Editorial Feedback – Revision 3

Dear Colleen and Co-Workers,

I received one last review from one of the four reviewers who evaluated your manuscript so far. While the reviewer is excited about your dataset and strongly suggests that it be published (same as all previous reviewers), the reviewer again (as the majority of reviewers before) emphasizes that there is (still) too much focus on microbial siderophores. I like to highlight one particular paragraph from the reviewer's feedback:

"The prime focus of the manuscript remains on siderophores. This risks disparaging contribution of other microbially-derived constituents of the ligand pool to Fe cycling at hydrothermal systems (For instance EPS, e.g. Mahieu et al., 2024), as well as other ligand groups. Refractory ligands are hypothesized of importance in transport from hydrothermal systems (e.g. Hassler et al., 2020), with DOM constituents considered weaker such as humics shown reflected in more strongly binding classes (e.g. Slagter et al., 2019). The latter is now touched on in a single line in §2.2 (191-192), but I feel it needs to be stated more strongly that siderophores are an informative but fractional explanation of microbial mobilisation of Fe."

This one-sided interpretation risks disparaging the contribution of other microbially-derived constituents of the ligand pool to Fe cycling at hydrothermal systems, as well as other ligand groups and classes.

Looking back on the manuscript history it feels we make only baby steps with every revision, which also starts exhausting the willingness of the reviewers to review the manuscript again. There were in total 4 reviewers involved and 3 of them found that the focus is too strongly on microbial siderophores without providing clear evidence for this argument (as there could be different explanations).

I am therefore taking the review into my own hands after your next revision and urge you to tone down the focus on microbial siderophores in the title, abstract, and interpretations. Please see the reviewer's comments for more guidance.

Please note that none of the reviewers excluded the possibility that microbial siderophores are involved (neither do I). All we ask for is to base the interpretations on what your analyses can tell. Microbial siderophores are one of the possible explanations, but not the only one. These different possible explanations need to be equally discussed (unless you can safely discard one or more - but I saw no evidence for that from the reviewers' evaluations of your methods).

I believe it will make your manuscript stronger if you openly discuss the different possible explanations. I am sure there will be future studies (either by yourself or by other groups) that will build on this observation and narrow the analytics to decipher the role microbial siderophores play.

Let me know if you need more advice. I really like to see this manuscript published soon. It takes only a minor step :-)

All the best
Tina

Dear Tina,

Thank you for your helpful comments on the manuscript. We are sorry to hear that you feel that we have not adequately addressed the reviewer comments in this process, and that the manuscript has only incrementally improved. While we have recognized the reviewers' comments that weak ligands are important for thinking about iron cycling in seawater, we have respectfully disagreed with the reviewers that this should be the focus of the manuscript. The focus of our manuscript, because it is the data that we actually have, is primarily about strong ligands and siderophores. We cannot change the focus of our paper to be about weaker ligands because discussing further about them would be entirely speculative, and the main data we have is siderophore concentrations, identities, and community composition of bacteria capable of producing siderophores. We do not feel we have overstated the importance of siderophores, and we try to point out in the manuscript that our focus is on these compounds is because 1) it is novel that we have detected them at all (this has never been done before), 2) their distributions match those of the strong ligand pool (despite them being detected in low concentrations, a caveat we recognize and point out several times), and 3) we saw a connection in our data between ligand identities with taxa capable of producing these compounds.

Regardless, in this version we have edited the abstract, introduction, and the first section of the results to include a few more sentences about weaker ligands. The most recent reviewer stated that only one line of the manuscript mentioned weak ligands, however almost all of section 2.1 in our manuscript discusses other contributors to the ligand pool as were mentioned by past iterations of the reviews. We are discussing in the manuscript that we think siderophores are possible contributors to the strong ligand pool that we observed to be coupled to dissolved iron in the neutrally-buoyant plume, but we do not discount the fact that other weaker ligands are present as well, and that other strong ligands are also likely present. It is worth noting that our paper also focuses on the neutrally-buoyant plume, where weak ligands were not dominant in any of the samples. Weak ligands were only dominant in the samples that were closest to the vent source. We discuss this at length in section 2.1, and also in section 2.4 (first paragraph). In our concluding paragraph we state, "*In this work, L_1 ligands were tightly coupled to dFe in neutrally buoyant plumes along the MAR and the presence of siderophores in these samples provided evidence for the first time, that at least some of these ligands are microbially produced.*" This is the main point of our manuscript, and we believe our data supports this, and that we do not believe that we are trying to argue that siderophores are comprising the entire iron-binding ligand pool in seawater, if this in fact the main concern of you and the reviewer.

If you believe that the major focus of our manuscript should not be on siderophores, then we are afraid we would have to move the paper elsewhere, because simply, that is the data we have and it is the main point of our paper. We too, are exhausted by this review process, and we simply can only write our paper about the data we actually have. We appreciate your willingness to consider this revision of the manuscript. Thank you.

Report# 1 – Revision 3

The prime focus of the manuscript remains on siderophores. This risks disparaging contribution of other microbially-derived constituents of the ligand pool to Fe cycling at hydrothermal systems (For instance EPS, e.g. Mahieu et al., 2024), as well as other ligand groups. Refractory ligands are hypothesized of importance in transport from hydrothermal systems (e.g. Hassler et al., 2020), with DOM constituents considered weaker such as humics shown reflected in more strongly binding classes (e.g. Slagter et al., 2019). The latter is now touched on in a single line in §2.2 (191-192), but I feel it needs to be stated more strongly that siderophores are an informative but fractional explanation of microbial mobilisation of Fe. Apart from what is here classed as L1, the fact that there is such a large binding potential in the weaker ligands found at the MAR sites is a result worth discussing as well. While touched on in §2.1, the discussion culminates in impacts of the L1 class and siderophores, leaving the reader somewhat wondering about the rest of the ligand pool.

The correlation sought with microbial production, via the still relatively novel application of genome mining for production pathways, remains a deeply valuable contribution. The focus here on certain siderophore related pathways is entirely defensible in the face of the state of knowledge and as the authors rightly indicate, is currently limited to known sequences just as the measurement of siderophores is limited to known compounds and/or functional groups. Together with the comprehensive titration dataset, the underlying data for the present manuscript must most definitely be published.

It remains however of concern to this reviewer that the interpretation remains so solely focused on elucidating and in particular exhibiting just the role of a minute constituent of the ligand pool, in the face of such a rich dataset at large. While underestimation is argued, even an order of magnitude greater representation still has the implications for Fe cycling, based on the present correlations, getting ahead of what is supported by these findings.

We thank the reviewer for their feedback. Please see the above comments to the editor regarding the discussion of weaker ligands and data collected in this study.

We have also added additional citations to expand to the weaker ligand discussion section in section 2.1 (Line numbers are reported for track-change accepted version)

Line 79: Fitzsimmons et al 2017, Fitzsimmons and Boyle 2014, Yucel et al 2011, and Lough et al. 2019

Line 80-81: Hawkes et al. 2013, Mahieu et al. 2024; Kleint et al. 2016, Hassler et al. 2020, and Slagter et al. 2019

Line 118-119: Slagter et al 2019, Hassler et al. 2020, Mahieu et al. 2024, and Hawkes et al. 2013b

Small remarks

- The wording of the last sentence of the introduction (93-94), implying importance of the role of the small siderophore fraction in hydrothermal systems by their presence. Please justify the importance of the role with some more elaboration.

We have removed these last two sentences from the introduction. These last two sentences discussed results and conclusions that are already further discussed within our results/discussion and conclusions sections below.

- The cited preprint of Lough et al. 2022 does not present ligand concentrations, yet is cited for ligand-dFe correlation;

We thank the reviewer for catching this mistake and have removed it from that citation.

- The last sentence of §2.3 asserts that previous insight underestimated siderophore production, waylaid by the finding of production pathways here. This underestimation would benefit from citation.

We have added the citation Li et al (2014), which discusses the first study where microbial transcripts for Fe uptake, biosynthesis, and regulations were found in the Guaymas Basin hydrothermal plume.

The sentence now reads as follows: "Thus, finding genera capable of producing only a subset of the siderophores characterized is not surprising. The observation that a significant portion of the *in-situ* microbial community is capable of synthesizing siderophores (**Fig 3**) suggests that siderophore production is more widespread in the deep ocean than previously believed and contribute to the "microbial iron pump" in hydrothermal plumes (Li et al., 2014)"

Li, M., Toner, B. M., Baker, B. J., Breier, J. a, Sheik, C. S., and Dick, G. J.: Microbial iron uptake as a mechanism for dispersing iron from deep-sea hydrothermal vents., Nat. Commun., 5, 3192, https://doi.org/10.1038/ncomms4192, 2014.

- "...will aid in constraining the biogeochemical importance of microbial feedbacks in impacting the hydrothermal dFe supply to the deep ocean" (279 onward). This sentence has already been going for a while and the authors have lost me. Please clarify what is meant with microbial feedbacks impacting HT dFe transport.

We have edited sentence to improve clarity:

Line 292-293 "How these complexes may facilitate the exchange of Fe between dissolved and particulate phases (Fitzsimmons et al., 2017), and whether siderophores are present across additional hydrothermal vent systems will aid in constraining biogeochemical cycles of the hydrothermal dFe supply to the deep ocean."

- §3.2 - Given the overnight equilibration with 10uM SA, has care been taken to minimise and account for the formation of $Fe(SA)_2$ as per Gerringa et al. (2021)?

Yes, the procedure took similar precautions to minimize $Fe(SA)_2$ while ensuring enough time for all Fe to associate with SA before analysis as reported in Moore et al. (2021), Bundy et al. (2018) and Mahieu et al. (2024). Mahieu et al. (2024) also notes that once sufficient conditioning has been achieved, using SA at higher concentrations than 5 μM also gives accurate data for model ligands.

Bundy, R. M., Boiteau, R. M., McLean, C., Turk-Kubo, K. A., McIlvin, M. R., Saito, M. A., Mooy, B. A. Van, and Repeta, D. J.: Distinct Siderophores Contribute to Iron Cycling in the Mesopelagic at Station ALOHA, Front. Mar. Sci., 1–15, https://doi.org/10.3389/fmars.2018.00061, 2018.

Mahieu, L., Omanović, D., Whitby, H., Buck, K.N., Caprara, S. and Salaün, P., 2024. Recommendations for best practice for iron speciation by competitive ligand exchange adsorptive cathodic stripping voltammetry with salicylaldoxime. *Marine Chemistry*, *259*, p.104348.

Moore, L. E., Heller, M. I., Barbeau, K. A., Moffett, J. W., and Bundy, R. M.: Organic complexation of iron by strong ligands and siderophores in the eastern tropical North Pacific oxygen deficient zone, Mar. Chem., 236, 104021, https://doi.org/10.1016/j.marchem.2021.104021, 2021.

Bib:

Gerringa, L.J.A., Gledhill, M., Ardiningsih, I., Muntjewerf, N., Laglera, L.M., 2021. Comparing CLE-AdCSV applications using SA and TAC to determine the Fe-binding characteristics of model ligands in seawater. Biogeosciences 18, 5265–5289. https://doi.org/10.5194/bg-18-5265-2021

Hassler, C., Cabanes, D., Blanco-Ameijeiras, S., Sander, S.G., Benner, R., 2020. Importance of refractory ligands and their photodegradation for iron oceanic inventories and cycling. Mar. Freshw. Res. 71, 311. https://doi.org/10.1071/MF19213

Mahieu, L., Whitby, H., Dulaquais, G., Tilliette, C., Guigue, C., Tedetti, M., Lefevre, D., Fourrier, P., Bressac, M., Sarthou, G., Bonnet, S., Guieu, C., Salaün, P., 2024. Iron-binding by dissolved organic matter in the Western Tropical South Pacific Ocean (GEOTRACES TONGA cruise GPpr14). Front. Mar. Sci. 11, 1304118. https://doi.org/10.3389/fmars.2024.1304118

Slagter, H.A., Laglera, L.M., Sukekava, C., Gerringa, L.J.A., 2019. Fe-Binding Organic Ligands in the Humic-Rich TransPolar Drift in the Surface Arctic Ocean Using Multiple Voltammetric Methods. J. Geophys. Res. Oceans 1491–1508. https://doi.org/10.1029/2018JC014576