Rebuttals of Manuscript PCIMicrobiol #49 entitled: “Long-term sulphide mitigation through molybdate at shrimp pond bottoms”

The manuscript specified above has been revised. The remarks of the reviewers have been incorporated. We appreciate the effort of the reviewers and their suggestions have allowed us to improve the quality of the paper. Please find our replies to the comments of the reviewers in the section below. Some minor changes to enhance grammar and overall readability are not marked.

Legend to the layout of this document:

Comments of the reviewers (all, complete) are shown in a box in italics.

The replies of the authors to the comments of the reviewers are shown in plain text. Changes in the revised manuscript are referred to with the page number and starting line. ~~Deletions are crossed out~~ and **additions are shown in bold.**

Reviewer #1:

The manuscript titled "Long-term Sulphide Mitigation through Molybdate at Shrimp Pond Bottoms" details the effects of molybdate addition (5 and 25 mg/L of $Na_2MoO_4.2H_2O$) as a strategy to reduce H_2S production by sulfate-reducing microbial communities in shrimp aquaculture ponds. While sulphide production was not completely prevented, molybdate addition delayed the onset of sulphide production and shifted the sulphide production zone to deeper sediment layers in the tanks. Unexpectedly, molybdate addition correlated with an increased abundance of putative sulfate-reducing populations affiliated with Desulfobacterota.

Overall, the study is interesting, the experimental rationale is solid, and the manuscript is well-written. However, I have one concern regarding the content and another regarding the form.

Answer: We would like to thank Reviewer #1 for her/his appreciation of our research paper.

Concerning scientific content, I have a question about the various processes associated with the biogeochemical sulfur cycle in the ponds. It is noted that H_2S concentrations, in the µM range, are lower in molybdate-treated ponds, while residual sulfate concentrations, in the 1000-1400 mg/L range, decrease similarly in both treated and untreated ponds. This raises questions about the different processes involved in the biogeochemical sulfur cycle in the ponds beyond sulfate reduction and their respective importance. H_2S concentration results from various processes (sulfate reduction, sulfur oxidation, pH-dependent precipitation, liquid/gas equilibration phenomena, etc.). Although these are mentioned in the discussion section (lines 360-414), the manuscript's clarity and impact would be enhanced by explicitly stating and discussing these processes, perhaps through a diagram of hypothetical sulfur-associated processes with/without molybdate. This would also clarify the interpretation of results for non-specialized readers.

Answer: As accurately highlighted by Reviewer #1, we already extensively discussed the potential mechanisms behind the impact of molybdate towards H_2S control. We would like to avoid repeating text book information on sulphate reduction, as this is already extensively discussed in several of the papers to which we refer, including potentially relevant figures. Since we are working with an open air system (as is the case in real pond systems) with the possibility of H_2S escaping, it is not possible to make accurate sulphur mass balances, and we prefer to avoid speculation on this matter, which is the second reason we prefer not to include such a figure. However, we included a clear statement on this matter in the Discussion section (1).

(1) Page 19 Line 424: **Overall, it is clear that the biogeochemical sulphur cycle in such a pond system involves various processes, *i.e.*, sulphate reduction, sulphide/sulphur (re-)oxidation, precipitation of metal sulphides, and production of polysulphides. Due to the nature of the open air system (as is the case in real pond systems) in the current study, with the possibility of H_2S escaping, it is not possible to make accurate sulphur mass balances.**

In terms of form, I find the manuscript clear and well-written, except for one main concern: the effect of molybdate addition and the operational applicability of the results seem overstated in the abstract and conclusion sections. While molybdate does affect the onset and localization of microbial sulfate-reducing activity in the sediments (e.g., Figure 3), its effect decreases over time and has limited impact on dissolved O_2 and H_2S concentrations in the bulk (Figures 1 and 2). The real benefit of the study lies in insights into the effect of molybdate addition rather than as a proposed operational mitigation strategy, which would require further studies and optimization. I recommend the authors remain more balanced regarding operational applicability in the abstract and conclusion sections and rephrase sentences such as:

- "In conclusion, molybdate acted as a long-term mitigation strategy against sulphide accumulation by directly influencing the microbial community in a shrimp pond system." (abstract)
- "Overall, molybdate can serve as a more environmentally friendly option compared to other conventional strategies for mitigating sulphide production in shrimp pond systems." (conclusions)

Answer: We agree with this comment, and we have updated these statements in the Abstract and Conclusions sections accordingly (1)-(3):

(1) Page 2 Line 38: Molybdate addition **partially** mitigated H_2S production in the sediment, and delayed its transfer to the bulk liquid by pushing the higher sulphide concentration zone towards deeper sediment layers.

(2) Page 2 Line 42: In conclusion, molybdate ~~worked~~ **has the potential to work** as ~~long-term~~ mitigation strategy against sulphide accumulation **in the sediment during shrimp growth** by directly steering the microbial community in a shrimp pond system.

(3) Page 22 Line 472: We showed that molybdate could be an effective mitigation agent against sulphide accumulation in shrimp ponds ~~as a long-term strategy~~, since it can be applied in a single dose, and at relatively low concentrations. Although~~,~~ sulphide production could not be avoided completely**, and only a temporal effect could be obtained,** molybdate reduced H_2S production in the sediment, and delayed its transfer to the water column by pushing the sulphide production zone towards deeper sediment layers. Molybdate induced a higher absolute abundance of Desulfobacterota, but this was not reflected in increased sulphide formation. Overall, molybdate ~~can~~ **has the potential to** serve as a more environmentally friendly option**,** compared to other conventional strategies**,** to mitigate sulphide production in shrimp pond systems.

More specific comments are as follows:
Title:
The title suggests addressing the effect of molybdate in long-term experiments corresponding to the final stages of shrimp growth. However, "Long term" as used in the title is misleading, as the mitigation appears to be temporary (see Figure 3) and its extent is limited (Figures 1 and 2). A rephrasing is suggested.

Answer: We agree with this point, hence, we suggested a new title (1):

(1) Page 1 Line 3: ~~Long-term sulphide mitigation through molybdate at shrimp pond bottoms~~ **Molybdate delays sulphide formation in the sediment and transfer to the bulk liquid in a model shrimp pond**

Materials and Methods:
This section is clear and well-described. The clear description of the level of replication in each type of experiment and measurement is appreciated.

Answer: We appreciate the positive comment of Reviewer #1.

Results:
Lines 253-255: The oxygen fluctuations affecting all pilots need clarification. Please mention possible causes or announce that possible cause are discussed latter in the discussion section.

Answer: As we have no direct explanation for this observation, we did not elaborate on this matter, as this would be mere speculation, which we prefer to avoid. As there were no differences in the bulk liquid oxygen profiles between the different treatments, we did not elaborate on this matter.

Lines 260-274: It is reported that H_2S concentrations are lower in molybdate-treated ponds, while residual sulfate concentrations decrease similarly in both treated and untreated ponds (Table 2). This necessitates a discussion on the different types of processes associated with the biogeochemical sulfur cycle in the ponds for clearer interpretation of the results.

Answer: We would like to refer to our reply to comment 2 of Reviewer #1. We extensively discussed the potential mechanisms behind the impact of molybdate towards H_2S control in the discussion, section 4.1. Since we are working with an open air system (as is the case in real pond systems) with the possibility of H_2S escaping, it is not possible to make accurate sulphur mass balances, and we included a statement about this in the Discussion section (see reply to comment 2).

Discussion:
Lines 378-390 and 403-414: The discussion is interesting, but it may not be fully understandable to a non-expert audience. A graphical scheme illustrating hypothetical sulfur-associated processes in the presence or absence of molybdate, distinguishing between processes supported by experimental data and those that are speculative, would aid the reader and enhance the manuscript's scientific impact.

Answer: We would like to refer to our replies to comment 2 and the previous comment for this matter.

Reviewer #2:

I was pleased to have the opportunity to review this interesting and workmanlike manuscript. The authors present the results of a carefully designed study, the results of which have been thoughtfully presented and discussed with clarity and modesty.

Answer: We would like to thank Reviewer #2 for her/his appreciation of our research paper.

In essence adding molybdate works in that it suppresses sulphide production but, for some reason the additive did not suppress the sulphate reducing bacteria (SRB).

Answer: Indeed, by adding molybdate, we could temporarily suppress the formation of sulphides and delay its transfer to the bulk liquid, but we could not fully eliminate or prevent sulphide formation.

The careful design included excellent replication of the sort that is most easily executed in a model system. However, it is not clear from the title that a model system has been used. I wonder if the authors would consider using the phrase "model shrimp pond". If only to telegraph to the reader that this is a study of a model and not a real system.

Answer: We agree with this point, hence, we suggested a new title (1):

(1) Page 1 Line 3: ~~Long-term sulphide mitigation through molybdate at shrimp pond bottoms~~ **Molybdate delays sulphide formation in the sediment and transfer to the bulk liquid in a model shrimp pond**

As noted above, the use of a model allowed high levels of control and replication. However, it may also have meant no sunlight, this in turn might affect photosynthesis and thus pH and oxygen. I assume algae normally grow in shrimp ponds but were absent from the model system. Would the authors care to comment on this? pH is important to sulphide toxicity and high pH might offset elevated sulphide levels.

Answer: As correctly pointed out by Reviewer #2, we indeed did not include the growth of (micro)algae and the supply of artificial or natural light. We avoided the growth of photosynthetic organisms to focus directly on the sulphate reduction/sulphide formation process in this model approach. We have commented on this in the text (1)-(2):

(1) Page 7 Line 132: To avoid excessive water evaporation, the beakers were put in a transparent plastic box with a non-airtight lid in a temperature-controlled room at 28 ± 1˚C without active aeration. **No artificial or natural light was foreseen to avoid the growth of microalgae and keep a focused approach towards sulphide formation and oxygen depletion.**

(2) Page 17 Line 373: **In a real pond system, also the growth of microalgae could play a critical role, as they (1) enable *in situ* formation of oxygen, and (2) by consuming CO_2, they could provoke an increase in pH, which could reduce H_2S toxicity, but increase ammonia toxicity. They can even actively contribute to an improved water quality (Huang et al., 2022). However, the direct involvement of microalgae in our model system would strongly add to the complexity of sulphide formation, because of their multi-level impact on the shrimp pond nutrient dynamics, so we eliminated the possibility for photosynthetic growth from our model by not supplying natural or artificial light.**

Huang, C., Luo, Y., Zeng, G., Zhang, P., Peng, R., Jiang, X. and Jiang, M. 2022. Effect of adding microalgae to whiteleg shrimp culture on water quality, shrimp development and yield. Aquaculture Reports 22, 100916.

The study itself was conducted over 60 days which I understand is the period it takes to grow and harvest shrimp. However, I am not sure if this is a "long term" study. However, this is a minor point and to some extent dependent on ones personal point of view.

Answer: This period of 60 days represents the final 60-day period of a typical 90-day shrimp growth cycle, as briefly explained at the end of the Introduction section. We agree that the term long-term might be misleading here, we adjusted this in the manuscript, and also in the title (1)-(9):

(1) Page 1 Line 3: ~~Long-term sulphide mitigation through molybdate at shrimp pond bottoms~~ **Molybdate delays sulphide formation in the sediment and transfer to the bulk liquid in a model shrimp pond**

(2) Page 2 Line 34: We used an experimental shrimp pond model to simulate the organic waste accumulation and sulphide formation ~~in a long-term experiment (61 days)~~ **during the final 61 days of a full shrimp growth cycle**.

(3) Page 2 Line 42: In conclusion, molybdate ~~worked~~ **has the potential to work** as ~~long-term~~ mitigation strategy against sulphide accumulation **in the sediment during shrimp growth** by directly steering the microbial community in a shrimp pond system.

(4) Page 4 Line 94: The applicability of molybdate as a remediation strategy towards sulphide formation in aquaculture, however, strongly depends on its ~~long-term~~ **lasting** effect during a 90-days shrimp growth cycle.

(5) Page 4 Line 97: The objective of this study was to determine the ~~long-term~~ **duration and magnitude of the** effect of molybdate towards H_2S mitigation in response to the gradual accumulation of organic waste during a full shrimp growth cycle.

(6) Page 7 Line 145: Two different concentrations of 5 (M5) and 25 (M25) mg/L of sodium molybdate ($Na_2MoO_4.2H_2O$, Sigma Aldrich, St. Louis, Mo., US), were compared with a control treatment (no molybdate addition) for ~~a long-term experiment of~~ **the last** 61 days **of a shrimp growth cycle**.

(7) Page 18 Line 384: These limitations substantiate the importance of a ~~long-term~~ **lasting** strategy to mitigate sulphide production in shrimp pond aquaculture systems.

(8) Page 18 Line 403: In the current study, the molybdate was only partially reduced, both in the M5 and M25 treatments, and the production of H_2S in the sediment and its transfer to bulk liquid could not be fully prevented ~~in the long-term experiment~~.

(9) Page 22 Line 472: We showed that molybdate could be an effective mitigation agent against sulphide accumulation in shrimp ponds ~~as a long-term strategy~~, since it can be applied in a single dose, and at relatively low concentrations.

The methods are very well described and I commend the authors for the use of flow cytometry to get some actual numbers, as well as the high levels or replication. However, I did wonder if *use of ANOVA was preceded by a study of the underlying distribution of the data to determine if it was indeed normally distributed.*

Answer: I assume that the author refers to the repeated measures analysis of variance as described in section 2.6. Given the size of the datasets on which the repeated measures ANOVA test was carried out (each sample contained on average 2,922 ± 876 OTUs per sample, as explained in the manuscript), the Central Limit Theorem applies here, hence, no normality testing was required. Concerning the PERMANOVA test that we used, since this is a is a non-parametric multivariate statistical permutation test, no normality is required.

The results are nicely presented, and the oxygen and sulphide profiles are fascinating. It is not always easy to compare the different test conditions as they are in different panels of the same graph. Have the authors considered comparing the sulphide profiles for experiment on a given day on the same graph? It might be no improvement, but if it was possible the differences between experiments would be clearer.

Answer: We indeed considered this, but as this did not improve the clarity of the data (overlapping graphs), we opted to combine the data per treatment and not per time point.

The discussion is sensible and to the point and contains excellent suggestions as to the underlying cause of the strange increase in SRB. I did wonder if the increased diversity might also be associated with an increase in evenness that in turn would permit more SRB to be detected. However, suspect this is a minor point give the finding that the number of SRB increased.

Answer: We did carry out the α-diversity analyses in which we focused on the Hill numbers (Hill, 1973), but as this did not result in clear and consequent (relevant) differences between treatments or timepoints, we did not include those data in the main manuscript.

Hill, M.O. 1973. Diversity and Evenness: A Unifying Notation and Its Consequences. Ecology 54(2), 427-432.

However, given that this is a model study, it might be worth adding a sentence or two about the implications of using molybdate at full scale. For example, how much molydate might be required and what tradeoffs might need to be considered. However, any discussion should not be at the expense of the admirable brevity of the present discussion.

Answer: Since this is only a first study using a lab-scale model, we prefer not to elaborate too much on trade-offs to be considered on this matter for full scale applications. It is our view that further research, *e.g.*, in systems exposed to artificial sunlight in which algae growth is possible and actual shrimp are included.