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Interactive comment on "Insignificant effects of elevated CO_2 on bacterioplankton community in a eutrophic coastal mesocosm experiment" *by* Xin Lin et al.

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The manuscript addresses the research question if bacterial communities in eutrophic coastal areas will be affected by elevated CO2 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 CO2 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.

Response: 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 CO2. 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 CO2 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 CO2. 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 (0.01um) 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.

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.

Response: 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 CO2 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 6-7). 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 CO2 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 CO2 with non-adaptive species outcompeted in semi-batch phytoplankton cultures prior to the experiment. A discussion or mentioning of this is missing.

Response: 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 17 Line 6-9).

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.

Response: 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 12).

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.

Response: 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 CO2 on BCC were not statistically tested prior to day 6 when CO2 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 CO2 on bacterioplankton community in a eutrophic coastal mesocosm experiment".

Response: 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 CO2 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?

Response: 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.

Repsonse: 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 pCO_2 was 650 uatm. How relevant are control mesocosms where the pCO_2 concentration is lowered? Despite it changing the carbon chemistry, seawater with 400 ïĄ■atm seems not to reflect the eutrophic coastal environment in the Wuyuan Bay during January and is therefore a questionable control to test the hypothesis.

Response: We agree that 400 uatm 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 uatm) and treatment (1000 uatm) was used for us to better observe the effects of elevated CO_2. So we suggest that choosing 400 uatm 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.

Response: 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 21-22).

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?

Response: 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.

Response: 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).

Response: 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 12-13).

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

Response: The detailed DNA extraction protocol: 1. Wash the filter with 1 ml of lysis

buffer described in (Francis et al., 2005) and 10 ul of lysozyme (100 mg/ml), vortex and incubate at 37 degrees for 30 minutes. 2. Add 5 ul RNase A (10 mg/ml), incubate at 37 degrees for 30 minutes. 3. Add 20 ul proteinase K 4. Add 220 ul 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.

Response: 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 14 Line 14-22).

Results page 10, line 11. Additional to pCO2 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.

Response: The pH value has been added in Figure 2 with pCO2 levels. The results section that contained passages of discussion has been moved to the discussion sec-

tion 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 Table 2.

Response: 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.

Response: 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.

Response: 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.

Response: 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. Because of this shift to natural bacteria, we think the results about the bacterioplankton community composi-

tion under the HC and LC conditions can be generalized, as on Page 15, line 17.

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.

Response: 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.

Response: 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.

Response: 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.

Response: 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 19-22).

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

Response: 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?

Response: 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 "Firstly, 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.

Response: The diatom BCC came from the sum of two 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?

Response: 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.

Response: 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)?

Response: 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.

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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