

Reviewer #1

This is the second revision of the manuscript titled "Microbial strong organic ligand production is tightly coupled to iron in hydrothermal plumes" by Hoffman and coauthors. I was rev1 for the first revision.

I am undecided about how to proceed. On one hand, the authors replied to all my questions and introduced changes in the text, but on the other hand, most of those changes were merely cosmetic. None of the (IMO) main shortcomings of the manuscript have been corrected. I think that the data base must be published, specially the chromatographic part, but the rejection to recognize the limitations of the technique and the refusal to comment or amend the oversimplification of data and processes makes the revision process unnecessarily long and frustrating. This attitude brings a clear problem on the long run, some misconceptions are perpetuated just because they have been published. I suggest another round of revision.

1 The first matter is the "organicity" of ligands. Some lines have been added mid-way the manuscript about the possible presence of inorganic phases and erroneously these are restricted to oxyhydroxides. Why inorganic phases are necessarily "weak" ligands? I insist, CLE-AdCSV does not provide any insight about the nature of the fraction that is not labile after equilibration with a competing commercial ligand. And this is not arguable. The manuscript should clearly state that the analytical technique cannot discriminate between organic, inorganic or mixed phases and modify the abstract to reflect that the contribution of inorganic and mixed phases to the ligand concentration is likely. Such phases are described in most of the bibliography. It must also be noted that calculations of the "ligand" concentration are based in a 1:1 Fe:ligand ratio model and that in the presence of mixed phases or inorganic phases this is just a parametrization and possibly does not respond to the actual speciation.

After my comment in the initial review, no biological process, apart of siderophore release, which is clearly insufficient, has been presented as a possible source of organic ligand concentrations of tens of nM. There is no bibliography showing such ligand concentrations, even in the absence of phytoplankton. I contacted one of the first authors involved in the electrochemical speciation in hydrothermal plumes and this researcher confirmed me that despite the label of organic ligands, no test was carried out to study their "organicity". And the whole bibliographic body about the presence of persistent inorganic phases has been either ignored or just cited ignoring the part referred to stabilized inorganic phases. The study of hydrothermal vents is complex and requires to take into account that many precipitation processes are of very slow kinetics, especially at low temperatures and that metastable (as in non thermodynamically stable) phases may be present for very long periods (I recommend a reading of Stumm and Morgan's book). Furthermore, colloidal phases are included in the operational definition of dissolved iron from filtration by 0.2 μm . The authors argue throughout the manuscript in terms of equilibrium and this is not correct for colloids. (Fitzsimmons et al., 2017) (cited when denying the possible inorganic character of the ligands) described dFe in hydrothermal plumes as a mix of two components, one of them would be inorganic oxyhydroxides that would persist even when the plume is so dilute that most of iron is background ocean dFe. Many articles argue about the presence and persistence of nanopyrites. There is irony behind the use of an article titled "Hydrothermal vents as a kinetically stable source of iron-sulphide-bearing nanoparticles to the ocean" (Yücel et al., 2011) to argument the complete precipitation of inorganic iron. Nanopyrites and the onion model are also discussed in (Hawkes et al., 2013) (cited in this manuscript) when referring to components of the "inert" fraction of hydrothermal dFe. Pyrites are known for their high insolubility; aged oxyhydroxides become more and more refractories even to acidification protocols (Raiswell et al., 2010).

This discussion pertains not only to ligand concentrations but also to the so-called L1. If iron inorganic

phases are organized in more or less refractory clusters, it is highly probable that SA, especially when used at such a low alpha coefficient, cannot "peel" the colloid off during the overnight equilibration. Iron not available for ligand exchange would manifest as a complex with a very high K but limited to an upper threshold. This upper limit is caused by the limitation of the analytical technique known as the analytical window that forces K (or alpha if the authors prefer) into the analytical window. The possible presence of inorganic phases and their possible inert character should be added to the abstract and throughout the manuscript and the role of siderophores toned down.

Originally the manuscript was only focused on the stronger ligand results because of the short format, so now that the paper is in a longer format we have added several sentences to discuss the various caveats you have mentioned here. In the first section of the results and discussion, we have now discussed our weaker ligand data more thoroughly, and have added some discussion about the "onion" concept you have noted above (also discussed in Hawkes et al. 2013). Although it is well known in the literature that the voltammetric techniques employed here do not give any definitive information on the identity of the ligands detected, repeated studies have shown that the SA method we used give reliable ligand concentrations and conditional binding strengths for model ligands such as ferrioxamine B (Mahieu et al. 2024). Thus we believe that our forward titrations are giving reliable ligand concentrations and strengths for excess ligands in these systems, as this is what the method is measuring. We can not say however, what the chemical form of the dFe is and it is entirely possible that it is in various inert, colloidal, or inorganic phases that could be detected as a ligand in our total ligand measurements. We did also perform reverse titrations which should give a better indication of total ligands (including the bound dFe fraction), but similar caveats would also apply. We added additional details about this in the manuscript (Lines 144-175).

We have also toned down our discussion of siderophores throughout the manuscript, and have focused our discussion of siderophores on the fact that it is ecologically interesting that they are present in these systems and it is likely they play some sort of role in Fe transformations in this environment. We believe there is very strong evidence for this in nature, particularly in terrestrial environments, and the novelty of this work was that siderophores were detected at all and that they had similar distribution patterns as the L₁ ligands despite their much lower concentrations. As expanded upon in the manuscript (Lines 91-93; Lines 278-282), the extraction technique for siderophores has a very low extraction efficiency, we do not know all of the siderophores that could be produced in nature, and we were very conservative with our siderophore identifications in this study, so we think that the concentrations of siderophores we identified in this system is a lower bound. We have found siderophores in seawater in every sample we have ever analyzed, so we suspect that the importance of these compounds is only just being discovered.

2 The use of L1 as a proxy for siderophore concentration is also at least arguable and there is not consensus in the field as the text seems to point. Recent works by Slagter, Gerringa, Laglera and Sukekava have shown that the so-called L1 is present in Arctic waters where humics are overwhelmingly the majority of ligands, and that competing desferrioxamine B only extracts a fraction of iron bound to reference humic material. The possible ascription of non siderophore ligands to the so-called L1 pool has also been recently discussed by Mahieu and co authors. It is very likely that nanopyrrites and nano aged Fe oxyhydroxides may contribute to electrochemical L1 in hydrothermal plumes (this is considered in (Kleint et al., 2016) and for the case of copper complexes in (Sander et al., 2007)).

We agree with the reviewer that L₁ ligands can be more than just siderophores. We have included such arguments at line 57 in the introduction, and throughout section 2.2 when the chromatographic data is presented, and again in Lines 284-285.

3 The weight of the role of siderophores. In their response the authors state We have edited some sections of the manuscript to make it clear that we do not think that siderophores are a major part of the L1 pool, but rather that their presence in these systems is intriguing and suggests that the other strong ligands we observed in the neutrally-buoyant plumes might also be microbially-produced (whether or not they are siderophores). This line of argument is impeccable but not compatible with this line in the introduction: “The tight coupling between strong ligands and dissolved iron within neutrally buoyant plumes across distinct hydrothermal environments, and the presence of dissolved siderophores with siderophore-producing microbial genera, suggests that biological production of ligands exerts a key control on hydrothermal dissolved iron concentrations”. Here again, it is conferred a controlling role to a group of substances found at a maximum of 4% of the ligand concentration and the possible contribution of inorganic colloids and nanoparticles, which presence is commonly accepted, completely ignored. The ecological importance of siderophores is huge but at the concentrations shown here, and in the absence of other organic candidate to contribute to organic ligands, the data base is far from even suggesting the biological control of dFe by microbial exudation or lysis products.

See our additional comments on siderophores above, but we have toned down the language around this throughout the manuscript.

4 The trend to self citation has not been corrected at all. In the response, the authors argue in favour of the relevance of their bibliography and the need to keep the number of citations low. Case by case this makes sense but when I go to the bibliography and see that authors of the weight in the iron speciation community such as Gerringa and van den Berg or in the study of hydrothermal vents such as Sander are cited a single time this does not look right. A reader not specialized in the field would think that the authors here are almost the only ones worth to be cited. The result is the absence of works that do not align with their vision of the topic. More specifically, the bibliography about the presence of inorganic phases in hydrothermal plumes has been ignored or badly cited (omitting such parts).

An illustrative example was my comment about the two self citations used to support the description of iron transition in estuaries. From the early works of Boyle and Sholkovitz, many authors have used electrochemical techniques to find ligands in estuaries and made more contributions than the authors here: van den Berg, Laglera, Whitby, Yang, Muller, Waeles/Pernet Coudrier, etc. . Furthermore, many of these studies actually indicate that iron is exported from estuaries as humic complexes, therefore, implying that, if only the iron bound to strong ligands survives the estuarine transition, as the authors suggest, a significant part of humics must belong to L1. This is also discussed in (Slagter et al., 2019; Sukekava et al., 2024). I guess that since this presence of refractory DOM reduces the theoretical contribution of siderophores to the so-called L1 concentrations, citations of these articles are not in the interest of the authors.

The authors have the logical aim for this work to transcend, but I do not think that ignoring previous effort by other authors in the field and not discussing but ignoring bodies of bibliography that do not concur with their vision of the topic, is the best approach.

Ignoring previous work in the CSV literature was not intentional, and as previously stated, was initially due to constraints from the formatting restrictions and the short format of the paper. However, now that we have lengthened the manuscript, we can more fully address the concern the reviewer has expressed above. Given this, we have added discussion and citation of additional papers from the large body of literature in the electrochemical community. Given that this manuscript is not a review of the CSV

methodology, we are unable to cite every previous work using electrochemistry techniques in aquatic/marine environments. We have instead focused our citations around hydrothermal systems. Some examples are noted here:

Line 57: Sentence that begins with “...open ocean” now includes Whitby et al 2024, Whitby et al 2020, and Misumi et al 2013.

Line 61: Sentence that begins with “...removal” now contains citations Fishwick et al 2014 and Aguilar-Islas et al 2010

Line 75-77: Sentence that begins with “Previous studies have both looked at...” now also includes the work of Kleint et al. 2016, Hawkes et al. 2013, and Sander and Koschinsky 2011

Line 102-103: Sentence that begins with “...in neutrally-buoyant hydrothermal plumes...” citations Hawkes et al. 2013b, Wang et al. 2022, and Bennett et al 2008 were added.

Line 124: Sentence that begins with “...layers of coordination bonds, forming complex...” now has Mackey and Zirino 1994 cited.

Line 126: Sentence that begins with “...bind Fe, but not in traditional organic coordination bonds” now has Fitzsimmons et al 2017 and Honeyman and Santschi 1989 cited.

Line 136-138: Additional discussion in section 2.1 around the weaker ligands also has highlighted work from Kuhn et al. 2014 and Muller 2018, in addition to Kleint et al 2016, Hawkes et al. 2013, and Sander and Koschinsky 2011 (all previously mentioned above).

Line 162-163: Sentence that begins with “in-situ production by bacteria within the neutrally buoyant plume” now also contains citations from Dick et al. 2013, Li et al. 2014, and Sheik et al 2015.

Line 206: Sentence that begins with “...also contributing to...” now has Laglera and van den Berg 2009 cited

Line 272-273: Sentence that begins with “...organic compounds are common” now also include citations from Fitzsimmons et al 2017, Hoffman et al 2018, Toner

Line 276-278: Sentence that begins with “Given the evidence from particulate Fe studies in neutrally-buoyant plumes..” now also includes Toner et al. 2009, Yücel et al. 2011 Fitzsimmons et al. 2014 and 2017, Hoffman et al. 2018, German and Seyfried 2014, and Holden et al 2012

5 From the revised version: The strong coupling between dFe and ligands was only observed at sites where L1 ligands were detected. Some sampling locations, such as in the buoyant plume or closer to the vent orifice, contained high concentrations of weaker ligands ($\log K_{Fe^2, FeL_{cond}} < 12$, Table S2) with no correlation to dFe. This is consistent with these environments likely being dominated by inorganic forms of Fe as hydrothermal fluids initially mix with oxygenated seawater

This is based in assuming that inorganic iron can only be ascribed to a weak ligand group. The refractory character of iron oxyhydroxides (for instance to competition by an added ligand) is obviously a matter of aging as the aggregates move from pure hydroxides (fresh ferrihydrite) to oxyhydroxides. In this process, not only iron aggregates (in the sense of increasing size) but also becomes more refractory, which also relates to the onion model by Mackey and Zirino. There are Raiswell references (Raiswell et al., 2010) where different oxyhydroxides are subjected to the same extraction protocols including acidification with

a enormous range of efficiencies, almost of them low. Nanopyrites are insoluble and should also be refractory to ligand competition.

We have rephrased this section to point out that various inorganic phases (e.g. oxyhydroxides, nanopyrites) are likely more dominant fractions of the iron pool closer to the vent orifice, so the lack of correlation of dissolved iron with organic ligand parameters is not surprising. We did not mean to imply that these inorganic phases would be only detected as part of the weaker ligand pool. As far as we know, there is no definitive answer as to whether all inorganic iron phases are inert to ligand exchange with SA or not, and thus with the data we have it is impossible to assign inorganic iron phases to a particular operational ligand class (see section 2.1).

6 from the response by the authors: and all ligand parameters reported were confirmed to be within the analytical window of the SA measurements,

This is no control of quality. Please revisit classic works from Apte and van den Berg for definitions of analytical window and its extent. The alpha coefficient of FeSA defines the CENTER of the analytical window. The WIDTH of the analytical window is given by the analytical error. All ligands can only be resolved in the absence of data error. The analytical error, even if small, prevents the accurate determination of tiny concentrations of FeL (for weak ligands) or FeLad (for strong ligands) and such ligands are not properly characterized. As a result, alpha values and therefore log K values of strong ligands are forced to fall into a window (centred around alpha FeLad) and do not correspond to the real stability of the complex. This is a limitation of the technique independently of the quality of the analysis. For the case of copper titrations this is explained in (Laglera et al., 2013; Pižeta et al., 2015) and references there in. This means that ligands of alpha coefficient stronger than the upper limit of the analytical window are detected (these ligands have not exchanged iron with the added ligand) but K is brought down so alpha falls into the analytical window. Ligands of weaker alpha are simply not measured. So, that ligand solutions fall close to the alpha of the artificial ligand is not surprising. However, in this manuscript there is something else, log alpha values of ligands fall approximately from 1.3 to 4.9 (more than 4 orders of magnitude, table in supplement) and the centre of the window should be around 1.7 from 10 uM SA and K and B values from (Abualhaija and van den Berg, 2014). The upper limit of the analytical window should be around 3.7, which implies that some of the values cannot be “confirmed”.

We have added some data quality flags in the supplementary table 2 and 3 for all data points, and to specifically point out which datapoints might be on the edge or outside of the analytical window.

7 Forward and reverse titrations should appear in different tables. Since the definition of the analytical window is different, the distribution of analytical error throughout the titration and the statistical weight of data during the data treatment is very different, forward and reverse titrations cannot return the same solution for the same sample if there is more than one ligand present. This is evident from works where both methods have been used in natural samples for copper (Nuester and van den Berg, 2005; Santos-Echeandía et al., 2008; Wiwit et al., 2021).

We have separated out the reverse titration (supplementary table S3) and forward titration data (supplementary table s2) results into two supplemental tables.

8 The bibliography of the tracked changes version has not been updated since at least the Hassler paper is missing

We thank the reviewer for catching this and have corrected this in the reference list with the previous and updated references added.

9 from the response to reviewers: Operationally defining classes of organic ligands by their conditional stability constant is an accepted and preferred strategy by the field in the application of CLE-AdCSV methods (Gledhill and Buck 2012).

This is simply not true. The definition and ascription of K values to classes (as in L1 siderophores, L2 HS and L3 EPS or similar) is the preferred strategy by a part of the field, mostly the authors in here. Other authors do not use it or directly challenge it (Mathieu, Laglera, Sukekava, Gerringa, Slagter recent manuscripts cited above)

We appreciate the reviewer's notes regarding this debate within the field. However, for clarity of discussion throughout the manuscript will be using the operationally defined classes put forward in Gledhill and Buck, (2012) and have been careful throughout the manuscript about ascribing specific ligands (e.g. siderophores, humics) to certain operational ligand classes or clear about the caveats when doing so.

10 from the response to reviewers: We are not sure which inorganic ligands the reviewer is specifically referring to, but if they are referring to the formation of inorganic iron oxy(hydro)oxides, then the CLE-AdCSV method would not artificially detect these compounds as “organic ligands” because the side reaction coefficient of these is $\alpha = 10$ or 11 which is outside of the analytical window used in this work.

The discussion about the presence of metastable phases, nanopyrite and aging of oxyhydroxides I wrote above is of relevance here. This inorganic alpha is not exactly the strength of the inorganic complexation, it is the ratio $Fe^{3+}:Fe'$. This alpha is summed to log K if the constant is referred to Fe^{3+} instead of Fe' . It does not make full chemical sense because includes the insoluble $Fe(OH)_3$ as part of the inorganic complexes and therefore it cannot be used to compare organic/inorganic complexation.

Thank you for pointing this out.

11 Section amended: “Over the last few decades, observations and modelling efforts have increased our understanding about the critical role organic ligands play in the cycling, transport, and utilization of trace metals (Tagliabue et al., 2017; Buck et al., 2018; Bundy et al., 2018; Moore et al., 2021). Organic ligands in seawater have a wide range of sources, which are only just beginning to be understood, but observations suggest that microbial production of siderophores, humic-like substances and exopolysaccharides are some of the major contributors (Hassler et al. 2017), linking microbial activity to impacts on Fe cycling. For example, microbial communities can influence Fe cycling in environments ranging from hydrothermal plumes (Cowen and Bruland, 1985; Cowen et al., 1990) to the open ocean (Lauderdale et al., 2020).”

I would add the expression “in deep waters”. In upper waters there are sources of organic ligands more important than microbial production of ligands. Phytoplankton cultures and phytoplankton blooming waters have shown substantial ligand increments (Boye et al., 2005; Boye and van den Berg, 2000; Kondo et al., 2008; Laglera et al., 2020), riverine humics are also an important source of ligands in areas

of the open ocean (van den Berg, Whitby, Laglera/Sukekava, Yang, Slagter/Gerringa, Muller, Dulaquais manuscripts).

We have added some additional details on sources specific to the deep ocean in the introduction.

With the rest of the manuscript I agree with the changes performed.

- Abualhaija, M.M. and van den Berg, C.M.G., 2014. Chemical speciation of iron in seawater using catalytic cathodic stripping voltammetry with ligand competition against salicylaldehyde. *Marine Chemistry*, 164: 60-74.
- Boye, M. et al., 2005. Major deviations of iron complexation during 22 days of a mesoscale iron enrichment in the open Southern Ocean. *Marine Chemistry*, 96(3-4): 257-271.
- Boye, M. and van den Berg, C.M.G., 2000. Iron availability and the release of iron-complexing ligands by *Emiliania huxleyi*. *Marine Chemistry*, 70(4): 277-287.
- Fitzsimmons, J.N. et al., 2017. Iron persistence in a distal hydrothermal plume supported by dissolved-particulate exchange. *Nature Geoscience*, 10(3): 195-201.
- Hawkes, J.A., Connelly, D., Gledhill, M. and Achterberg, E.P., 2013. The stabilisation and transportation of dissolved iron from high temperature hydrothermal vent systems. *Earth and Planetary Science Letters*, 375: 280-290.
- Kleint, C., Hawkes, J.A., Sander, S.G. and Koschinsky, A., 2016. Voltammetric investigation of hydrothermal iron speciation. *Frontiers in Marine Science*, 3: 75.
- Kondo, Y. et al., 2008. Organic iron (III) complexing ligands during an iron enrichment experiment in the western subarctic North Pacific. *Geophysical Research Letters*, 35(12).
- Laglera, L.M., Downes, J. and Santos-Echeandía, J., 2013. Comparison and combined use of linear and non-linear fitting for the estimation of complexing parameters from metal titrations of estuarine samples by CLE/AdCSV. *Marine Chemistry*, 155(0): 102-112.
- Laglera, L.M. et al., 2020. Iron organic speciation during the LOHAFEX experiment: Iron ligands release under biomass control by copepod grazing. *Journal of Marine Systems*, 207: 103151.
- Nuester, J. and van den Berg, C.M.G., 2005. Determination of metal speciation by reverse titrations. *Analytical Chemistry*, 77(1): 11-19.
- Pižeta, I. et al., 2015. Interpretation of complexometric titration data: An intercomparison of methods for estimating models of trace metal complexation by natural organic ligands. *Marine Chemistry*, 173(0): 3-24.
- Raiswell, R., Vu, H.P., Brinza, L. and Benning, L.G., 2010. The determination of labile Fe in ferrihydrite by ascorbic acid extraction: Methodology, dissolution kinetics and loss of solubility with age and de-watering. *Chemical Geology*, 278(1-2): 70-79.
- Sander, S.G., Koschinsky, A., Massoth, G., Stott, M. and Hunter, K.A., 2007. Organic complexation of copper in deep-sea hydrothermal vent systems. *Environmental Chemistry*, 4(2): 81-89.
- Santos-Echeandía, J., Laglera, L.M., Prego, R. and van den Berg, C.M.G., 2008. Copper speciation in estuarine waters by forward and reverse titrations. *Marine Chemistry*, 108(3-4): 148-158.
- Slagter, H.A., Laglera, L.M., Sukekava, C. and Gerringa, L.J.A., 2019. Fe-binding Organic Ligands in the Humic-Rich TransPolar Drift in the Surface Arctic Ocean using Multiple Voltammetric Methods. *Journal of Geophysical Research: Oceans*, 124: 1491-1508.
- Sukekava, C.F., Downes, J., Filella, M., Vilanova, B. and Laglera, L.M., 2024. Ligand exchange provides new insight into the role of humic substances in the marine iron cycle. *Geochimica et Cosmochimica Acta*, 366: 17-30.
- Wiwit et al., 2021. Wide-range detection of Cu-binding organic ligands in seawater using reverse titration.

Marine Chemistry, 230: 103927.

Yücel, M., Gartman, A., Chan, C.S. and Luther, G.W., 2011. Hydrothermal vents as a kinetically stable source of iron-sulphide-bearing nanoparticles to the ocean. Nature Geoscience, 4(6): 367-371.

Reviewer #2

The current manuscript is a good advance in terms of Fe-biogeochemistry and the role of microorganisms in hydrothermal plums.

The critical point here is that the authors measured Fe-binding ligands considering the dFe concentration in the plume. However, a fraction of this dFe can be colloidal and not be absorbed on the Hg drop. Did the authors check the dFe-labile by the voltammetric system? If this inorganic fraction of Fe is not reactive with the drop, the LFe can be overestimated.

In addition, is also a critical point is the low efficiency of the solid-phase extraction technique, that is around 10%. Please, discuss about it in the discussion section.

We test the lability of the dissolved iron at the end of each forward titration, and we do not believe the excess ligands are overestimated based on our estimates of labile iron in the majority of our samples. We did also perform reverse titrations on some samples where dissolved iron was particularly high (see supplementary tables) and a forward titration failed (indicating that dissolved iron was greater than the ligand concentration). The reverse titrations give an estimate of the labile iron, that seemed consistent with what others have observed in hydrothermal systems (Hawkes et al. 2013).

We thank the reviewer for this insight on the solid phase extraction method (SPE). We discuss the efficiency of the SPE and implications for our dataset between line 278-282.

1- Line 71 - mistake where gap is written.

This has been fixed.

2- Add the information about the equilibration time between Fe and SA. And about this time, did you ensure that was enough to achieve the equilibration?

At line 397-398 we have added "Samples were then equilibrated overnight before being measured on the BASi". Overnight equilibration times have shown to be sufficient for exchangeable dissolved iron (Moore et al., 2021; Bundy et al., 2016; Abualhaja and van den Berg, 2014).

Reviewer#3

It would be my suggestion to either revise the manuscript to widen the scope of the analysis to the entire width of the dataset (i.e., include L2 and L3 classes and describe their roles more completely, as to further motivate the assertions made on the L1 class), or walk back the assertiveness of statements to their implications for the specific parts of the microbial community these relate to, as opposed to Fe cycling in bulk.

We thank the reviewer for this comment. Now that the paper is no longer in the shorter format, we have expanded the discussion to incorporate discussion of the entire dataset as well as adjust our language in certain sections. This is mostly in section 2.1.