural bacterioplankton community. Even if the bacteria found in the mesocosms were of marine origin, the initial community composition is unknown and not shown to be similar among the mesocosms. Therefore the results and study are not reproducible.

RE: We appreciate the reviewers' comments and suggestions on the manuscript. Oceanic systems are open to the air with continuous exchanges of substances and microbes. In our experimental system, the mesocosms were open and aerated with filtered air of different levels of CO_2. Therefore, these mesocosms are subject to fluctuating environmental conditions and comparatively (relative to indoor or closed large-scale cultures) closer to natural conditions, other than the manipulated CO_2 levels. What we were trying to test were the basic principles of how a bacterial community changes along with phytoplankton growth under the influence of elevated CO_2. To investigate this, we used a model bacterial community composed of taxa originally associated with the cultured algal inoculum, combined with the natural marine assemblage that inevitably entered the mesocosms from sea spray, etc. It would have been impractical to cultivate the large volumes of axenic phytoplankton we would have needed to inoculate the mesocosms without adding any bacteria from

the phytoplankton cultures. At any rate, in the end the bacterial taxa present largely resembled those found in the natural community, suggesting the resident marine bacterial assemblage was able to dominate over the added cultivated bacteria.

We agree that if in situ natural phytoplankton and bacterioplankton communities were used in this mesocosm experiment, it would more closely reflect the effects of ocean acidification on the mixed natural phytoplankton and bacterioplankton communities. Considering the number of studies that have been done on the model phytoplankton responses to OA that have been carried out in laboratory, we felt it would a useful intermediate step to use model phytoplankton species to initiate the mesocosm studies before using natural communities. Therefore, we used filtered (0.01μm) seawater that did not have any bacteria in all the mesocosms in the beginning. Then we inoculated phytoplankton culture containing bacterioplankton into the mesocosms. Bacterial populations developed gradually with air-sea exchanges. We believe that using filtered seawater with inoculated isolates was reasonable and logistically practical for our experiment.

Our experiment was designed as an intermediary step between laboratory and natural community field experiments, with isolates of non-axenic phytoplankton being added to filtered natural waters. In this way, we were able to investigate the effect of OA on phytoplankton and bacterioplankton in eutrophic coastal waters while minimizing the complexity of shifting compositions of natural phytoplankton communities. That is, all the mesocosms start from the same point in terms of BCC or phytoplankton composition. The correlated data on phytoplankton using this mesocosm system entitled “Carbon assimilation and losses during an ocean acidification mesocosm experiment, with special reference to algal blooms” will soon be published at Marine Environmental Research (in press).

BCC in our study could be the combined result of a combination of the inoculated phytoplankton, air-sea exchange and sampling. Previous mesocosm experiments started with natural communities also had BCC from air-change and sampling. The important point is that each mesocosm has the same BCC, as in previous mesocosm studies. The dynamics of bacterioplankton throughout previous mesocosm studies

were also due to the combination of the original bacterioplankton community added in the mesocosm bags in the beginning and any outside bacterioplankton that entered during the experiment..

Furthermore, bacteria were not detectable by flow cytometry in the filtered seawater just before inoculation. Three species of non-axenic phytoplankton with bacterioplankton were mixed and then inoculated into each mesocosm bag. So the initial bacterioplankton community was considered the same among all mesocosms.

We revised the manuscript and double checked the data and their interpretations to further explained the reasons that we used filtered seawater for our eutrophic coastal seawater mesocosm experiment as well as the strengths and weaknesses of this experimental design (Page 7 Line 14-22, Page 8 Line 1-3).

In fact, samples of the initial days are missing. The BCC after 4 days looks different between mesocosms, yet 3 replicates are missing in the figures, results section and statistical analysis without mentioning.

RE: We tried to do sampling at day 2 but the samples were not successfully collected, probably due to very high concentration of TEP (Transparent Exopolymer Particles) which easily blocked the polycarbonate filter for bacterioplankton collection. According to the bacterioplankton abundance data in Yibin Huang et al (entitled "responses of phytoplankton and bacterial metabolism to CO_2 enrichment during a coastal mesocosm experiment", under revision after first-round review for Limnology and Oceanography), the bacterioplankton abundance was very high at day 2 and day 4 which may be associated with high TEP concentration (Sugimoto *et al.*, 2007, Ramaiah *et al.*, 2000). We also tried to do sampling at day 4. But eventually we successfully extract enough DNA for sequencing only from bag 1, bag 7 and bag 6. So some replicates were missing in the Figure 3. The replicates of HC and LC were mentioned in material and method section (Page 6 line 4-5). The replicates have been mentioned again in statistical analysis, result section and figure legends to make it easier for the readers.

Generally, it appears bizarre that a study addressing the BCC response to elevated CO_2 filters away all seawater bacteria before inoculating the water with non-axenic phytoplankton lab cultures. Phytoplankton culture parameters possibly selected for a fast-growing bacterial community that was adapted to phytoplankton bloom conditions and variation in water pH due to phytoplankton respiration processes. This would mean that the studied BCC was likely preconditioned to fluctuations in CO_2 with non-adaptive species outcompeted in semi-batch phytoplankton cultures prior to the experiment. A discussion or mentioning of this is missing.

RE: This is a very good point. We agree that the bacterioplankton originated from phytoplankton culture likely outcompeted other non-adaptive species in semi-batch phytoplankton cultures prior to the experiment. We have added some sentences in the discussion to address this point (Page 16 Line 19-22).

Data about other microbial measurements, such as bacterial activities or cell counts, are missing – questioning if bacterioplankton actually was the initial target of the study. Did the authors develop the network method themselves as references in the method section about networks are missing? In that case the method should have been validated.

RE: Bacterial activities and bacterial cell abundance data were shown in another paper (Yibin Huang et al, under revision of Limnology and Oceanography). We did not develop the network analysis method by ourselves. We followed the network construction methodology described in Wang *et al.*, 2016. The reference for network construction and analysis has been added to the method and material section (Page 10 Line 7).

The flaws of experimental design, setup and continuous samplings are complemented by insufficiently described materials and methods. Text and style of the manuscript are poor: several references are misplaced, missing or incorrectly cited in the reference list. The text contains word/grammar mistakes, wordautocorrect errors and the style of the text is inconsistent throughout the manuscript.

RE：We improved the materials and method section to clarify the experimental design and sampling. The references have been rearranged carefully. The text has been revised carefully and the English has been polished.

Specific comments. The title is misleading. The effects of elevated CO_2 on BCC were not statistically tested prior to day 6 when CO_2 concentration actually differed between treatments and the bacterioplankton community was artificially induced by contamination. I doubt that the authors' results support the statement "Insignificant effects of elevated CO_2 on bacterioplankton community in a eutrophic coastal mesocosm experiment"

RE: We have replaced the previous title with a new title "Interactive network configuration maintains bacterioplankton community structure under elevated CO_2 in a eutrophic coastal mesocosm experiment".

We agree that if the data prior to day 6 were shown in the manuscript, the conclusion would be more solid. It's a pity that we only successfully obtain several samples for sequencing at day 4 due to the reasons mentioned above. The pH values were statistically different from day 0 to day 10. So our results and analysis were still meaningful.

Although the pH was maintained at the target pH value throughout the experiment, this doesn't mean that all the results based on mesocosm experiments were meaningless. In the natural environment, pH increases gradually throughout the phytoplankton bloom. Our experiment and previous mesocosm experiments could be considered as the phytoplankton bloom initiated with different CO_2 concentration/pH.

Methods: page 5, line 18. What was the purpose of filtering the seawater for the mesocosms if the aim of the study was to study the bacterioplankton community?

RE: As mentioned above, we wanted to minimize the complexity of shifting compositions of natural phytoplankton communities and using filtered seawater was reasonable and practical for our eutrophic coastal seawater mesocosm experiment. Furthermore, according our unpublished data, the bacterioplankton in phytoplankton

cultures played important roles under ocean acidification which were usually ignored in previous studies. So we think the effects of ocean acidification on bacteriplankton in phytoplankton cultures is worth to be investigated in a larger scale experiment, which was our original purpose. However, as noted above the bacterioplankton from natural environment gradually became dominant in the mesocosm bags. So actually, the bacterioplankton we studied in this paper were mainly bacterioplankton from the natural environment.

If the majority of the bacteria originated with the phytoplankton cultures, why does the community composition in Fig S.1 look very different from the community composition of the mesocosms at day4? At day4, the class distribution of LC mesocosms shows nearly 50% Epsilonbacteria in D4.1, while no Epsilonbacteria are reported from the coccolithophore or diatom cultures.

RE: The results suggest that the outside bacterioplankton replaced the bacteria originating in the phytoplankton culture and became the dominant bacterioplankton in the mesocosm over day 0 to day 4. So Fig S.1 looks very different from the community composition of the mesocosms at day 4.

page 5, line 20. The in situ seawater pCO2 was 650 μatm. How relevant are control mesocosms where the pCO2 concentration is lowered? Despite it changing the carbon chemistry, seawater with 400 μatm seems not to reflect the eutrophic coastal environment in the Wuyuan Bay during January and is therefore a questionable control to test the hypothesis.

RE: We agree that 400 μatm may not reflect the eutrophic coastal environment in the Wuyuan Bay during January. However, the system we used was an intermediary step between laboratory and natural community, not a natural environment experimental system even though filtered eutrophic seawater was used. So the bigger contrast between control (400 μatm) and treatment (1000 μatm) was used for us to better observe the effects of elevated CO_2. So we suggest that choosing 400 μatm as the control in our study was reasonable.

page 6, line3. How did the pH change over time and when were samples taken? During phytoplankton blooms, this has major importance as pH changes with respiration during the day and can shift largely over the course of 24 hours.

RE :The samples in this study were collected at about 10 am each time while the other parameters were also measured simultaneously. We agree that the pH variation over the course of 24 hours should be considered during the phytoplankton blooms. It was pity that we did not collect bacterioplankton samples over the course of 24 hours. The comment “In future studies, it would be also worthwhile to sample over a diel cycle in order to understand the cyclic variability in pH and whether this affects short term changes in bacterioplankton community structure.” has been added in the discussion section (Page 21 Line 13-15).

page 6, line 8. Mesocosms were bubbled with air containing 1000 ppm and 400 ppm CO_2, yet differences in CO_2 concentrations could not be maintained throughout the experiment. Why?

RE: When phytoplankton bloom occurred and phytoplankton cells reached high concentration, the consumption of CO_2 was much higher than during the early stage. So this meant that the CO_2 concentrations could not be maintained when phytoplankton entered into logarithmic growth stage. For indoor semi continuous ocean acidification experiments with CO_2 bubbling, the cultures have to be diluted periodically to maintain the cell concentration and thus control the CO_2 concentration. But such dilution was not possible in this mesocosm experiment considering the big volume of seawater in each mesocosm bag.

Page 7, line 3. Can the authors show that the bacterial community composition at the beginning of the experiment was the same in all mesocosm bags? If not, their hypothesis cannot be tested! page 7, line 14. BCC at day zero or 1 was not sampled.

RE: At the beginning of this experiment, no bacteria were detected prior to phytoplankton inoculation. The phytoplankton culture with bacterioplankton were

evenly distributed into each bags for inoculation. So we considered the bacterial community composition at the beginning of the experiment was the same or similar in all mesocosm bags. As for day 0, no detectable bacterioplankton were detected before inoculation. We agree that it is better to show the data at day 2, but unfortunately we were unable to collect samples due to the technical limitations mentioned above.

page 7, line 18. Sequential filtering prior DNA extraction – missing discussion about the majority of bacteria not being included in the results (particle attached and algae associated/attached bacteria were filtered away).

RE: We agree with you that the majority of the particle attached and algae attached bacteria were filtered out by sequential filtering. Consequently, the bacterioplankton in our study did not include these bacteria. This has been added to the discussion section (Page 21 Line 4-7).

page 7, line 19. Which DNA extraction protocol was used? phenol/chloroform method?

RE: The detailed DNA extraction protocol:

1. Wash the filter with 1 ml of lysis buffer described in (Francis *et al.*, 2005) and 10 μl of lysozyme (100 mg/ml), vortex and incubate at 37 degrees for 30 minutes.
2. Add 5 μl RNase A (10 mg/ml), incubate at 37 degrees for 30 minutes.
3. Add 20 μl proteinase K
4. Add 220 μl GB solution from Bacteria DNA extraction kit (Tiangen DP302)
5. Follow the Bacteria DNA extraction kit's instruction to finish the DNA extraction.

The method description is insufficient.

page 8, line 9. The QIIME pipeline is not sufficiently described. How many raw sequences were obtained? How many samples were sequenced/passed quality control? Which pipeline parameters were used? How was the phylogenetic tree produced? What kind of tree is it? Section 2.5 is missing references, parameter description or validation of the method, the link to the sequencing center IEG is insufficient here.

RE: When the sequencing finished, we need to filter the raw data to secure the quality of our data, which mainly including:

1) Cut the polluted adapter;

2) Remove low quality reads, specifically reads with average quality less than 19, based on the Phred algorithm;

3) Remove the reads with N base exceeding 5%.

Finally 2972070 raw reads were obtained in total from all the samples and 2365844 reads passed quality control (see Supplementary Table 1), the average of clean read rate was 79.65%.

According to the reference database, the representative sequences for each OTU were aligned using PyNAST (Caporaso *et al.*, 2010), finally the phylogenetic tree was generated from the Graphlan (Langille *et al.*, 2013) using information on both the relative abundance and phylogenetic relationship of observed species. The missing references have been added to the method section (Page 9 Line 12-18

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Results page 10, line 11. Additional to pCO_2 levels, the measured pH should be shown in a graph. The results sections contain many passages of discussion that should not be included here (for example page 11, line 19 or page 14, line 16). page 11, line 16.

RE: The pH value has been added in Figure 2 with pCO_2 levels. The results section that contained passages of discussion has been moved to the discussion section or rephrased. The structure of this manuscript has been rearranged.

How many sequences were included in the results? How many reads were obtained per sample? Why were some replicates not included in the results? page 12, line 20. Was the BCC tested for differences prior to day6? If so, results are not described or included in Table2.

RE: The raw reads and the clean reads of each sample were shown in supplementary table 2. As mentioned above, probably due to high concentration of TEP, all the

samples at day 2 were not successfully collected and only a few samples at day 4 were successfully collected probably.

On page 12, some bacteria phyla were selected for analysis, does it mean that the rest was ignored in analysis after this point and in the network analysis? How similar/different are mesocosm replicates? Inter-treatment variability seems to be very high, possibly coupled to initial differences in bacterial communities in the different mesocosms.

RE: All the bacteria phyla were analyzed in the network analysis. We agreed that inter-treatment variability was high. This mesocosm experiment was conducted outdoors and the mesocosm enclosures were exposed to fluctuating environmental factors which led to high inter-treatment variability. Previous mesocosm experiments also have similarly high inter-treatment variability, which is very hard to avoid for outdoor mesocosm experiments. We did sampling every two days which also can introduce outside bacteria randomly. So we think the high inter-treatment variability was due to the mesocosm experiment itself, rather than to initial differences in bacterial communities in the different mesocosms.

page 14, line 12. Naming of OTUs is weird (e.g. OTU 4331023), the high numbers suggest many OTUs, but only 4992 were reported. Can the authors support the results with bacterial abundance data? If certain bacteria increase/decrease in relative abundance, is this due to a change in community composition or an overall increase/decrease in cell numbers? This would stress the effect of the phytoplankton bloom on bacterial growth and BCC

RE: The OTU IDs in our study were IDs in Greengene database.

The increase/decrease of certain bacteria in relative abundance is due to a change in community composition, not an overall increase/decrease in cell numbers. There was no big variation in the cell density from Day 12 to Day 32 according to Yibin HUANG et al (Limnology and Oceanography, under revision). However, our data showed a big variation in community composition between day 13 and day 29. All

above information indicated that bacteria increase/decrease in relative abundance was due to the change in community composition, not the overall increase/decrease in cell numbers.

The discussion is too short, selective and does not truly discuss the results in a broad perspective. For example: Page 15, line 17. If the BCC resulted from phytoplankton culture inoculum, the bacteria were adapted to growth alongside phytoplankton in cultures and closed containers and resulting pH ranges due to phytoplankton respiration (possibly for several years, depending on when phytoplankton strains were isolated, non-adapted bacteria would have been outcompeted prior to the experiment). Therefore, the results should not be generalized but discussed in this perspective.

RE: We agree with the reviewer that the inoculated bacterioplankton along with the phytoplankton probably have outcompeted the non-adapted bacteria prior to the experiment. It seems though that the environmental bacterioplankton from outside through tubes, sampling and sea air exchange became dominant in the mesocosms from day 0 to day 4, because the bacterioplankton composition at day 4 and day 6 were very different from the bacterioplankton composition in the original phytoplankton cultures, including some which were not detected in the phytoplankton cultures at all. This suggests the local bacterioplankton outcompeted the bacterioplankton from the phytoplankton cultures at an early stage of the mesocosm experiment. Everything mentioned above has been added to the discussion section (Page 16 line 18-22, Page 17 line 1-3). Because of this shift to natural bacteria, we think the results about the bacterioplankton community composition under the HC and LC conditions can be generalized, as on Page 17, line 20-22.

page 17, line 22. The authors “speculate that the stimulation of growth of Flavobacteria could have been due to the enhanced activation of proteorhodopsin under the HC treatment at the early stage of diatom bloom”. This is pure speculation based only on selective reading of the literature and has no place here in the absence of any evidence of expression of proteorhodopsin.

RE: We agree that this is just speculation without proteorhodopsin expression data in our study. We have rephrased this description.

Figures: Figure 1 is not relevant for the manuscript.

RE: We think showing the location of the experiment site is important for the whole manuscript. We want to show Wuyuan Bay is in the city center and strongly influenced by human activity. To address this comment though, this figure has been moved to supplementary data.

In Figure 2, SE or SD (description missing in Figure legend) should be shown both upwards and downwards.

RE: SD with upwards and downwards has been added in Figure 2. The description of SD has been added in the Figure 2 legend as well.

Figure 3 misses a description of replicate numbers. Why does day 4 only have one replicate? It would aid the reader to have spaces between the different days. Interreplicate variability is apparent, mesocosm 8 for example has a distinct BCC compared to other LC mesocosms (increase of Phaeobacter over time), however this is not discussed in the paper.

RE: The replicate numbers have been added in the Figure 3 legend. As mentioned above, we tried to collect the samples and extract DNA from all mesocosm bags but we only successfully extracted enough DNA from bag 1 and bag 6 at day 4 for sequencing.

Extra space between different days have been added in Figure 3. We agree that mesocosm 8 has distinct BBC compared to the other LC mesocosms. We think the high inter-replicate variability was due to the experimental environment. The increase of Phaeobacter in mesocosm 8 was a random issue in this mesocosm experiment. The discussion about the distinct BBC in mesocosm 8 has been added in the discussion (Page 17 Line 7-13).

Figure 4, which information does this figure show that are not visual in Figure 3? How many replicates were included?

RE: Figure 3 showed the overview of community structure at different taxonomic levels of all the samples. But it is not easy to get information about the change of certain bacteria groups throughout the experiment. Figure 4 showed clearly the change of Bacteroidetes in contrast with Proteobacteria at the phylum level; Flavobacteria in contrast with Alphabacteria at the class level; Flavobacteriales in contrast with Rhobacteriales at the order level; and Flavobacteriaceae in contrast with Rhodobacteriaceae at the family level. 3 replicates were included except the missing samples at day 4 and day 6 for Figure 3 and Figure 4.

Figure 5, which data were used for the network? Which day/replicates? How are differences in replicate numbers accounted for? How are "OTUs with importance" evaluated?

RE: We used all the data we have from each bag on each day, except some samples that were missing on day 4 and day 6 for network analysis. The sequencing data from each mesocosm bag throughout the experiment at different time points were considered as different replicates with time series. For example, the sequencing data from mesocosm bag 1 with time series at day 4, day 6, day 8, day 19 and day 29 were considered as HC1. Mesocosm 1, 6 and 8 were three replicates for HC treatment and mesocosm 2, 4 and 7 were three replicates for LC treatment. The main text about network construction in method and material section has been revised as "First, the similarity matrices of the relative abundance of OTUs in LC and HC conditions were created respectively using Pearson correlation coefficient across time points with biological replicates by a random matrix theory (RMT)-based approach".

OTUs with high relative abundance were defined as OTUs with importance. OTU 572670 with 21402 reads from all the samples, OTU 558885 with 5780 reads, OTU 190052 with 42525 reads, OTU107130 with 12892 reads, OUT 572670 with 21402 reads, OUT 4331023 with 7845 reads were considered as OTUs with importance (see supplementary table 2)

Fig S1, how representative is the diatom BCC if it comes from two species? Is it the sum/average of cultures? Replicates? When were samples taken? During inoculation or before/after the experiment? BCC likely changes throughout the course of phytoplankton growth (as shown by the authors in the mesocosm experiment) and can affect the BCC of the inoculum.

RE: The diatom BCC came from the sum of two diatom species of culture. The phytoplankton culture samples were taken after the inoculation in order to investigate the roles of phytoplankton culture BCC in the whole mesocosm experiment. It cannot be denied that it would have been better to collect the bacterioplankton from the phytoplankton just before inoculation. We think the BCC of phytoplankton culture should be stable over the short term, because the phytoplankton cultures were maintained in semi-continuous culture with artificial seawater.

Fig S2, the Figure text is not sufficient. How was the tree generated? What kind of tree is this? Is it rooted? Which parameters were used when it was generated? Is it relevant?

RE: PyNAST method (Caporaso, et al.,2010) and Graphlan software (Langille, et al., 2013) were used to construct the phylogenetic unrooted NJ tree as mentioned above. The legend of Fig S2 has been revised.

S5, the figure illustrates that the bacterioplankton diversity is widely spread in the early days of the experiment, and it is obvious that replicates at day 4 are missing. Yet a discussion of these results is missing in the text.

RE: The explanation of missing data at day 4 has been mentioned above, and added in the methods and materials section.

S6, The figure legend is misleading. The PCA legend does not show the different mesocosm replicates and they are replicates (at the same day)?

RE: The legend of Fig. S6 has been revised to clarify that each symbol presents the

average value of the HC and LC treatments with three replicates at different days. For example, HC-D13 presents the average value of HC2, HC4, HC7 at day13.

Referee #2

The goal of this study was to assess the effect of ocean acidification (OA) on the bacterial community during an "induced phytoplankton bloom" in a coastal area. The coastal water was filtered onto 0.1 μm (but some bacteria were present at the start of the experiment) then three xenic phytoplankton cultures were added to the mesocosms. Despite the massive sequencing work, there are important points that have not been addressed by the authors in the experimental design as well as in the sampling and analysis steps thus weakening the paper. The authors do not show the community structure of the "contaminated water" at the beginning of the experiment (prior phytoplankton amendment) and this is a critical point in order to be able to state whether there is an effect or not of OA on bacterial community structure. It would be important to discuss how different the contaminated water community was in comparison to the bacterial community associated with the phytoplankton strains.

RE: We appreciate the comments from reviewer #2. The description of the experimental design, sampling and analysis have been strengthened in the revised manuscript..

Our experiment was designed as an intermediary step between laboratory and natural community field experiments, with isolates of non-axenic phytoplankton being added to filtered natural waters. In this way, we were able to investigate the effect of OA on phytoplankton and bacterioplankton in eutrophic waters while minimizing the complexity of shifting compositions of natural phytoplankton communities. In other words, we aimed to study the effects of ocean acidification on some model phytoplankton species and phytoplankton culture-originated bacterioplankton in a larger scale experiment compared to the lab experiment. Therefore, this experiment could not truly reflect the effects of ocean acidification on field natural phytoplankton and bacterioplankton communities. The outdoor mesocosm system was not sterile,

and it was impossible to avoid the bacteria from outside through sampling and air-sea exchange during the experiment. Our data showed that the local bacterioplankton communities were very different from bacterioplankton originated from phytoplankton culture by day 4 based, on the comparison of the bacterioplankton community at day 4 and the original bacterioplankton community. And some bacterioplankton that were not detected in the original phytoplankton culture appeared in samples collected at day 4. Therefore, we conclude that the environmental bacterioplankton outcompete the phytoplankton-originated bacterioplankton from day 0 to day 4. Since the day 2 data were lacking, it seems likely that the environmental bacterioplankton became dominant even before day 4. This suggests the bacterioplankton studied in this paper were mainly natural bacterioplankton. The points mentioned above have been added to the results and discussion section. We agree that it is important to discuss the contaminated water community in comparison to the bacterial community associated with the phytoplankton by showing the bacterial community structure at day 2 and day 4. We tried to do sampling at day 2 and day 4. But eventually we could successfully extract enough DNA only from bag 1, bag 6 and bag 7 at day 4 for sequencing, probably due to high concentration of TEP (Transparent Exopolymer Particles) (Sugimoto *et al.*, 2007, Ramaiah *et al.*, 2000).

Bacteria were not detectable by flow cytometry in the filtered seawater prior to inoculation. Three species of non-axenic phytoplankton with bacterioplankton were mixed and then inoculated into each mesocosm bag. Because the mixture added was the same, we considered the initial bacterioplankton community was similar in each mesocosm bag.

We described the experimental design in a more detailed way to clarify why we used this approach in the revised manuscript. The limitations of our experimental design and approaches were also pointed out in the manuscript (Page 7 Line 14-22, Page 8 Line 1-3).

I would encourage the authors to present also the bacterial abundance data (the authors say that bacteria were present in the "contaminated water and I assume that

they have counted them) that will be very useful to understand the bacterial dynamic and response to OA. Furthermore, the DOC and POC data should be included here since the authors state that data those have been packaged in another paper.

RE: We agree that it is better to discuss the correlation between bacterioplankton abundance and community structure in the manuscript as well as DOC and POC data in this paper.

The bacteria abundances were shown in Yibin Huang et al entitled “responses of phytoplankton and bacterial metabolism to CO_2 enrichment during a coastal mesocosm experiment” (in the second round revision at Limnology and Oceanography). DOC and POC data were shown in Nana Liu et al (in press at Marine Environmental Research).

The section Environmental parameters and experimental timeline is confusing. The authors could consider to include a table that summarizes the nutrient trends and if possible other important data (bacteria count, viral count, phytoplankton count, DOC and POC)

RE: Sorry for the confusion. We agree that the nutrient trends, bacteria abundance data, phytoplankton abundance data, DOC and POC data are important for supporting our main results (Viral counting was not done in this mesocosm experiment). However, these data were packaged in other papers either published or under revision as mentioned above. We think it is not appropriate to use these data directly in this paper. We have cited these papers containing bacteria counts, phytoplankton counts, and DOC and POC data.

Some graphs in the main text and in the SI are not very informative such as phylum distribution and genus distribution graphs and confuse the message of the paper. The SI material needs more explanation and for instance the PCA graphs do not show very clearly the findings.

RE: We improved the legends of the supplementary figures and the text to make them more informative. For example, the software used to construct the phylogenetic tree and the type of phylogenetic tree has been added into the legend of Fig.S2. The

explanation of different replicates of the HC and LC treatment has been clarified in the legend Fig. S7 (PCA graph).

It would be useful that the authors would comment the use of their primers in the light of the Environ Microbiol. 2016 May;18(5):1403-14. doi:10.1111/1462-2920.13023. Epub 2015 Oct 14: Every base matters: assessing small subunit rRNA primers for marine microbiomes with mock communities, time series and global field samples by Parada et al.

RE: The choice of primers amplifying 16S genes is crucial. Sequencing depth, high coverage of the taxa of interest, the ability to compare results with prior studies, accuracy in relative abundances and the phylogenetic resolution of the sequenced PCR products should be considered when choosing suitable primers (Parada *et al.*, 2016) We used primers 341F (5'-CCTACGGGNGGCWGCAG-3') and 805R (5'-GACTACHVGGGTATCTAATCC-3') targeting the 16S V3-V4 region, which has successfully been applied in previous studies (Hugerth *et al.*, 2014). Thus we used 341F/805R primers that were well accepted for bacteria diversity studies. For our study, using 341F/805R was appropriate considering the ability to compare results with prior studies, accuracy in relative abundances and the phylogenetic resolution of the sequenced PCR products. The paper mentioned above mainly discussed about the primers 515F-Y/926R and 515F-C/806R targeting the 16S V4-V5 region. The advantage of these two pairs of primers is that it should match bacteria as well as archaea. Therefore, the archaea were missing in our data set based on the primers 341F/805R we used in this study. We think primers 515F-Y/926R are better candidates because of their better coverage and their sequences have been validated in Parada *et al*. Thus we think 515F-Y/926R will be useful for future bacteria diversity studies. The limitations of the primers used in this study has been added to the discussion section (Page 21 Line 5-6).

The English and the structure of the paper should be revised.

RE: The text and the structure has been revised carefully.

Reference:

Caporaso, J. G., Bittinger, K., Bushman, F. D., Desantis, T. Z., Andersen, G. L., and Knight, R. 2010. PyNAST: A flexible tool for aligning sequences to a template alignment. Bioinformatics, 26: 266–267.

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Interactive network configuration maintains bacterioplankton community structure under elevated CO_2 in a eutrophic coastal mesocosm experiment

Xin Lin[†*1], Ruiping Huang[†1], Yan Li[1], Futian Li[1], Yaping Wu[1,2], David A. Hutchins[3], Minhan Dai[1], Kunshan Gao[*1]

Institutions:

[1]State Key Laboratory of Marine Environmental Science, Xiamen University (Xiang An Campus), Xiamen 361102, China.

[2]College of Oceanography, Hohai Uuniversity, No.1 Xikang road, Nanjing 210000, China.

[3]Department of Biological Sciences, University of Southern California, 3616 Trousdale Parkway, AHF 301, Los Angeles, CA 90089-0371, USA.

[†] These authors contributed equally to this work.

Correspondence to: Xin Lin (xinlinulm@xmu.edu.cn, TEL: +865922880171);

Kunshan Gao (ksgao@xmu.edu.cn, TEL: +865922187963)

Abstract

There is increasing concern about the effects of ocean acidification on marine biogeochemical and ecological processes and the organisms that drive them, including marine bacteria. Here, we examine the effects of elevated CO_2 on the bacterioplankton community during a mesocosm experiment using an artificial phytoplankton community in subtropical, eutrophic coastal waters of Xiamen, Southern China. Through sequencing the bacterial 16S rRNA gene V3-V4 region, we found that the bacterioplankton community in this subtropical, high nutrient coastal environment was relatively resilient to changes in seawater carbonate chemistry. Based on comparative ecological network analysis, we found that elevated CO_2 hardly altered the network structure of high abundance bacterioplankton taxa, but appeared to reassemble the community network of low abundance taxa. This led to relatively high resilience of the whole bacterioplankton community to the elevated CO_2 level and associated chemical changes. We also observed that the Flavobacteria group, which plays an important role in the microbial carbon pump, showed higher relative abundance under the elevated CO_2 condition during the early stage of the phytoplankton bloom in the mesocosms. Our results provide new insights into how elevated CO_2 may influence bacterioplankton community structure.

Key words: elevated CO_2; mesocosm; bacterioplankton community; ecological network; Flavobacteria

1 Introduction

It is well established that ocean acidification is being caused by increased uptake of anthropogenically-derived carbon dioxide in the surface ocean. Consequently, it is predicted that under a "business-as-usual" CO_2 emission scenario, the present average surface pH value will drop 0.4 units over the next century (Gattuso et al., 2015). Despite a growing interest in the importance of the roles of marine bacterioplankton in ocean ecosystems and biogeochemical cycles, our current understanding of their responses to ocean acidification is still limited. Over half of autotrophically-fixed oceanic CO_2 is processed by heterotrophic bacteria and archaea through the microbial loop and carbon pump (Azam, 1998; Jiao et al., 2010). Furthermore, marine bacterioplankton play an essential role in marine ecosystems and global biogeochemical cycles central to the biological chemistry of Earth (Falkowski et al., 2008). The null hypothesis is that elevated CO_2 will not affect biogeochemical processes (Liu et al., 2010; Joint et al., 2011), however more investigation is required. Ocean acidification mesocosm experiments provide good opportunities to explore the responses of marine bacteria to elevated CO_2. Mesocosm studies conducted in the Arctic Ocean, Norway, Sweden and the coastal Mediterranean Sea using natural phytoplankton communities have often found that elevated CO_2 has little direct effect on the bacterioplankton community (Zhang et al., 2013; Ray et al., 2012, Roy et al., 2013; Baltar et al., 2015). In contrast, phytoplankton blooms induced by high CO_2 can sometimes have significant indirect effects on heterotrophic microbes, thus altering bacterioplankton community structure (Allgaier et al., 2008, Hutchins and Fu, 2017).

Although most mesocosm studies have showed that elevated CO_2 had an insignificant impact on bacterioplankton community structure, microcosm experiments have demonstrated that small changes in pH can have direct effects on marine bacterial community composition (Krause et al., 2012). Ocean

acidification experiments using natural biofilms showed bacterial community shifts, with decreasing relative abundance of Alphaproteobacteria and increasing relative abundance of Flavobacteriales (Witt et al., 2011). Coastal microbial biofilms grown at high CO_2 level also showed different community structures compared to those grown at ambient CO_2 level in a natural carbon dioxide vent ecosystem (Lidbury et al., 2012). Ocean acidification also affects the community structure of bacteria associated with corals. It has been reported that the relative abundance of bacteria associated with diseased and stressed corals increased under decreasing pH conditions (Meron et al., 2011). A very limited number of studies focused on the effects of ocean acidification on isolated bacterial strains have also been reported. Under lab conditions, growth of *Vibrio alginolyticus*, a species belonging to the class Gammaproteobacteria, was suppressed at low CO_2 levels (Labare et al., 2010). In contrast, stimulation of growth was observed for one Flavobacteria species under high CO_2 levels (Teira et al., 2012).

Taken together, results from mesocosm, microcosm and cultured isolate experiments indicate a potentially complex interaction between different groups of marine bacteria in response to elevated CO_2. One promising method to elucidate these types of complex interactions is network analysis. Ecological network approaches have been successfully applied to investigate the complexity of interactions among zooplankton and phytoplankton from different trophic levels during the Tara Oceans Expedition project (Lima-mendez et al., 2015; Guidi et al., 2015). Elucidating the complex interactions between bacterioplankton and other marine organisms under anthropogenic perturbations will increase our understanding of their impact in a holistic way. Previous studies using ecological network analysis showed that elevated CO_2 significantly impacted soil bacterial/archaeal community networks, by decreasing the connections for dominant fungal species and reassembling unrelated fungal species in a grassland ecosystem (Tu et al., 2015). It was also reported using ecological network analysis that

elevated pCO_2 did not significantly affect microbial community structure and succession in the Arctic Ocean, suggesting bacterioplankton community resilience to elevated pCO_2 (Wang et al., 2016).

It has been reported that eutrophication problems in coastal regions lead to complex cross-linkages between ocean acidification and eutrophication (Cai et al., 2011). The occurrence of ocean acidification combined with other environmental stressors such as eutrophication can potentially produce synergistic or antagonistic effects on bacterioplankton that differ from those caused by ocean acidification alone. Although there are some reports from mesocosm experiments describing the response of bacteria to elevated CO_2, there are limited studies on how the bacterial community responds to ocean acidification in eutrophic marine environments. In this study, Illumina sequencing of the V3-V4 region of the bacterial 16S rRNA gene was used to explore the effects of ocean acidification on bacterioplankton community composition and ecological network structure in a eutrophic coastal mesocosm experiment.

2 Methods

2.1 Mesocosm setup and carbonate system manipulation

The mesocosm experiment was conducted in the FOANIC-XMU (Facility for the Study of Ocean Acidification Impacts of Xiamen University) mesocosm platform located in Wuyuan Bay, Xiamen, Fujian province, East China Sea (N24°31′48″, E118°10′47″) during the months of December 2014 and January 2015 (Fig. S1). Each transparent thermoplastic polyurethane (TPU) cylindrical mesocosm bag was 3 m deep and 1.5 m wide (~4000 L total volume). After setting up the mesocosm bags within steel frames, in situ seawater from Wuyuan Bay was filtered through a 0.01μm water purifying system and used to simultaneously fill eight bags within 24 hours. The initial in situ seawater pCO_2 in Wuyuan Bay was ~650 μatm, due to the active decomposition of land-sourced organic compounds. In order to reach the target low pCO_2 associated with ambient air (400 ppm), Na_2CO_3 was added to each mesocosm to

increase dissolved inorganic carbon (DIC) and total alkalinity (TA) by 100 μmol/L and 200 μmol/L respectively, based on carbonate system calculations (Lewis and Wallace, 1998). To adjust seawater to projected end of this century seawater conditions of ~1000 ppm CO_2, about 5 L of CO_2 saturated filtered seawater was added to 4 mesocosms (#2, #4, #7, #9), collectively considered to be the HC treatment, while the other 4 mesocosms (#1, #3, #6, #8) were considered to be the LC treatment. Throughout the experiment, HC mesocosms and LC mesocosms were bubbled with air containing 1000 ppm and 400 ppm CO_2, respectively supplied by a CO_2 Enrichlor (CE-100B, Wuhan Ruihua Instrument & Equipment Ltd, China) at a flow rate of 4.8 L per minute.

Two diatoms, *Phaeodactylum tricornutum* CCMA 106 from the Centre for Collections of Marine Bacteria and Phytoplankton of the State Key Laboratory of Marine Environmental Science (Xiamen University, China), and *Thalassiosira weissflogii* CCMP 102 from the Provasoli-Guillard National Center for Culture of Marine Phytoplankton (CCMP, USA), as well as the coccolithophorid *Emiliania huxleyi* CS-369 from the Commonwealth Scientific and Industrial Research Organization (CSIRO, Australia) were used as inoculum to construct a model phytoplankton community. The effects of ocean acidification on these phytoplankton species mentioned above have been intensively studied in the lab at physiological, biochemical and molecular levels. However, it is difficult to extrapolate the response of these species to ocean acidification in natural complex environments based on laboratory single species experiments (Busch et al., 2015). Our experiment was designed as an intermediary step between laboratory and natural community field experiments, with isolates of non-axenic phytoplankton being added to filtered natural waters. In this way, we were able to investigate the effect of OA on phytoplankton and bacterioplankton in naturally eutrophic waters while minimizing the complexity of shifting compositions of natural phytoplankton communities. Correlated data about the effects of ocean

acidification on the artificial phytoplankton community using the same mesocosm system are available in (Jin et al., 2015) and Liu et al. (in press).

The initial concentration of both *P. tricornutum* and *T. weissflogii* was 10 cells/mL, and *E. huxleyi* was added at 20 cells/mL. The phytoplankton cultures were not axenic. The bacteria community composition in the inoculated phytoplankton culture is shown in Fig. S2. Bacteria were not detectable by flow cytometry in the filtered seawater just before inoculation. The three species of non-axenic phytoplankton with bacterioplankton were mixed and then inoculated into each mesocosm bag. Thus, we considered the initial bacterioplankton community to be the same or similar in each mesocosm bag. The mesocosm and the CO_2 bubbling system were not sterile and not completely closed during the experiment. Therefore, natural bacterioplankton were undoubtedly introduced into the mesocosm system through sampling and air-sea exchange, and the bacterioplankton community in this mesocosm experiment was derived from both the bacteria added with the inoculated phytoplankton culture, and the natural local prokaryotic assemblage.

The use of the natural phytoplankton and bacterioplankton communities in this mesocosm experiment would better represent the effects of ocean acidification on natural phytoplankton and bacterioplankton communities. However, considering the highly eutrophic in situ seawater in Wuyuan Bay, it was impractical to use the in situ seawater with the in situ natural community (bacterioplankton, phytoplankton, zooplankton) directly without filtration, because of the dense phytoplankton bloom that could be induced within several days, making the pCO_2 very difficult to keep under control. Alternatively, we would have had to dilute 4 tons of seawater in the mesocosm bags at least every two days to maintain the cell density and CO_2 concentration. Furthermore, considering a number of studies on the typical phytoplankton responses to OA that have been carried out in laboratory, it was indeed a

natural progression for us to use typical model phytoplankton species to initiate the mesocosm studies before using natural communities. Therefore, using the filtered seawater with inoculated isolates was reasonable and logistically practical for our experiment.

2.2 Bacteria sampling, filtration and sample selection

A total of 500 mL to 2 L of water, depending on bacterial concentration, was collected from the mesocosms. Six of the mesocosms (HC: #2, #4, #7 and LC: #1, #6, #8) were chosen for further study. Samples from days 4, 6, 8, 10, 13, 19, and 29 were collected in this study due to time, personnel and equipment constraints. Sequential size fractionated filtration (2 μm and 0.2 μm polycarbonate filters) by peristaltic pump was used to filter seawater collected from the mesocosm bags. We tried to do sampling at day 2, but the samples were not successfully collected, probably due to very high concentration of TEP (Transparent Exopolymer Particles) which easily blocked the polycarbonate filter. Some replicates were missing at day 4 because we were able to successfully extract enough DNA for sequencing only from bag 1, bag 7 and bag 6, also probably due to high TEP at day 4. It has been reported that high TEP concentration was associated with high bacteria biomass (Sugimoto et al., 2007, Ramaiah et al., 2000). According to the bacterioplankton abundance data in Yibin Huang et al. (in review), the average bacterioplankton abundance was 9.71 $\times 10^9$ cells/ml and 3.15 $\times 10^9$ cells/ml at day 2 and day 4 respectively.

2.3 DNA extraction, 16S rDNA V3-V4 region amplification and Illumina MiSeq sequencing

Samples collected by 0.2 μm polycarbonate filters as described above were washed with PBS buffer and then centrifuged at 9600g to obtain a cell pellet. A previously described DNA extraction protocol (Francis et al., 2005) was utilized with some modifications, using the columns for DNA purification from a bacteria DNA extraction kit (Tiangen DP302, China). Amplification, library construction and

sequencing were performed offsite at ANNOROAD using the DNA samples isolated as described above. Primers were 341F (5'-CCTACGGGNGGCWGCAG-3') and 805R (5'-GACTACHVGGGTATCTAATCC-3'), targeting the V3-V4 hyper variable regions of bacterial 16S rRNA gene. The PCR amplification condition was as follows: initial denaturation at 95°C for 3 min, 25 cycles of denaturation at 95°C for 30 s, annealing at 55°C for 30 s and extension at 72°C for 30 s, then final extension at 72°C for 5 min. DNA library construction and sequencing followed the MiSeq Reagent Kit Preparation Guide (Illumina, USA).

2.4 Sequence assignment and sequence statistics analysis

Clean paired-end reads were merged using PEAR (Zhang et al., 2014). The remaining raw sequences were distinguished and sorted by unique sample tags. Unique operational taxonomic units (OTUs) were picked against Greengenes database (http://greengenes.lbl.gov/cgi-bin/JD_Tutorial/nph-16S.cgi) (McDonald et al., 2012) at 97% identity. OTUs with less than 2 reads were not considered. According to the reference database, the representative sequences for each OTU were aligned using PyNAST (Caporaso et al., 2010a). Finally, the phylogenetic tree was generated from the Graphlan (Langille et al., 2013) using information on both the relative abundance and phylogenetic relationship of observed species. QIIME 1.8.0 was used for sequence analysis including OTUs extraction for bacterioplankton community structure analysis, OTUs overlapping analysis, species diversity, species richness analysis and Principal Components Analysis (PCA) (Caporaso et al., 2010b). Bacterioplankton community composition differences were assessed by Unweighted UniFrac distance using QIIME 1.8.0 as well. Dissimilarity tests were based on the Bray-Curtis dissimilarity index using analysis of similarities (ANOSIM) (Clarke, 1993), non-parametric multivariate analysis of variance (ADONIS) (Anderson, 2001), and multi-response permutation procedures (MRPP) (Mielke et al., 1981). Observed

species, Chao index, Shannon index and Simpson index were used for estimating the community diversity. Analysis of variance (ANOVA) followed by T-test was performed to determine any significant differences between HC and LC treatments.

2.5 Ecological network construction and analysis

As previously described, ecological network construction and analyses were performed based on the relative abundance of OTUs in HC and LC treatments with three biological replicates (http://129.15.40.240/mena/, Wang et al., 2016). The sequencing data from each mesocosm bag with time series throughout the experiment were considered as different replicates. First, the similarity matrices of the relative abundance of OTUs in LC and HC conditions were created respectively using Pearson correlation coefficient across time points with biological replicates by a random matrix theory (RMT)-based approach. Cut-off values were determined according to R^2 of power-law larger than 0.8 and equal between two manipulations to construct network structure. In order to ensure the constructed networks were not random, biologically meaningless networks, 100 networks from the same matrix were constructed and randomized. This resulted in the experimental networks being different from random networks judging by significantly higher modularity, clustering coefficient and geodesic distance (Table 1). Then, module separation was produced using greedy modularity optimization, and *Z-P* values for all nodes were calculated. In addition, to compare networks, the network connection was randomly rewired and network topological properties were calculated. Finally, the bacteria network interaction was visualized by Cytoscape v.3.3.0. The *Z–P* plots were constructed based on within-module (*Z*) and among-module (*P*) values of each node derived from ecological network analysis. Ecological network analysis is a novel RMT-based framework for studying microbial interactions. A node in ecological network analysis shows an OTU and a link demonstrates a connection between two OTUs. The shortest

path between nodes is indicated by geodesic distance. Since the network constructed by OTUs can be separated into several sub-communities, or modules, the modularity value indicates how well a network can be divided into different sub-communities. Clustering coefficients demonstrate how well an OTU is connected with other OTUs, while average clustering coefficients indicate the extent of connection in a network.

3 Results

3.1 Environmental parameters and experimental timeline

The initial inorganic nitrogen, PO_4^{3-}, and SiO_3^{2-} concentrations were 70–75 μmol/L, 2.5–2.6 μmol/L, and 38–39 μmol/L, respectively. Except for SiO_3^{2-}, nutrient concentrations decreased with rapid growth of the phytoplankton and reached low concentrations by day 15. The dissolved total inorganic nitrogen dropped from an initial concentration of 74.9 ± 2.87 μmol/L to 57.2 ± 4.37 μmol/L in the HC condition and 72 ± 5.90 μmol/L to 53.6 ± 5.60 μmol/L in the LC condition by day 8, and reached low concentrations by day 15 (average 3μmol/L in LC and average 6μmol/L in HC).

The pCO_2 in this study was calculated from DIC and pH by the CO2SYS Program (Lewis and Wallace, 1998). The initial pCO_2 of 373.0 ± 43.9 μatm (pH_{NBS}: 8.18 ± 0.02) in the LC treatment and 1296.0 ± 159.6 μatm (pH_{NBS}: 7.75 ± 0.04) in the HC treatment increased and reached a peak value of 922.5 ± 142.0 μatm (pH_{NBS}: 7.74 ± 0.08) in the LC treatment at day 8 and 1879.6 ± 145.4 μatm (pH_{NBS}: 7.49 ± 0.05) in the HC treatment at day 4. After reaching the peak, the pCO_2 values of both treatments decreased and were no longer statistically different from day 13 onwards due to rapid CO_2 uptake by the phytoplankton, despite air containing 1000 ppm CO_2 being continuously bubbled into the HC treatments (Fig. 1 a, b). *P. tricornutum* and *T. weissflogii* were the dominant species throughout the whole phytoplankton bloom in both HC and LC conditions. Chlorophyll *a* (Chl*a*) concentration and diatom cell

densities were used to identify changes in the diatom bloom following inoculation (Fig. 1c, Liu et al., in press). Chl*a* concentration increased from 0.23 ± 0.12 μg/L to 5.33 ± 1.82 μg/L in the LC conditions, and from 0.19 ± 0.07 μg/L to 5.75 ± 1.17 μg/L in the HC conditions from day 4 to day 9. Thereafter, Chl*a* concentration increased significantly and peaked at 109.9 ± 38.04 μg/L in the LC treatment and 108.6 ± 46.07 μg/L in the HC treatment at day 15. Subsequently, Chl*a* concentrations in both treatments were maintained at high concentrations until day 25 and decreased progressively afterward. The bloom process identified by cell concentration of *P. tricornutum* and *T. weissflogii* was similar with that illustrated by Chl*a* concentration. The growth of these two diatom species entered into logarithmic phase from day 2. Cell density reached highest concentration at day 15 and day 19 for *T. weissflogii* and *P. tricornutum* respectively, and then dropped down slowly. The coccolithophore *Emiliania huxleyi* largely disappeared from the experimental mesocosms. A comprehensive description of phytoplankton cell density, Chl*a* concentration, particle organic carbon (POC) and particle organic nitrogen (PON) during the experiment is given in Liu et al. (in press).

3.2 Overview of sequencing analysis

Following sequencing, 828524 high quality sequences were kept after processing (Table. S1), and 39.3% of assembled reads were successfully aligned with the database. As a result, a total of unique 557 OTUs were generated after clustering at a 97% similarity level. 49.1% of OTUs were classified to genera level with high taxonomic resolution (Table. S2). The phylogenetic tree was constructed based on the sequences derived from all of the samples (Fig. S3). The bacterioplankton from all of the samples in this study were identified as members of Bacteriodetes or Proteobacteria phylums. The most dominant OTUs were Alphaproteobacteria, Rhodobacterales, Rhodobacterceae and Sediminicola at class, order, family and genus level respectively (Fig. S4). The most abundant sequences at class, order, family and

genus levels accounted for 43.4 %, 42.6 %, 41.7% and 32.8 % of all sequences respectively.

3.3 Bacterioplankton community structure throughout the phytoplankton bloom

The bacterioplankton community structure in the mesocosm bags was very different from that in the originally inoculated phytoplankton cultures by day 4. For instance, some bacterioplankton phyla not detected in the original phytoplankton culture were observed in the samples collected on day 4. This may indicate that the bacterioplankton from the natural environment gradually became dominant in the mesocosm bags from day 0 to day 4. For example, Epsilonbacteria appeared in the mesocosms at day 4, while no Epsilonbacteria were detected in the coccolithophore or diatom cultures. Nearly 50% of the bacterioplankton in the mesocosms were composed of Epsilonbacteria in D4.1 (Fig. S2, Fig. 2).

Bacterioplankton community structure underwent dynamic changes during the diatom bloom in both the HC and LC treatments, varying significantly at different stages of the phytoplankton bloom (Fig. 2). At the phylum level, the bacterioplankton were dominated by Proteobacteria, while the relative abundance of Bacteroidetes was very low when nutrients were replete and diatom biomass was not high. However, Bacteroidetes increased dramatically as diatom biomass increased, and began to drop down after reaching a peak at day 10 (Fig. 2 and Fig. 3). In contrast, Proteobacteria began to increase after reaching their lowest concentration at day 10.

The Alphaproteobacteria, Flavobacteria, and Gammaproteobacteria classes with high abundance in all samples were selected for further analysis. The proportion of the Gammaproteobacteria class from the Proteobacteria phylum was very high at the beginning of the experiment (50.2 ± 13.8 % in the HC treatment and 44.1 ± 6.4 % in the LC treatment at day 6) and decreased throughout the duration of the experiment. On the other hand, the Alphaproteobacteria class, also from the Proteobacteria phylum, decreased from initially high proportions (46.9 ± 13.2 % in the HC treatment and 43.9 ± 11.6 % in the LC

treatment) at day 6 to low proportions at day 10 (27.2 ± 2.8 %) in the HC treatment, but remained almost unchanged (44.6 ± 7.5 %) in the LC treatment and increased to 63.2 ± 27.3 % in the HC treatment and 60.8 ± 32.7 % in the LC treatment at day 29 (Fig. 2 and Fig. 3). The relative abundance of the Flavobacteria class from the Bacteroidetes increased from the beginning and reached a peak at day 10 (52.2 ± 4.2 % in the HC treatment and 24.8 ± 16.9 % in the LC treatment), then dropped down until day 19 (19.9 ± 2.2 % in the HC treatment and 18.0 ± 15.4% in the LC treatment) (Fig. 2 and Fig. 3). The proportional variation of the Flavobacteriales order and the Rhodobacterales order showed similar trends with the Flavobacteria class and the Alphaproteobacteria class, respectively, as shown in Fig. 2 and Fig. 3.

3.4 The effects of elevated CO_2 on bacterioplankton community structure

Bacterial community structures of the HC and LC treatments were compared at different sampling time-points (Fig 2), and a dissimilarity test based on ANOSIM, MRPP and ADONIS methods showed that no statistically significant differences were observed (Table 2). PCA analysis also agreed with the dissimilarity test (Fig. S7). The bacterioplankton community diversity in all samples was estimated by observed species, Chao index, Shannon index and Simpson index. Rarefaction curves showed no remarkable differences in community diversity between HC and LC, regardless of the time point (Fig. S5). In general, bacterioplankton community diversity in both HC and LC treatments followed the same trend, in that it peaked at day 10 and declined for the remainder of the experiment (Fig. S6).

Although the general trend of bacterioplankton community structure variation was similar in both the HC and LC treatments as described above, some groups of bacterioplankton showed different responses to elevated CO_2 at some time points. Notably, Bacteroidetes (predominantly Flavobacteria) had a higher average proportion in the HC treatment (52.2 % of Bacteroidetes and 52.2 % of Flavobacteria) than in the

LC treatment (25.2% Bacteroidetes and 24.8% Flavobacteria) at the early stage of the diatom bloom at day 10 (p=0.049 and 0.053 respectively). In contrast Proteobacteria, especially the Alphaproteobacteria, were observed to have lower proportion in the HC treatment (47.8 % of Proteobacteria and 27.2% of Alphaproteobacteria) than in the LC treatment (74.8 % of Proteobacteria and 44.6% of Alphaproteobacteria) at day 10 (p=0.049 and 0.019 respectively, Fig. 3). At a higher taxonomic level, Flavobacteriales demonstrated higher relative abundance in the HC treatment (52.2 %) compared to the LC treatment (24.8 %) at day 10 (p=0.053), while for Rhodobacterales the inverse pattern was observed (p=0.020). Moreover, Flavobacteriaceae were observed to have a relatively higher ratio in the HC treatment (50.3 %) compared to the LC treatment (24.0 %) at day 10 (p=0.053), whereas Rhodobacteraceae demonstrated the opposite pattern (p=0.021, Fig. 3). It is notable that Alteromonadales, belonging to the Gammaproteobacteria, had a higher ratio in the HC treatment compared to the LC treatment at day 19 and day 29, although this was not statistically significant (p=0.24 and 0.34 at day 19 and 29 respectively).

3.5 The effects of elevated CO_2 on bacterioplankton community interactions

Both HC and LC networks were dominated by Alphaproteobacteria, Gammaproteobacteria and Flavobacteria, suggesting their vital roles in maintaining stability of microbial ecosystems under both HC and LC conditions. The observation of more negative links compared to positive links indicates the dominant relationship among bacterioplankton is competitive rather than mutualistic under both the HC and LC treatments. The average connectivity and clustering coefficient of the network were higher in the HC treatment than in the LC treatment, while geodesic distance and modularity value was higher in the the LC treatment. Bacterioplankton formed more modules under the LC treatment, but were densely connected in less modules under the HC treatment (Table 1, Fig. 4). However, as shown in Fig. 4, the

links among the OTUs with high abundance, 558885 (Rhodobacteraceae), 572670 (Rhodobacteraceae), 190052 (Flavobacteriaceae), 107130 (Flavobacteriaceae) and 4331023 (Rhodobacteraceae), were positive in both HC and LC.

Interestingly, some nodes that were sparsely distributed in independent modules in the LC network formed dense modules with high connectivity in the HC network (Fig. 4). As the OTUs connected within a module, they could be considered as a putative bacterioplankton ecological niche (Zhou et al., 2010). It is plausible that elevated CO_2 disrupted the connection between different bacterioplankton community niches, but enhanced alternative connections among species within certain ecological niches. Within module connectivity (*Zi*) and among-module connectivity (*Pi*) indexes were used to identify key module members (Olesen et al., 2007, Fig. 5). In an ecological context, the peripherals may represent specialists, while module hubs and connectors may be considered more as intra-module and inter-module generalists respectively. Network hubs are usually considered as super-generalists (Deng et al., 2012). It is interesting that the numbers of connectors that are considered as generalists were reduced, whereas module hubs were increased under the HC treatment. However, two network hubs, the super-generalists that are more important than module hubs and connecters, were detected in the LC network but not in the HC network (Fig. 5).

4 Discussion

This study was designed to bridge the gap between lab cultures and field studies, with isolates of non-axenic phytoplankton being added to filtered natural waters. The lab conditions possibly have selected for a fast-growing bacterial community adapted to live with semi-continuous phytoplankton culture. Therefore, the inoculated bacterioplankton were likely preconditioned to lab conditions in semi-continuous phytoplankton cultures prior to the experiment. However, the bacterioplankton from

the natural environment gradually became dominant in the mesocosm bags from day 0 to day 4, based on the comparison of the community at day 4 and the original community in the phytoplankton cultures.

In this mesocosm experiment, significant variation in community structure was observed through the whole diatom bloom process, suggesting that the diatom bloom was a major driver for bacterioplankton community structure dynamics in both the HC and LC treatments. This finding is in line with previous mesocosm experiments and field observations (Allgaier et al., 2008, Teeling et al., 2012). Along with the phytoplankton bloom process, the inter-replicate variation of bacterioplankton community became more apparent, which was inevitable for an outdoor mesocosm experiment. For example, the bacterioplankton community in mesocosm bag 8 was dominated by *Phaeobacter*. *sp* at day 29, which was distinct from the other mesocosm bags. According to the phytoplankton data mesocosm bag 8 was probably contaminated with dinoflagellates at a late stage of the algal bloom, likely resulting in a different bacterioplankton community structure compared to the others.

Although effects of elevated CO_2 on bacterioplankton communities have been reported (Allgaier et al., 2008; Tanaka et al., 2008; Wang et al., 2016; Zhang et al., 2013; Ray et al., 2012; Roy et al., 2013; Baltar et al., 2015; reviewed in Hutchins and Fu, 2017), how marine bacteria communities react to the occurrence of elevated CO_2 in eutrophic seawater is still uncertain. This mesocosm study comprehensively investigated the effects of elevated CO_2 on bacterioplankton community structure and networks using Illumina sequencing and ecological network analysis in the context of eutrophication. Compared to the effects of the phytoplankton bloom, ocean acidification did not strongly influence the bacterioplankton community structure. The results indicate that bacterial abundance and community structure at different taxonomic levels were generally similar between the HC and LC treatments at the

different diatom bloom stages, in line with previous ocean acidification mesocosm bacterioplankton community studies (Tanaka et al., 2008; Wang et al., 2016; Zhang et al., 2013; Ray et al., 2012; Roy et al., 2013; Baltar et al., 2015). Differences in bacterioplankton community diversity between the HC and LC treatments were also not remarkable. These results suggest the possibility that the whole bacterioplankton community has a certain degree of resilience to elevated CO_2, which is consistent with a previous stated hypothesis (Joint et al., 2011).

It has previously been proposed that the observed insignificant effects of ocean acidification on coastal bacterioplankton may be due to their adaptation to strong natural variability in pH in coastal ecosystems, where amplitudes of >0.3 units from diel fluctuations and seasonal dynamics are commonly seen (Hofmann et al., 2011). The comparative ecological network analysis in this study to some extent explains the resilience of the bacterioplankton community to elevated CO_2 levels. According to the present study, substantial numbers of OTUs that were sparsely distributed in different and small modules in the LC network became connected with each other and formed fewer modules in the HC network, implying elevated CO_2 has the potential to reassemble the bacterioplankton community (Fig. 4). The positive relationship among these principal components were almost unaltered in the network analysis, suggesting that the positive relationships among them were robust in the face of CO_2 changes, thus contributing to whole community stability (Fig. 4). It has also been reported that sparsely distributed fungal species were reassembled into highly connected dense modules under long-term elevated CO_2 conditions (Tu et al., 2015).

It is noteworthy that the OTUs involved in possible community reassembly were not very abundant, whereas the relationship between the abundant OTUs was virtually unaltered by elevated CO_2 in this study. Although elevated CO_2 promoted the reassembly of the bacterioplankton community, the network

constructed by abundant OTUs which are usually considered as the foundation of the whole bacterioplankton community was still stable in response to elevated CO_2. This to some extent led to maintenance of bacterioplankton community structure under the ocean acidification stimuli in the context of eutrophic conditions. Additionally, these data indicate that more negative than positive relationships between OTUs were observed in both HC and LC treatments, which is consistent with a previous ocean acidification mesocosm study conducted in the Arctic Ocean (Wang et al., 2016). It was proposed that a community with more competitors would be more stable and yield less variation under environmental fluctuations (Gonzalez and Loreau, 2009). Therefore, it could be speculated that the dominant competitive relationship between bacterioplankton species in this mesocosm experiment helped the whole bacterioplankton community to adapt to pH perturbations, with less variation in total biomass and diversity.

Although the effects of elevated CO_2 on bacterioplankton community structure were not significant, the proportion of some groups of bacterioplankton varied between the HC and LC treatments in the early stages of the diatom bloom. Elevated CO_2 significantly increased the proportion of Flavobacteria (dominated by Flavobacteriales) in the HC treatment at day 10, when the diatoms cells began to grow rapidly. In contrast, the HC treatment had negative effects on the growth of Alphaproteobacteria compared to the LC treatment. The results reported here are in line with previous reports about the response of Flavobacteria to ocean acidification in biofilm and single species experiments (Witt et al., 2011; Teira et al., 2012). Flavobacteria are considered as the “first responders” to phytoplankton blooms because they specialize in attacking algal cells and further degrading biopolymers and organic matter derived from algal detrital particles (Kirchman, 2002; Teeling et al., 2012). Flavobacteria are especially good at converting high molecular weight (HMW) dissolved organic matter (DOM) to low molecular

weight (LMW) DOM using the highly efficient, extracellular, multi-protein complex TonB-dependent transporter (TBDT) system, based on previous in situ proteomics and metatranscriptomics data (Teeling et al., 2012). Higher abundance of Flavobacteria under elevated CO_2 means more HMW DOM could be degraded and so enter into the carbon cycle (Buchan et al., 2014). Based on the results reported here, it can be speculated that increased amounts of Flavobacteria under the elevated CO_2 treatment in eutrophic seawater could promote the TBDT system to break down HMW DOM and lead to improved efficiency of the Microbial Carbon Pump (MCP), and possibly further influence the carbon storage in the ocean (Jiao et al., 2010). It has also been postulated that the Flavobacteria-originated, light-driven proton pump proteorhodopsin could be involved in dealing with ocean acidification and pH perturbation (Fuhrman et al., 2008). Recent metatranscriptomic data further emphasize the role of proteorhodopsin in pH homeostasis in bacterioplankton under elevated CO_2 (Bunse et al., 2016; Gómez-Consarnau et al., 2007). The underlying mechanisms underlying the enhanced growth of Flavobacteria under elevated CO_2 need further investigation in the future.

Interestingly, Flavobacteria in our study showed higher abundance in the HC treatment in the early phytoplankton bloom stage. However, a negative relationship between CO_2 level and relative abundance of Bacteroidetes based on terminal restriction fragment length polymorphism (T-RFLP) method was observed in a mesocosm experiment conducted in the Arctic region with low nutrient levels (Roy et al., 2013). Moreover, the effects of elevated CO_2 on bacterioplankton community interaction webs in this study were not observed in previous mesocosm work in the Arctic Ocean (Wang et al., 2016; Roy et al., 2013). The results of the current study showed that the effects of elevated CO_2 in the context of eutrophication were different compared to elevated CO_2 on bacterioplankton community networks in a mesocosm study carried out in the oligotrophic Arctic Ocean. The data here and previously reported,

seemingly contradictory results highlight the importance of including the combined effects of ocean acidification and other anthropogenic perturbations to interpret and predict the impact of global change on marine life.

In this study, the majority of the particle-attached and algae-attached bacteria were filtered out by sequential filtering. Additionally, the archaea were not included in our data because we used the primers 341F/805R, which do not target archaea. Therefore, the community structure of particle-associated bacteria and all archaea were not investigated in our study. Furthermore, a simplified model phytoplankton community was used in this study, composed of the two diatom species *P. tricornutum* and *T. weissflogii* in both LC and HC treatments. It is possible that the similarity of the two bacterial communities in the two treatments was due to the similar composition and quality of DOM produced by these two diatoms. With a more diverse natural phytoplankton community experimental system, perhaps different phytoplankton taxa would have dominated in the HC and LC treatments, leading to different bacterial communities. In future studies, it would also be worthwhile to sample over a diel cycle in order to understand the cyclic variability in pH, and whether this affects short term changes in bacterioplankton community structure.

Conclusion

Elevated CO_2 was not a strong influence on bacterioplankton community structure compared to the diatom bloom process, based on 16S V3-V4 region Illumina sequencing. Based on ecological network analysis, elevated CO_2 appeared to reassemble the community network of taxa present with low abundance, but barely altered the network structure of the bacterioplankton taxa present with high abundance. It is this differential sensitivity of common and rare groups to carbonate chemistry changes that may largely explain the resilience of the bacterioplankton community to elevated CO_2.

Author contributions

Conceived and designed the experiments: K. Gao, X. Lin, M. Dai. Performed the experiments: R. Huang, X. Lin, Y. Wu, Y. Li and F. Li. Analysed data: R. Huang and X. Lin. Wrote the paper: X. Lin. Revised the paper: D. Hutchins and K. Gao. All authors reviewed the manuscript.

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Competing interests:

The authors declare no competing financial interests.

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Figure legends

Figure 1 Temporal variations of pCO_2 (a), pH_{NBS} (b) and Chl*a* (c) during the whole experiment. The pCO_2 was calculated from DIC and pH using the CO2SYS program. Data are the means ± SD, n=3.

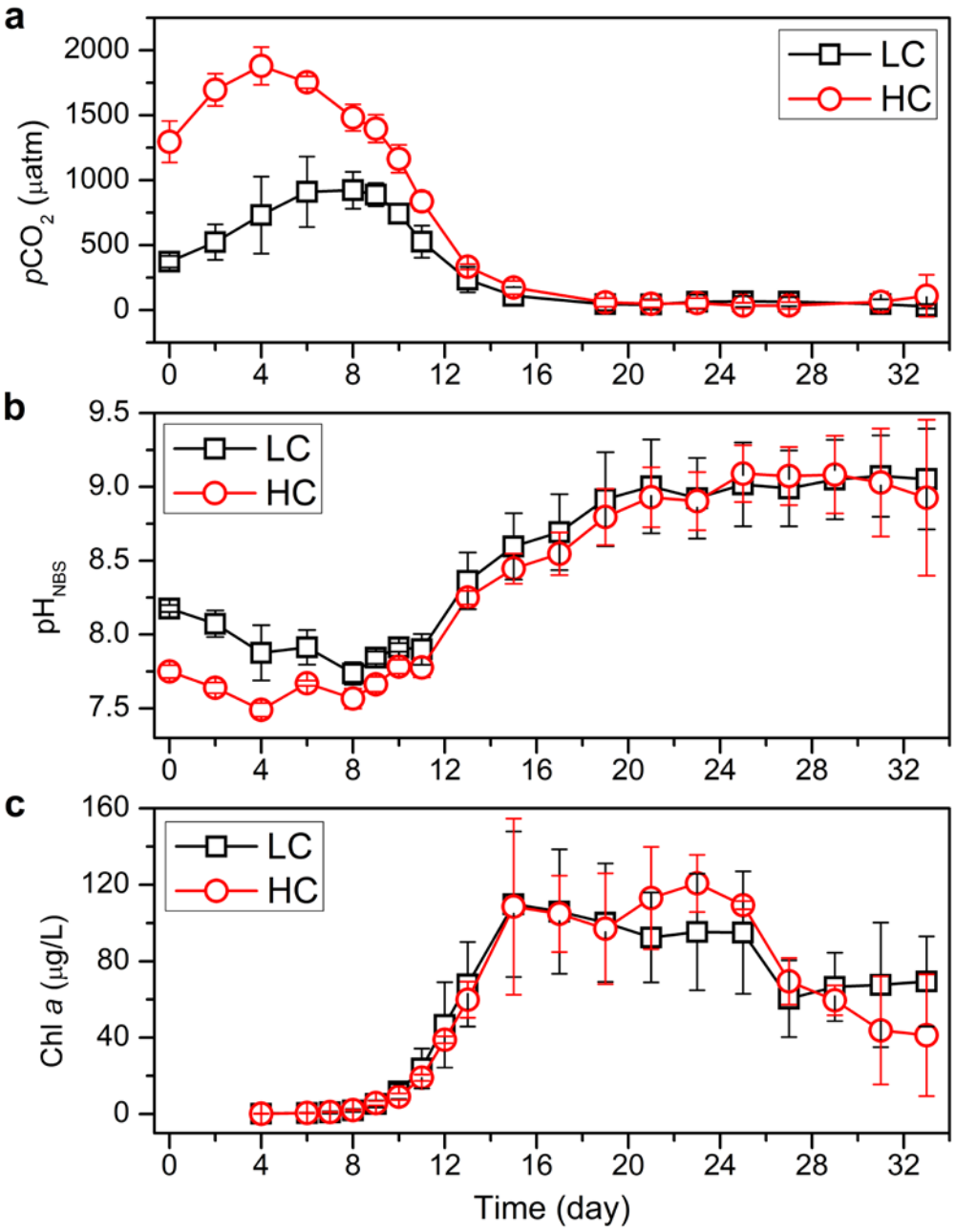
Figure 2 Bacterioplankton community structure overview at different taxonomic levels during days 4, 6, 8, 10, 13, 19 and 29 (#1, #6, #8) under LC and HC (#2, #4, #7). X-axis represents sample name (for example, D4.1 refers to bacterioplankton in mesocosm bag 1 collected at day 4) and the Y-axis represents relative abundance of different groups of bacterioplankton.

Figure 3 The relative abundance over time of primary taxa of the bacterioplankton community; HC in red and LC in black. Proteobacteria (a) and Bacteroidetes (b) are phylum level; Flavobacteria (c) and Alphabacteria (d) are class level; Flavobacteriales (e) and Rhodobacteriales (f) are order level; Flavobacteriaceae (g) and Rhodobacteraceae (h) are family level. Data are the means ± SD (n=3), and the asterisk represents a difference at $p < 0.05$.

Figure 4 Bacterioplankton network interactions under LC (a) and HC (b) conditions. Each node represents an OTU. Node colors demonstrate different taxon. Each line connects two OTUs. A blue line indicates a negative interaction between nodes, suggesting predation or competition, while a red line indicates a positive interaction suggesting mutualism or cooperation. OTUs with importance are marked with OTU identification numbers.

Figure 5 Sub-modules in ecological network analysis under LC (a) and HC (b) conditions. Each dot

represents an OTU. The *Z–P* plot shows OTU distribution based on their module-based topological role according to within-module (*Z*) and among-module (*P*) connectivity. The nodes were defined as module hubs with $Zi > 2.5$ and $Pi < 0.625$, which were more closely connected within the module, while the connectors were nodes with $Zi < 2.5$ and $Pi > 0.625$ were more closely connected to nodes in other modules. Network hubs are super-generalist with a $Zi > 2.5$ and $Pi > 0.625$. The other nodes were considered peripheral.



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2 **Figure 1**

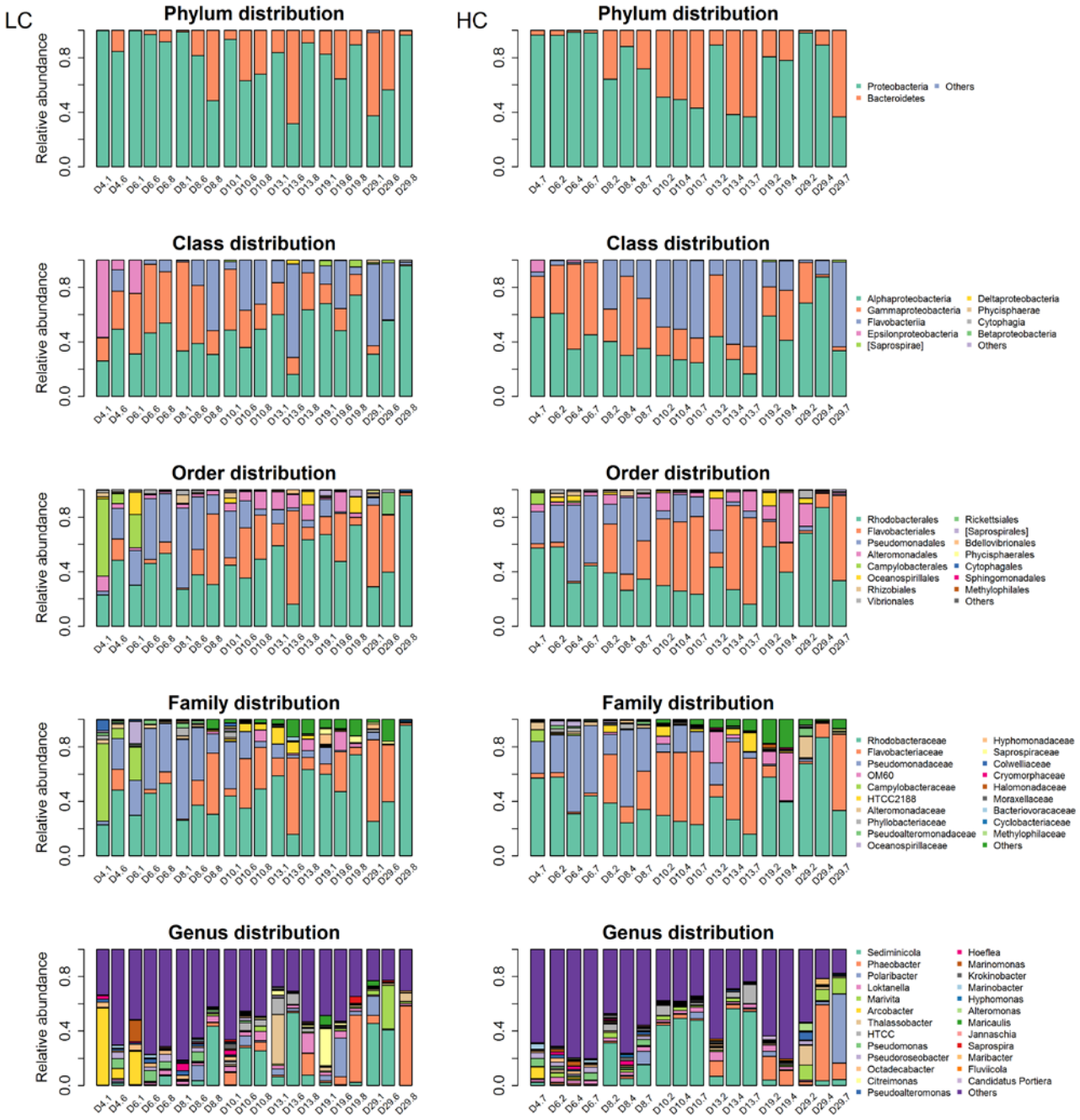
LC
HC
Phylum distribution
Relative abundance
Proteobacteria
Bacteroidetes
Others
Class distribution
Alphaproteobacteria
Gammaproteobacteria
Flavobacteriia
Epsilonproteobacteria
[Saprospirae]
Deltaproteobacteria
Phycisphaerae
Cytophagia
Betaproteobacteria
Others
Order distribution
Rhodobacterales
Flavobacteriales
Pseudomonadales
Alteromonadales
Campylobacterales
Oceanospirillales
Rhizobiales
Vibrionales
Rickettsiales
[Saprospirales]
Bdellovibrionales
Phycisphaerales
Cytophagales
Sphingomonadales
Methylophilales
Others
Family distribution
Rhodobacteraceae
Flavobacteriaceae
Pseudomonadaceae
OM60
Campylobacteraceae
HTCC2188
Alteromonadaceae
Phyllobacteriaceae
Pseudoalteromonadaceae
Oceanospirillaceae
Hyphomonadaceae
Saprospiraceae
Colwelliaceae
Cryomorphaceae
Halomonadaceae
Moraxellaceae
Bacteriovoracaceae
Cyclobacteriaceae
Methylophilaceae
Others
Genus distribution
Sediminicola
Phaeobacter
Polaribacter
Loktanella
Marivita
Arcobacter
Thalassobacter
HTCC
Pseudomonas
Pseudoroseobacter
Octadecabacter
Citreimonas
Pseudoalteromonas
Hoeflea
Marinomonas
Krokinobacter
Marinobacter
Hyphomonas
Alteromonas
Maricaulis
Jannaschia
Saprospira
Maribacter
Fluviicola
Candidatus Portiera
Others

Figure 2

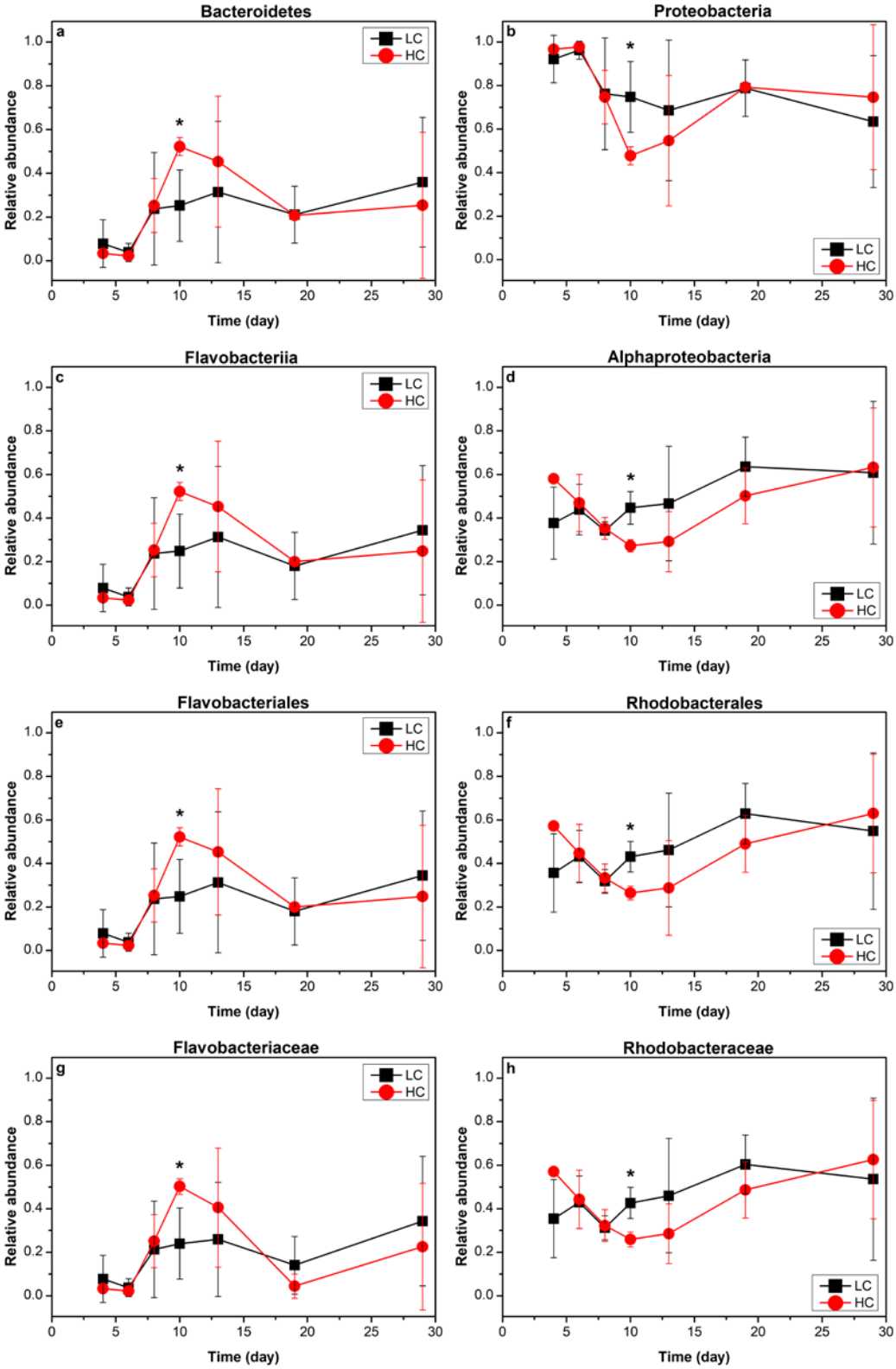
Bacteroidetes
a
Proteobacteria
b
Flavobacteriia
c
Alphaproteobacteria
d
Flavobacteriales
e
Rhodobacterales
f
Flavobacteriaceae
g
Rhodobacteraceae
h
LC
HC
Relative abundance
Time (day)
*

Figure 3

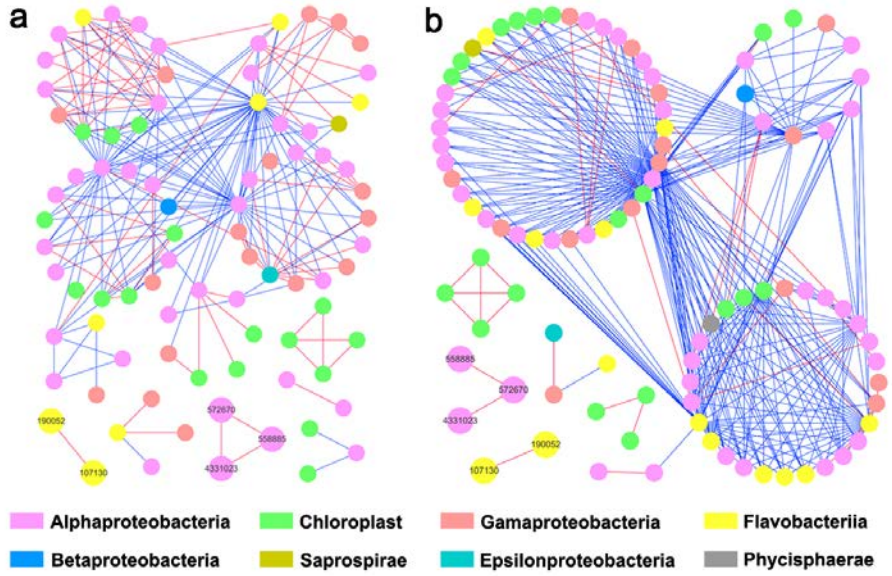
a
b
190052
572670
558885
107130
4331023
Alphaproteobacteria
Betaproteobacteria
Chloroplast
Saprospirae
Gamaproteobacteria
Epsilonproteobacteria
Flavobacteriia
Phycisphaerae

Figure 4

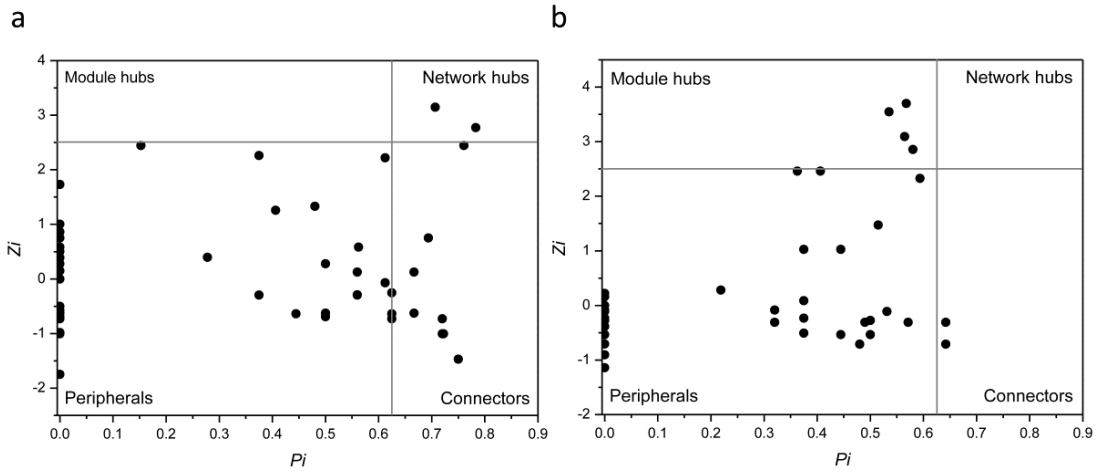

Figure 5

Table 1 Topological properties of the bacterioplankton communities as represented by molecular networks under HC and LC treatments; also their rewired random networks.

	Experimental network							**Random network**		
	Total nodes	**Total links**	**R2 of power-law**	**Average clustering coefficient (avgCC)**	**Average connectivity**	**Harmonic geodesic distance (HD)**	**Modularity**	**Average clustering coefficient (avgCC)**	**Harmonic geodesic distance (HD)**	**Modularity**
LC	85	209	0.817	0.402	0.625	3.397	0.414	0.424 +/- 0.023	2.187 +/- 0.049	0.249 +/- 0.010
HC	96	310	0.817	0.448	0.714	2.956	0.303	0.292 +/- 0.023	2.306 +/- 0.059	0.323 +/- 0.008

Table 2 Dissimilarity tests of bacterial communities under HC and LC treatment at various time points.

	Anosim		**MRPP**		**Adonis**	
Time	R	P-value	δ	P-value	R^2	P
day6	-0.111	0.602	0.3952	1	0.15447	1
day8	0.111	0.284	0.438	0.6	0.2	0.5
day10	0.037	0.613	0.4929	0.7	0.17829	0.7
day13	0.111	0.309	0.412	0.5	0.19714	0.5
day19	0	0.693	0.4336	0.3	0.28263	0.3
day29	-0.259	1	0.4513	0.9	0.15517	0.9