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Interactive comment on "Impacts of biogenic polyunsaturated aldehydes on metabolism and community composition of particle-attached bacteria in coastal hypoxia" *by* Zhengchao Wu et al.

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General comments

1. The manuscript is generally well written with proper language although some sentences need improvements. Results are well presented in the figures, but some complement of error bars are needed.

Response: Thanks to the reviewer for constructive comments. The manuscript has been proofread by a native English speaker to correct grammar errors and to improve

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the written language. We have redone these figures by including the error bars in the revised manuscript.

2. I lack a convincing motivation to the importance of PUAs compared to the multitude of other organic compounds, and the importance of particle-attached bacteria as compared to free-living. No direct comparison with other organic compounds or free-living bacteria is done in the study.

Response: The reviewer is right that there are other organic compounds that may also likely affect bacterial respiration, such as 2-n-pentyl-4-quinolinol (Long et al., 2003) and acylated homoserine lactones (Hmelo et al., 2011). Meanwhile, a perennial bloom of PUA-producing diatoms in the PRE mouth (Wu and Li, 2016) should argue for the importance of PUAs for microbial activity here compared to many other organic compounds. The reviewer is also right that free-living bacteria are important for community respiration in the ocean. However, our focus here is on the coastal transition zone, where particle-attached bacteria could be more important. In the revised manuscript, we have provided data of FLB respiration compared to the bulk bacterial community respiration in the hypoxic waters (FLB respiration account for only 25-30% of the total bacterial community).

3. A general importance of PUAs and particle attached bacteria should be tuned down in the discussion and conclusion. The effects of PUA on particle-attached bacteria is still of value as such.

Response: OK. We have rewritten the relevant sentences in the discussion and the conclusion sections as suggested by the reviewer.

4. A short-coming of the experimental design is a lack of true replication of the treatments.

Response: We actually have two replicates for each treatment. The original text was not well written. We have clarified this in the revised manuscript.

5. In addition, the fact that only one season has been investigated.

Response: The hypoxia could only occur during the summer in our study region. Therefore, a seasonality of hypoxia suggested by the reviewer may not be unnecessary.

6. I also miss proper measurement of the abundance, acidity and taxonomy of free-living bacterial to put the claimed influence of particle attached bacteria in perspective.

Response: We have added data of the size-fractionated bacterial respiration rates (for both free-living and particle-attached bacteria) in the hypoxic waters of station Y1 to the revised manuscript along with the bulk bacteria taxonomy data, although we do not have measurements for free-living bacteria abundance and taxonomy.

7. A similar argument for the lack of other organic compounds in the study.

Response: The reviewer is right about that there are other organic compounds that may also likely affect bacterial respiration. In the revised manuscript, we have compared PUAs with other organic compounds that would potentially affect bacterial activities in the hypoxia, such as 2-n-pentyl-4-quinolinol (PQ) and acylated homoserine lactones (AHLs). " A perennial bloom of PUA-producing diatoms in the PRE mouth (Wu and Li, 2016) should indicate the importance of PUAs for microbial activity here compared to many other organic compounds, such as 2-n-pentyl-4-quinolinol (Long et al., 2003) and acylated homoserine lactones (Hmelo et al., 2011).".

8. The conclusions must therefore be made more cautious, specific and these shortcomings commented on. Some speculative statements in the discussion and conclusion section need to be removed or rephrased.

Response: We agree with the reviewer on this. In the revised manuscript, we have rewritten the discussion and the conclusion sections.

9. There are parts of the method descriptions that need to be clarified, better specified or added. A major revision in this spirit is required to motivate publication.

Response: We have carefully addressed all these parts of descriptions concerning the methodology based on the reviewer's comments.

Detailed comments

10. r. 13-14 There is an extensive literature on eutrophication driven hypoxia in e.g. the Baltic Sea since 4 decades (cf. Cloern 2001). Please rephrase sentence accordingly

Response: Agree. In the revised manuscript, the sentence has been rewritten as " Eutrophication-driven coastal hypoxia is of great interest for decades".

11. r. 15. Do you mean ": : :water mainly dominated: : :".

Response: Agree.

12. r. 21 Please change "activity" to "..e.g. bacterial respiration and growth: : :) and revise the sentence.

Response: Done.

13. r. 35 Change to "..deoxygenation is also tightly: : :"

Response: Done.

14. r. 43-45 In most aquatic environments free living bacteria are dominating in numbers as well as biomass (e.g. Kirchman 2008). The reference is not convincingly showing that particle-associated bacterial dominate in terms of abundance or how the growth of particle associated bacteria was measured. Please revise the message. This also question the focus on particle associated bacteria in the manuscript.

Response: We agree with the reviewer that free-living bacteria are most dominant in many parts of the ocean. Meanwhile, our focus is on the high turbid coastal transition zone where particle-attached bacteria can be relatively more important. We have added a new reference of Lee et al (2015) to this sentence to show the dominance of PAB in some coastal regions. Lee, S., Lee, C., Bong, C., Narayanan, K., Sim, E.:

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The dynamics of attached and free-living bacterial population in tropical coastal waters, Mar. Freshwater Res., 66, 701-710, 2015.

15. r. 49-51 In many cases free-living bacteria dominate the respiration (Robinson and leB Williams 2005). Both types of bacteria is preferably studied. This question the general relevance of the study.

Response: The reviewer is right about that free-living bacteria are important for community respiration in the open ocean. Meanwhile, what we focused on is the coastal transition zone, where particle-attached bacteria could be more important. Our field data indeed suggested that FLB respiration accounts for only 25-30% of the bulk bacterial community respiration in the hypoxic waters at station Y1. Nevertheless, we have rewritten these sentences to emphasize more on the general relevance of our study for both types of bacteria.

16. r. 64-65. However, many other organic compounds may drive the bacterial respiration. Please provide some reference showing to that extent PUF is contributing to bacterial respiration.

Response: We agree with the reviewer on this point. References for PUA contribution to bacterial metabolisms have been provided in the revised manuscript. “The strong effect of PUAs on bacterial growth, production, and respiration has been well demonstrated in the laboratory studies (Ribalet et al., 2008) and the field studies (Balestra et al., 2011; Edwards et al., 2015).”

17. r.68-76 I would prefer more explicit research questions to be addressed in this paragraph for clarity and coupling to performed experiments.

Response: Ok. We have rewritten this paragraph.

18. r. 84-85 Please define here what depths that were used for middle and bottom water categories (e.g. figure 6).

Response: The middle layer was at 12 m with the bottom layer at 25 m (4m above the

seafloor) for station Y1 (Figure 6). We have clarified this in the revised manuscript.

19. r. 87-88 Please define the abbreviations pPUA and dPUA.

Response: Agree. The pPUAs and dPUAs have been defined in the revised manuscript.

20. r. 97 Filtration and freezing of nutrient samples may release nutrients from broken cells.

Response: The influences of filtration and freezing/thaw on nutrient concentration should be negligible due to high nutrient concentration in the coastal system. They will only affect the oligotrophic open ocean waters with nanomolar nutrients (Li QP and Hansell DA, Anal Chim Acta, 611, 68-72, 2008).

21. r.124-129 How is the centrifugation and resuspension of particles influencing their morphology, attachments of PUAs and PABs?

Response: We believe that particle morphology and the attachments of PUAs and PABs will not be influenced by low-speed centrifugation (3000 rpm for one minute) or by a gently shaking for resuspension. The same approaches have been used to study particle-attached bacteria and particle-related compounds on sinking particles (Hmelo et al., 2011)

22. r. 131-132 Pleas provide the recovery efficiency of particle attached PUAs after the preparation procedure described.

Response: The recovery for the particle-adsorbed PUA should be 100% as the supernatants after centrifugations have all been added back to the final 50 ml centrifuge tube. We have clarified this in the revised manuscript.

23. r. 136-137. Please provide a reference where the method is validated.

Response: Done. References have been added to the revised manuscript. The protocol is modified from those of Edwards et al. (2015) and Wu and Li (2016).

24. r. 146-147 Freeze thawing may relate PUAs from living cells also.

Response: The reviewer is right that free-thawing would release PUAs from living cells. What we actually mean in the text is that we use the same determination method for undisrupted and disrupted PUAs although they are pre-treated differently (one with direct extraction method and the other with the freeze-thaw method). Anyway, we have clarified this in the revised manuscript.

25. r. 152 Should it be nmol per some volume or particle unit?

Response: Agree. It is the concentration of the undisturbed PUAs in the 50 mL sampling tube. We have clarified this in the revised manuscript.

26. r.160 Please specify what is meant by clean. What was the washing procedure?

Response: It means sterile. We have rewritten this in the revised manuscript. Generally, these bottles have been soaked in 10% HCl for 24 h, rinsed with deionized water for several times, and sterilized before use.

27. r.162 As presented here there was no true replication of the treatments?

Response: We actually have two replicates for each treatment. The sample in the 1-L Nalgene bottle had been transferred to two 0.5 L bottles for each treatment. We have clarified this in the revised manuscript.

28. r. 167 How does methanol included in the procedure affect bacterial abundance and activity? Any control or test for this? Please comment on relevance for natural conditions.

Response: The methanol has been added as a cosolvent for PUA (Franze et al., 2018). The methanol concentration of 0.05% in our experiment should not have a large effect on bacteria, since the previous study suggested that bacterial strains would not be significantly affected by methanol at a level below 1% (Patterson and Ricke, 2015). Patterson J.A., and S.C. Ricke, S.C., (2015) Effect of ethanol and methanol on growth

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of ruminal bacteria Selenomonas ruminantium and Butyrivibrio fibrisolvens, Journal of Environmental Science and Health, Part B, 50:1, 62-67.

29. r. 176-179 Give some information on how close to natural conditions these final concentration of PUAs are.

Response: This PUA level was close to the hotspot PUAs concentration of 240 ïAŲmol L−1 found in a station near the PRE and was also comparable to the hotspot concentration of ∼60 ïAŲmol L−1 found in the western and subarctic North Atlantic (Edwards et al., 2015). We should emphasize that the concentration of PUA in the water-column is inhomogeneous due to the presence of particles. The hotspot concentration of PUA associated with these particles should be the PUA concentration in the volume of the water parcel taken up by the aggregation particles.

30. r.180 it is not obvious that turbidity will be detected if cells remain below about 109 cells cm3. Were the cell concentration measured by direct microscopy?

Response: We did not measure cell concentration during the experiments. The experiment is designed to only qualitatively assess the PAB response to different types of carbon sources. The culture duration of over 30 days should be long enough for significant bacterial growth (say with cell concentration well exceed the detection limit) to show up if the organic substrate could be used as a carbon source (Dong et al., 2015).

31. r. 181 Please provide a description on incubation conditions and length.

Response: Done. These experiments were performed in dark at room temperature for over 30 days. We have clarified this in the revised manuscript.

32. r. 185-196 The description of methodology is unclear. Please make clear if and how free-living bacterial abundance was measured? How is the methanol treatment accounting for bacteria from breaking particles? In addition, please provide a reference validating this method. Give some measure of the precision of the flow cytometer analysis and a relevant reference.

Response: We did not measure the free-living bacteria abundance. We only measure the abundance of the bulk water bacteria (>0.2 ïA■m) that includes both FLB and PAB. The method for bulk-water bacterial abundance has been added to the revised manuscript. We have also provided a reference for the flow cytometry method (Marie et al., 1997) as well as the relevant precision (CV%). The original text about methanol treatment was not well written, we have rewritten the sentence as “To break up particles and attached bacteria, 0.2 mL pure methanol was added to the 2 mL sample and vortexed”.

Marie, D., Partensky, F., Jacquet, S. and Vaulot, D.: Enumeration and cell cycle analysis of natural populations of marine picoplankton by flow cytometry using the nucleic acid stain SYBR Green I, Appl. Environ. Microbiol. 63, 186-193, 1997

33. r.198-202 As no relevant pre-filtration is used you may include other organisms than bacteria in the respiration estimate. Please clarify and rephrase as needed.

Response: The reviewer is right about this. We did not perform any pre-filtration at this step. Besides the PAB, the particle aggregates of >25 μm would likely be consist of some phytoplankton and microplankton. So, the BR could be somewhat overestimated in our experiment. Nevertheless, this effect could be relatively small, given that bacterial respiration has been generally considered as the major contribution for community respiration. In addition, our particle samples were collected from the subsurface layer, which had negligible phytoplankters (as indicated by very low chlorophyll-a) and thus less zooplankton compared to the surface layer.

34. r. 208-209 What was the final TCA concentration in the sample. This does not follow the common procedure. Neither use of ethanol.

Response: Done. The final TCA concentration is 5%. The procedure of TCA step, as well as the use of ethanol, is based on the previous publication (Huang et al, 2018, doi: 10.1016/j.scitotenv.2018.03.222). We have clarified this in the revised manuscript.

35. r. 241 However, the lack of true replicates for the treatment (i.e. replicate 1-L Nalgene bottles per treatment) question a reliable result from the t-test.

Response: As we have responded to the previous point of this reviewer on No. 27, we have two replicates for each treatment. The sample in the 1-L Nalgene bottle had been transferred to two 0.5 L bottles for each treatment.

36. r. 251, Figure1. Consider to reverse the colour palette. More logical to have blue for well oxygenated and red for hypoxia. Change “constant” to “similar”.

Response: Done. The figure has been revised as suggested by the reviewer. The word “constant” has been replaced by “similar” as well.

37. r. 261-263. Pleas provide a statistical test for the claimed difference and confidence intervals (error bars) in figure 4.

Response: Agree. In the revised manuscript, we have added statistical information to the difference for BR and BP. Error bars have also been provided in the revised figure.

38. r.265. Proved statistical results for the claimed difference between bacterial phyla. Do the same for other differences claimed throughout the manuscript.

Response: It is a typo. A statistical test is not doable for comparing different bacterial compositions. In the revised manuscript, we have corrected the sentence as “. . . was substantially different from those of X2 and X3”. In addition, statistical information has been checked for each comparison throughout the manuscript.

39. r. 273 Should it be Bacteroidetes also here?

Response: Yes. We have revised as 4% of Bacteroidetes.

39-2. Figure 5. Please present the type of error bars used. Same for all figures with error bars.

Response: Error bars are the standard deviations. We have clarified these in the figure

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legends of the revised manuscript.

40. r. 320-329 What can be considered significant differences as opposed to random variation in this analysis. Please motivate convincingly.

Response: Statistical information (t-value, n, and p-value) for comparing ïĄğ-pro percentage between control and treatments have been added to the revised manuscript. However, a statistical test is not doable for comparing the difference of the bacterial community compositions. We have replaced the word “significant” with “substantial” in the revised manuscript.

41. r. 335 One month is an extremely long incubation. How relevant is this for the application to the natural environment?

Response: Bacterial utilization of organic carbons may depend on the nature of the organic compound. Bacteria may need a longer period to utilize refractory organic matters (ALK and PAH). On the other hand, our experiment goal is to qualitatively assess the possibility of PUA as a carbon source for PAB growth. The color change can be more easily appreciated after one-month for both PAH and ALK and thus allow us to compare the bacterial responses to different organic compounds (PUA, PAH, and ALK). There was no bacterial growth in the PUA medium throughout the one month should provide strong evidence that PUA was not used as a carbon source.

42. r. 360 Would be more informative to use a unit per particle or mass of particles? Litre of particles is unclear.

Response: The concentration of PUA in the water-column is inhomogeneous due to the presence of particles. The hotspot concentration of PUA should be the PUA concentration in the volume of the water parcel taken up by the aggregation particles. Therefore, particle volume is more informative and allows a better comparison of the hotspot concentration with the bulk water concentration.

43. r. 365 This assumes that PUA is a major substrate among all other organic com-

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pounds. Please provide some references on this matter and discuss it critically.

Response: We should emphasize that our focus here is to explore its role as a signal substance for PAB metabolism rather than as an organic carbon substrate for PAB growth. Actually, PUA accounts for only a small part of the particulate organic carbon (1-16%, Edwards et al., 2015). The specific arrangement of two double bonds and carbonyl chain makes PUA not a group of labile organic carbon for bacterial utilization. Anyway, we have rewritten the sentence to clarify this in the revised manuscript.

44. r. 368 This should be compared with the biomass of free living bacteria. They may also be elevated in the hypoxic water. I find the lack of measurement of free-living bacteria a short coming in the context of claiming importance of PABs.

Response: The reviewer is right about that free-living bacteria (FLB) may also be elevated in the hypoxic water. Our field data suggested that FLB respiration accounts for only 25-30% of the bulk bacterial community respiration in the hypoxic waters. We have added the bacterial respiration data of FLB (Figure S1) and the data of the community composition of bulk bacteria (Figure S2) to the revised manuscript.

45. r. 372-373. How is respiration by particle-attached bacteria distinguished from protozoa, phytoplankton and larger zooplankton? This is typically difficult to achieve. Comment in a critical manner.

Response: We cannot distinguish BR from respiration by phytoplankton and microzooplankton (large-zooplankton has been picked off already). Nevertheless, this effect could be relatively small given that bacterial respiration has been generally considered as the major contributor for community respiration (Robinson and Williams, 2005). In addition, our particle samples were collected from the subsurface layer, which had negligible phytoplankters (as indicated by very low chlorophyll-a) and thus less zooplankton compared to the surface layer.

46. r. 376-390. Given the apparent lack of true replication of the treatments (i.e.

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replicate 1 L Nalgene bottles) the conclusions regarding treatment effects is highly uncertain. This needs a discussion.

Response: As we have responded to the previous point of this reviewer on No. 27, we have two replicates for each treatment. We have clarified this in the revised manuscript.

47. r. 389-390 Relevance in the natural environment assumes that the applied concentrations are relevant for those occurring in the natural environment. Please consider, discuss and modify the conclusion accordingly.

Response: We should emphasize that the concentration of PUA in the water-column is inhomogeneous due to the presence of particles. The micromolar level of PUA for incubation was chosen to represent the actual hotspot concentration of PUA (the PUA concentration in the volume of the water parcel taken up by the aggregation particles) not the mean PUA concentration (nanomolar level) in the bulk water. Anyway, we have clarified this and discussed them properly in the revised manuscript.

48. r. 391-392 I find it valuable to know if PUA stimulates bacterial activity whether as an organic substrate of metabolic signal substance. Please explain why only the latter would be ecologically important.

Response: We should note that PUA accounts only for a small percentage of the organic carbon (<16%, Edwards et al., 2015). Also, PUA can be toxic to some bacteria precluding its use as a carbon source. In addition, the specific arrangement of two double bonds and carbonyl chain make PUA not a group of labile organic carbon for bacterial utilization. Therefore, it has less ecological importance as a carbon substrate.

49. r. 392-394 Please use the same concentration unit for comparability of levels. Heptadienal alone used for the test may not be comparable to a mixture of different PUAs (i.e. concentration more than twice used in the combined concentration). Why was not the same mixture used for this experiment? Other methods like using labelled PUA and analyse for metabolism of those would better test the mechanism of PUA

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effect.

Response: Agree. We have changed the unit to 200 ïĄmol/L in the revised manuscript. One reason for using heptadienal alone (C7) in the experiment is its lower toxicity compared to the other two (C8 and C10). Thus, C7 may be more likely used by bacteria if it can serve as a carbon source. Also, C7 is generally the dominant PUA over the large area of our study regions (Wu and Li 2016). Therefore, we focus on C7 alone rather than the mixture of various PUAs to qualitatively assess the bioavailability of PUAs to bacteria.

50. r. 405-407 Please consider that a few species within the Alteromonas phylum may be responsible for the observed response. PUA metabolism might not be a function attributed to the whole phyla. Rephrase the discussion accordingly.

Response: We agree with the reviewer that various bacterial species within the genus Altermonas may respond differently to the PUA treatments. We have revised the sentence as " . . . Our result was well consistent with the previous finding of the significant promotion effect of 13 or 106 ïĄmolL-1 PUAs on Alteromonas hispanica from the pure culture experiment (Ribalet et al., 2008). An increase of PUAs would thus confer some of the ïĄğ–Pro (mainly special species within the genus Alteromonas, such as A. hispanica, Figure S2) a competitive advantage over other bacteria . . ."

51. r. 411-412 Please refer to what figure and test that show a difference between particle-attached and bulk bacteria.

Response: Ok. A figure has been provided in the supplement material to compare the community difference between particle-attached bacteria and the bulk bacteria (Figure S2A).

52. r. 427 How strongly are PUAs adsorbed to particles (i.e. chemical bonding)? How may this influence their potential to act as signalling molecules?

Response: It is still not well known about the mechanisms for PUA adsorption on par-

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ticles. PUA may form a robust microzone around the particle, which would persist in the boundary layer and remain stable for some time (Juttner 2005). Juttner, F. (2005) Evidence that Polyunsaturated Aldehydes of Diatoms are Repellents for Pelagic Crustacean Grazers, Aquatic Ecology. 39, 271-282.

53. r. 439-441 I have not seen any analyses of the lipoxygenase hypothesis in the study? It is thus speculative and should be removed. Focus on conclusion that can be derived from the performed study.

Response: Agree. The related sentences have been removed in the revised manuscript.

54. r. 442-445 As there was no true replicates this conclusion should be made more cautious.

Response: As we have mentioned in our response to the previous point of this reviewer in No. 27, we did have two replicates for each treatment although more replicates are limited by the labor intensity of the experiment.

55. r.455-460 If this section should remain it need to be moved to the discussion section.

Response: Agree. The related sentences have been moved to the last part of the discussion section in the revised manuscript.

56. r. 460-464 The sudden appearance of PUFA is not connected to the previous sentence?. Again, this part is highly speculative and not part of conclusion from the study. Remove or move parts to the discussion.

Response: Agree. The sentences have been moved to the discussion section in the revised manuscript. To avoid disconnection between them, we have revised the sentences as “Eutrophication results in intense algae bloom with phytoplankton carbon sedimentation and accumulation in the coastal sediment, including PUFA compounds derived from the lipid production. Oxidation of these PUFA-rich organic particles during

. . .”

57. Literature cited Cloern, J. E. 2001. Our evolving conceptual model of the coastal eutrophication problem. Mar. Ecol.-Prog. Ser. 210: 223-253, doi.

Robinson, C., and P. J. Le B Williams. 2005. Respiration and its measurement in surface marine waters, p. 147-180. In P. A. del Giorgio and P. J. Williams, le B [eds.], Respiration in aquatic ecosystems. Oxford University Press.

Response: Agree. The mentioned references have been cited in the revised manuscript.

Please also note the supplement to this comment:
https://bg.copernicus.org/preprints/bg-2020-243/bg-2020-243-AC1-supplement.zip

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