Concepción-Chile, February 27, 2024

Co-editors-in-chief
Biogeosciences Journal
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Dear Editors and reviewers,

We sincerely appreciate the time and effort that you and the reviewer have dedicated to evaluating the manuscript, titled "Picoplanktonic methane production in eutrophic surface waters", submitted for consideration in Biogeosciences.

We have received your response with some minor suggestions, which contribute to improve the quality of our work.

We have thoroughly reviewed and addressed each comment, implementing necessary adjustments throughout the manuscript. Some modifications were made, particularly in the Results and Discussions sections. While we appreciate the reviewer's suggestion for a more in-depth examination of the data, our responses to these comments are provided in the dedicated response section.

We express our gratitude for the valuable suggestions and efforts in enhancing the quality of our manuscript.

The following is a **list of changes** that were made in response to the reviewer's valuable comments:

1. **Material and methods**
- The transport of seawater for the experiments was described (2.6.1. dissolved methane).
- In the data analysis part, we have added the conversion of CH_4 molar fraction to dissolved concentration.
2. **Results and discussion**
- The size of the figure 3 has been improved.
- The net CH_4 production rates in the aggregated vial experiments with methylated and microcosm substrates were described.
3. **References**
- We have added a couple of references

Supplementary material
- The size of the figure S4 has been improved.
- Unit was added to the net CH_4 rate to tables S2 and S4.

The following section provides a detailed account of the responses to each comment, observation, recommendation, and suggestion made by the reviewer.

General comments from referee 1

1. Thank you for considering the comments I provided during the first round of revision. As a result, the paper has improved in clarity. In some cases, you took the time to explain some aspects that were not clear, in the response letter, yet you did not incorporate these changes in the text. Please consider that whatever was not clear to me, is also not clear to the readers.
   Answer: Thank you for your valuable feedback and for recognizing the improvements made to the manuscript since the first round of review. We appreciate your efforts in helping us enhance the clarity of our work.
   We acknowledge your concern regarding instances where changes discussed in the response letter were not fully incorporated into the text. We understand the importance of ensuring that all revisions are reflected in the manuscript to enhance clarity for readers. In response, we will diligently address each point you have highlighted and ensure that these changes are properly integrated into the text. We apologize for any oversights in this regard and are committed to rectifying them.
   We understand that reconciling conflicting reviewer comments can be challenging, and we appreciate your understanding in this matter. We will carefully review each comment and make the necessary adjustments to ensure that the manuscript meets the highest standards of clarity and coherence.
   If there are any specific areas from the first round that you believe still require attention, please do not hesitate to inform us. We are committed to addressing any outstanding issues to ensure the quality of the manuscript.

2. While reading the revised version, I made several more comments that should be addressed. In some cases, these are merely technical as minor word changes or increasing font sizes in figures, specifically Fig, 3 and S4 (but please inspect also other figures).
   Answer: Thank you for your thorough review of our manuscript. We appreciate your suggestion to improve the font size of all figures, and we will promptly implement this change.

3. As mentioned also in the previous round of comments, many of the patterns you describe are derived from changes between two time points. If these patterns had covered 3 or more time points they would have been more convincing. It is clear that you cannot redo the experiments at this point. Therefore, I suggest discussing this transparently.
   Answer: In our manuscript, we conducted two types of experiments: short-term experiments lasting 24 hours, aimed at exploring daily responses, and long-term experiments lasting over 60 hours, which allowed for a more comprehensive analysis of methane cycling dynamics.
   For the short-term experiments, we collected at least 7 measurements, including their respective replicates (standard deviations), for all variables, with a particular focus on CH_4. These results are presented in Table S2, where we highlight the recycling rates for the entire dataset and separately for light and dark periods. In the text, we have emphasized the patterns that caught our attention and may require further investigation by future researchers to provide more accurate explanations for the observed variability.
   The long-term experiments also involved a significant number of measurements and focused on assessing the effects of organic compound additions or picoplankton concentration on CH_4 accumulation/consumption patterns. The calculation of the cycling rate is provided in Table S4.

Based on your feedback, we have enhanced the discussion surrounding these findings. We have incorporated additional information, particularly from Tables S2 and S4, to provide a more comprehensive understanding of the results and further support our main findings.

4. I also suggested that when discussing and analyzing the patterns in Figs. 6 and 7 you normalize all data to the T0 of each treatment and each community type and represent the data as relative changes. I.e. T0 is 1 an increase would be 1.1, 1.5 etc whereas a decrease would be 0.x.
We have gone through the exercise of normalizing the data, the details are in the specific answers below, but we do not believe it is appropriate to normalize the data for concentrations since all the results are on the same scale.

5. Please see my detailed comments and suggestions on the annotated PDF of the revised manuscript.
We have reviewed the comments in the pdf.

6. A few comments on the supplementary material:

6.1 Caption of Fig. S3 change "and at surface in the B." to "and at surface in panel B."
We have changed that words.

6.2 Same caption, change "* represents a significant correlation of 0.05" to "* represents a significant correlation below or equal to 0.05" - assuming that you had indeed values below 0.05.
This has been corrected.

6.3 Table S1, and S3 - OD in µM should likely be DO - dissolved oxygen rather than optical density (OD). Please correct and change wherever this reoccurs.
Thank you for observing this inconsistency, indeed, DO is about dissolved oxygen. We have corrected it.

Specific comments from referee 1

Line 121, comment: rephrase: the organic methylated substrates Mpn and TMA.
Answer (line 121 line): this was modified.

Lines 123 to 124, comment: Remove this as this is an inaccurate assumption. Transportation in the dark only removes the activity of phototrophs. Unless you specify what is controlled temperature and how much it differs than ambient temperature, than also this part is not very informative. Last, it is not stated how much time were the samples in transport prior to further analysis.
Please try to provide explicit information as much as possible. Obviously what was done cannot be changed now, so for the sake of transparency just report it.
Answer (lines 122 to 130 lines): in the manuscript, we have provided additional information regarding the mode of transportation. However, we want to emphasize that the transportation of seawater prior to the onset of the experiments was conducted with the utmost care. The marine biology station is near to the sampling station, and we took great care in handling the samples. The longest procedure time involved the picoplankton concentration.

Line 139, comment: is this: Optical density, Oxygen demand, or a typo for dissolved oxygen?
Answer (144 line): DO is dissolved oxygen.

Line 293, comment: Please increase the font size of the text in the figure - axes and scale bar.
Answer (302): the font size of the figure is improved.

Line 360, comment: You explained this second SL in the response but not here. The reader has no idea what does this mean.
Answer (263 and 369 lines): We have updated this table from its previous version by removing the concentration of Chl-a, which was originally presented as ug L^{-1} (discrete unit) and in inventories. Instead, we have focused solely on inventories, integrating data by depth ranges. The term 'surface layer' (SL) is consistently used in the text, figure legend, and materials and methods section.

Line 424, comment: comprise of
Answer (435 line): this was corrected.

Line 457, comment: 1) Correct Arquea to Archaea. 2) I suggest adding here also Candidatus pelagibacter sp. see this paper https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5901744/
This would make a lot of sense in your case as you say that the water is highly dominated by Bacteria as compared to Archaea.
Answer (468 to 469 lines): 1. the word "Archaea" was corrected. 2. we have added *Candidatus pelagibacter spp*.

Line 461, comment: upwelling regions
Answer (473 line): the word regions have been added.

Line 475, comment: I am sorry - but the data for the MPn amended treatment in panel A does not seem reliable. Comparing to Panel B where we see a gradual increase in the dark period between 16 and 24 h from 5 nM to 20 nM, in panel A this increase occurs over 1 h and is followed by a decrease. You explanation "We hypothesize that in dark conditions, microorganisms might become more efficient at metabolizing dissolved organic carbon " does not make sense. If the increase was caused by dark it would be instantaneous. The current line is drawn between the last point in the light and the first point at the beginning of the dark period. Also if the efficiency is higher in the dark why was no additional MPn demthylated?
Since at this point there is no way that I can think off to support either options, i.e. true result or methodological artifact/error, I suggest mentioned the option that one cannot exclude that these values were offset by some error and the real increase was more gradual towards the second time point.
Answer (480 to 489 lines): In reference to Figure 5, depicting the short-term experiments, notable differences in budgets between March (summer) and May (fall) are evident. Particularly in Panel A, the observed jump is quite surprising but the values at time: 5 6 7 are really high compared to the light period but eliminating that value does not change the pattern.
While we acknowledge the possibility of a methodological error, we find it challenging to reconcile the notion that, if all experiments were conducted uniformly, contamination occurring after 12 hours seems unlikely. Our skepticism arises from the fact that each data point represents the outcome of three different vials, initially filled (108 in total), randomly selected for sorting, and then discarded after each use (across 3 replicates). Another potential factor could be a sensitivity issue with the Gas Chromatograph (GC). However, it is important to note that the samples were consistently analyzed using a standard, with one standard read every 12 vials in this case.
The variance in behaviour patterns between both periods could be is attributed to the distinct microbial compositions in Phase II and III and the different DOC supply among phases, as is discussed in the ms.
To enhance clarity, we have redrafted the discussion, delineating between the different periods and removing sentences that do not significantly contribute to our understanding. We have also taken into consideration the possibility of experimental errors.

Line 481, comment: change this to a different end product than CH_4.
Answer (497 line): It is correct. During the metabolization of TMA, other compounds such as ammonium, di- and mono-methylamines, can be formed.

Line 491, comment: 1) When you use the term Candidatus only this appears in italics and the genus and species name appear in normal font. 2) Pelagibacter ubique was deemed an incorrect name and changes to Candidatus Pelagibacter communis. (https://lpsn.dsmz.de/species/pelagibacter-communis)I suggest using the genus in general Candidatus Pelagibacter spp. (https://lpsn.dsmz.de/genus/pelagibacter).
Answer (508 line): we were unaware of the name change. This has been modified.

Lines 519 to 520, comment: When the treatments were compared, did you use the absolute numbers or did you use the relative changes from T0.
The latter would be the correct, option as to eliminate any influence of effect of the absolute concentration of the analysis of patterns.
In all experiments there is an increase in CH4 between 40 and 46h regardless of treatment. What would be the explanation of this common pattern?
Answer (517 to 519 lines and 537 to 539): Absolute values were utilized, i.e. the concentrations at each time of incubation.
Standardization is particularly valuable when comparing and evaluating variables with diverse units of measurement and scales. In our case, concerning data concentrations over incubations times, all experiments (treatments) were conducted on the same time scale (collected simultaneously), and the CH_4 concentration in no treatment falls outside an expected range of elevation or consumption. Anyway, we did the exercise for the April data. As depicted in the figure (Standard April), the CH_4 production patterns of the different treatments are the same as in Fig. 6 of the manuscript and the statistics analysis show significant differences between treatments (p=0.02). So, we will stick to the absolute data analysis (concentrations) and to reinforce our results we have added the rates net from 4S table.
Regarding the jump in concentrations observed at the 40 - 46 hours may be due a change in the internal pressure of the microcosm, as explained in the methodology (Fig. 1), all microcosms are were recirculated with the same zero air. At the time of the change of hoses to measure the CH_4 concentration in the spectrometer, there may have been a change in pressure, but we are not sure about them since the pattern is persistent in all microcosms (treatment). In addition, this pattern was attributed to a change in pressure. This observation is not extensively discussed because the focus of these experiments is on comparing the changes between treatments, and it is evident that the CC experiment stands out prominently.

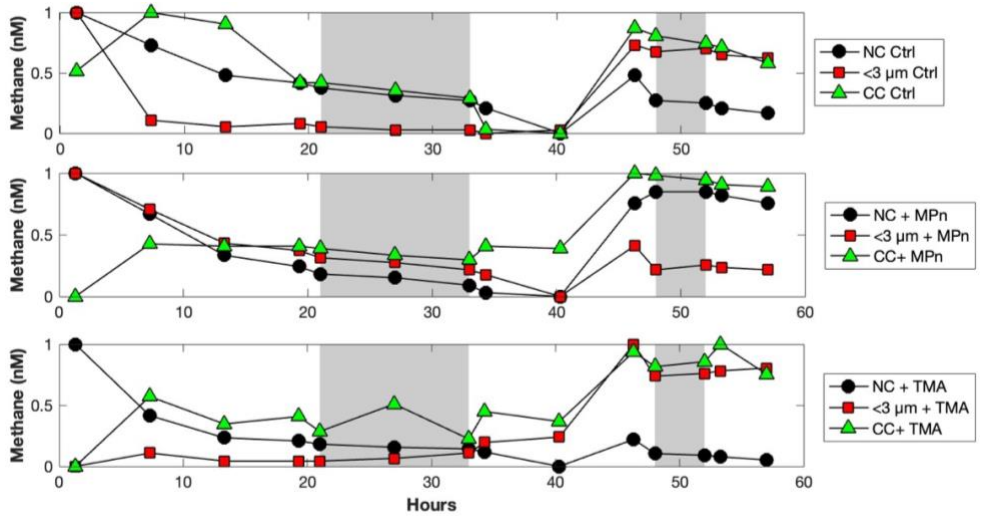

Fig. Standard April.

Lines 522 to 528, comment: There are two increase points T0-T8h and T40 and T46 - One has to question why there is no trend across any 3 or more time points.

There are clear differences between the three communities per experiment and in some cased between experiments, but as is I am not convinced about the significant difference between the CC among the three experiments. As suggested above, please normalize all the data to the starting point (i.e. T0 =1) and all the changes are relative to that - then the patterns can be clearer and better statistically analyzed.

Answer (245 to 248 lines and 539 to 549 lines): no pattern is found between more than 3 points because the CH_4 formation process is very varied in time (on the order of hours), this we corroborated with a previous vial experiment (results not shown), where sampling was done every two hours. Therefore, having microcosm (x 9) results at this resolution was methodologically difficult, so, to ensure that the microcosm reaches equilibrium before the spectrometer takes the sample to determine the CH_4 concentration, it was established to take the sample every 6 hours.

As previously mentioned, we conducted the exercise of normalizing the data and subsequently applied comparative statistics. All analyses were performed on the unprocessed measured data (concentrations), and the rates were also calculated using the actual data. With the normalized data, the comparison results between controls treatments ($p = 0.14$), between communities with MPn ($p = 0.14$) and communities treated with TMA ($p=0.02$). Again, we do not agree to normalize the data because the measurements are on the same scale and we do not see the presence of outliers. As detailed in the methodology, each point represents the plateau of the measurement and the average of about 150 measurements, hence the poor visibility of the SD (<1) in the graphs.

Lines 533 to 535, comment: Agreed - but you can also cite papers of organisms using MPN (to a lower extent) also in the presence of P.

Answer (556 to 558 lines): we have included a couple of references.

Line 556, comment: Here again there is the same interesting sudden increase just before the second dark period - in all treatments and community types. Also here the graphs and the analyses should be done on normalized values to eliminate the large difference in CH4 concentration at T0 between CC and the other treatments.

Answer (567 to 574 lines): For the September case, the normalized values and actual concentrations have the same pattern as in Figure 7 of the manuscript, however, the concentrated picoplankton community (CC) shows low concentrations (Fig. Standard September). We assume that this is because the CC started (T0) with values above the other communities, however the comparisons are valid within this group (CC), because the 3 treatments (CC control and aggregates with MPn and TMA) started with the same CH_4 concentration. The comparison with the other communities is also valid since they were under the same conditions. Moreover, we believe that normalizing or transforming the data would not allow us to provide experimental data of this nature, therefore, we leave the results with the CH_4 concentrations obtained and we have added the description of the 4S table concerning net rates and the difference between photoperiod. Again, abrupt changes in CH_4 production patterns may be due to a change in the internal pressure of the microcosm explained in one of the previous questions.

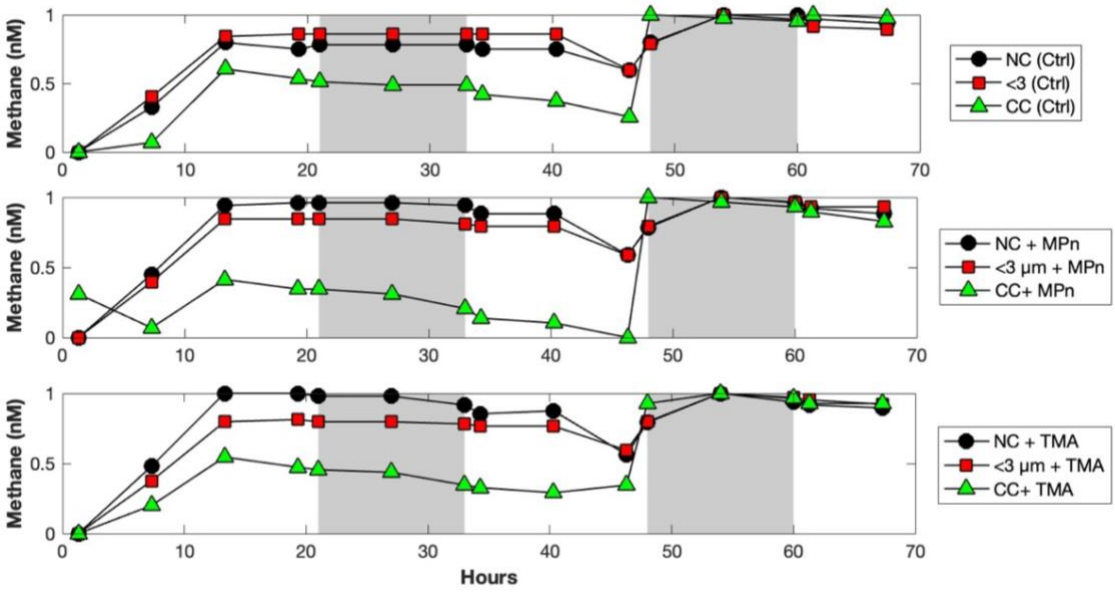

Fig. Standard September.

Line 571, comment: Please remove N. pumilus from the figure and substitute with a general term as MPn producing Bacteria and Archaea.
See reference in previous comment. Also note that Cyanobacteria can also produce MPn. https://linkinghub.elsevier.com/retrieve/pii/S0043135422003475
Answer (598 line): We modified the figure. We indicate the bacteria and archaea and who, e.g. *N. maritimus* and *Synechococcus spp*. which are very important in the study area but also ubiquitous.

Line 585, comment: I likely missed this - but I did not see any evidence to which part of the community metabolized the MPN or TMA. the fact that some increase occurred during dark does not mean that this was not done by the Cyanobacteria. These are also metabolically active in the dark.
Answer (612 line): We currently lack evidence regarding the specific microorganisms responsible for the demethylation of MPn or TMA. Our results are based on cytometry findings that reveal certain broad microbial groups. While cyanobacteria can produce MPn, we are uncertain whether they can cleave MPn; the necessary machinery for this step is provided by some heterotrophic bacteria. Therefore, we only mention that Synechococcus can produce CH_4 under light conditions through photosynthesis. We appreciate the consideration.

We appreciate once again the opportunity to improve our manuscript and are willing to make additional adjustments if necessary. We hope that these modifications will meet the expectations of the journal and the reviewer.

Yours sincerely,

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