Author Responses to RC1 (in *blue italics*)

Review of "Microbial strong organic ligand production is tightly coupled to iron in hydrothermal plumes" by Hoffman and coauthors:

The manuscript employs a combination of classical electrochemical methods (CLE-AdCSV) to measure iron ligand concentrations in solution with novel developments in chromatographic recognition and quantification of siderophores to shed further light in our understanding of iron speciation in the ocean, in this case in the plumes of hydrothermal vents. The goal of the chromatographic section is to detect a broader range of these compounds for which a careful description of the level of confidence in the assignment of molecular formulae is described. The manuscript is well-written and accessible to specialists.

However, although the experiment planning and the resulting database are extremely valuable for the scientific community, I have strong reservations about the interpretation of the results, particularly in the electrochemical part and I am going to suggest major changes. Whole sections should be thought through using the arguments hereafter and followed by rewriting of many sections. Accordingly, I would only consider this manuscript as a letter if those changes are implemented.

My major concern with the current version is the assumption that the outcome of CLE-AdCSV represents "organic" ligands' concentration and stability constant. Unfortunately, a wrong assumption, once established, might be challenging to reverse. In waters with high terrestrial dissolved organic matter (DOM) input or significant biological activity (resulting in ligand exudation), this assumption might be close to the actual situation. However, it cannot be universally granted. The technique involves introducing an artificial ligand that equilibrates with the sample (CLE step), extracting a fraction of the iron in solution, and then measuring the concentration bound to the artificial ligand (the electrolabile fraction, the AdCSV step). If there are no sequestering natural ligands, the artificial ligand recovers the entire iron concentration, and electrolabile iron equals total. If all iron is so refractory bound that it does not exchange at all overnight in contact with the artificial ligand, the concentration of electrolabile iron is zero, and all iron is considered complexed with a stability constant that should be infinite ($K \sim \propto$) but that the analytical window limitation of the technique forces to fall inside this window. Real samples exhibit an intermediate situation, with a partition between iron complexed to the original ligands and the added ligand, dependent on the concentration and stability constant of both ligands.

It is evident from this description that CLE-AdCSV cannot discriminate between organic and inorganic fractions, only between "labile" and "refractory" fractions competing for exchange with the added ligand. Historically, since the oceanographic studies in the 80s, researchers assumed that the partially exchanged fraction should mostly be of organic origin, especially since the technique was initially used for copper, which has no solubility issues. As the field started the study of iron in the 90s, this assumption was carried over and it was assumed that inorganic oxyhydroxides do not contribute to the partially exchangeable fraction, as their concentration is presumed to be very small and their stability very small compared to organic ligands. However, this is hardly compatible with the well known low solubility, formation of aggregates

characterized by very different reactivity as a function of aging, estuarine trapping and open ocean scavenging suffered by iron.

It is certain that the manuscript's assumption about the organic complexation of iron in hydrothermal fluids relies on the literature. However, this stems from the assumption that CLE-AdCSV experiments exclusively determine "organic" ligands (Buck et al., 2018; Kleint et al., 2016; Sander and Koschinsky, 2011). This assumption is highly unlikely in hydrothermal waters with surges of Fe(II) at substantial concentrations. Fe(II) quickly oxidizes (hours-days at the local temperature and pH), creating stable colloids that continue to grow until reaching a size that induces precipitation. And no local nanomolar concentration of ligands can prevent this precipitation of micromolar iron. In this scenario, if the sample for CLE-AdCSV contains any inorganic phase smaller than 0.2 µm, that does not undergo complete solubilization overnight by the extracting effect of the artificial ligand, it will be counted as a ligand and labelled as "organic". This is very unlike in this case. Some ligand concentrations presented here are of the same order of magnitude as those measured in cultures using cell growth media and an order of magnitude higher than iron ocean fertilizations. This is very suspitious.

In my opinion, considering that part of the ligands may be inorganic would explain 1) the high ligand concentrations, 2) the high correlation of ligands and dissolved iron independent of any biological variable, 3) and the high concentration of ligands in the absence of primary producers that could support the ligand production of bacteria. Actually arguing fot the presence of inorganic fractions in the definition of ligands, would drift from the current paradigm and add extra relevance to the manuscript.

Response: The reviewer brings up important historical context for CLE-AdCSV measurements for dissolved iron in seawater. We agree with the reviewer that inorganic dissolved iron (< 0.2 µm) is very important in hydrothermal environments (such as truly dissolved Fe(II); or small inorganic Fe colloids that pass through a 0.2 µm filter) and it was not our intention to emphasize that organically-bound dissolved iron is the only fraction that is important (or dominant) in hydrothermal systems. However, the relative importance of organic and inorganic iron speciation in hydrothermal vent systems is significantly impacted by where the samples are taken in the vent environment. The reviewer is correct that when iron is expelled from the vent orifice, it is present in micromolar concentrations and primarily in the reduced form, and upon mixing with oxygenated seawater the iron forms inorganic colloids (of various sizes) that precipitate next to the vent and are quantitatively lost from solution (Toner et al. 2009a, Yücel et al. 2011). However, as the plume becomes neutrally-buoyant and has mixed with oxygenated seawater, the iron that remains in the plume is largely the dissolved iron that "escaped" this flocculation process. The samples from this work are all primarily from the neutrally-buoyant plume, after much of the inorganic iron flocculation has occurred. Several studies have found that a significant fraction of this dissolved iron in the neutrally buoyant plume that remains after the initial precipitation of particulate iron near the vent source is organically complexed (Bennett et al. 2008, Sander et al. 2011, Fitzsimmons et al. 2017, Buck et al. 2018, Fitsimmons et al. 2014, Hawkes et al. 2013). Additional precipitation of iron can of course continue to occur during the advection of the neutrally-buoyant plume, however several studies (included those references above) have shown that the organically-bound fraction of dissolved iron is important in the neutrally-buoyant plume, even at (relatively) high concentrations of dissolved iron. Studies

which have examined both organic and inorganic speciation of dissolved iron in the same neutrally-buoyant plume have also noted the co-existence of both inorganic and organic forms of dissolved iron (Fitzsimmons et al. 2017). Indeed, even the particulate iron present in many neutrally-buoyant plumes is covered in organic compounds containing various functional groups (Hoffman et al. 2020, Hoffman et al. 2018, Toner et al. 2012, Toner et al. 2009b).

So while we recognize that inorganic dissolved iron is also very important to consider in these environments, we believe that these established methods for measuring organic iron speciation in seawater in the neutrally-buoyant plume are reasonable and our results align with several other papers that have already been published in this field. What is unknown however, is whether the some of the organic ligands that we observe are also associated with small inorganic colloids in a complex organic colloidal matrix. We suspect that in these environments there is a mixture of defined ligands such as the siderophores we identified via mass spectrometry, and also other organic ligands that are no more than functional groups present on natural organic matter that are capable of binding iron, whether also associated with a complex inorganic or organic colloid or not. We have added some additional details in the manuscript that makes it more clear that we are working primarily in the neutrally-buoyant plume of these systems, and that the complexity of the remainder of the organic ligands we measured with CLE-AdCSV is unknown beyond our operational definition of ligand classes. For example, the first paragraph of section 2.1 ends with,

"The strong coupling between dFe and ligands was only observed at sites where L_1 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 $K^{cond}_{Fe',FeL}$ < 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."

Further comments on the technical details on the CLE-AdCSV method specifically are noted in more detail below.

Other concerns:

- There is a non explicit, but implicit, argument pointing to siderophores as a "major" part of the so-called L1 (my interpretation). This is not based in literature where there is no evidence of such, it seems very unlikely (siderophore production is very energy demanding and bacteria requirements are not that high, and the bibliography used is very one sided to this argument. In my opinion the ecological importance of siderophores is paramount but, since the fraction of iron complexed by siderophores in minor (<2% with our current analytical tools), siderophores should not be considered a major driver of iron cycling. No author has found important concentrations of siderophores to justify this relevance (Bundy, Boiteau, Gledhill, Ahner and so on manuscripts)

<u>Response</u>: 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 L_1 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). Previous

studies have observed a tight coupling between strong ligands and dissolved iron in the neutrally buoyant plume even at great distances from the vent source (Buck et al. 2015, Buck et al. 2018), and this study adds to those observations by showing a similar relationship across many distinct vent systems and shows that a subset of these strong ligands are siderophores. While we do not argue that siderophores are not comprising the entire strong ligand pool that we observe, we suggest that their presence (and their similar relationship with dissolved iron) points to a mechanism for the tight coupling between ligands and iron in these systems. The detection of siderophores as part of the strong ligand pool suggests that at least some of the organic ligands binding dissolved iron in the neutrally-buoyant plume could be actively microbially-produced within the plume, and play a role in stabilizing dissolved iron in these systems. This observation is also consistent with rapid exchange between the dissolved and particulate phase in these systems (Fitzsimmons et al. 2017) and with genetic evidence that siderophores are actively produced in hydrothermal vents (Li et al. 2014). Additionally, for many reasons, we think the quantification of siderophores we present in this work are a lower bound on their concentrations and therefore their contribution to the total L_1 pool (see section 2.2). We have amended section 2.4 to make sure we are clear that we do not think all of the L_1 ligands are siderophores.

- The authors do not seem aware of the limitations of the technique and the limitation caused by the existence of an analytical window. In table S3 stability constants (K1 to K3) are spread in almost 5 orders of magnitude. Such accuracy is impossible, it would mean negligible analytical error. The analytical window is approximately 4 orders of magnitude wide (depends on the error, there is related bibliography from Apte, van den Berg, Pizeta, Laglera, Gledhill and Gerringa) and K fall inside the range, cannot fall on the very edges or beyond like in a few cases here. Could this be related to the use of salycilaldoxime as competing ligand (recent Geriinga et al BG manuscript)?

  Response: We are aware of the limitations of the analytical window in CLE-AdCSV measurements, and all ligand parameters reported were confirmed to be within the analytical window of the SA measurements. The analytical window is not only defined by the logK of the ligands, but instead by the side reaction coefficient, α, which is equal to the concentration of the ligands multiplied by the conditional stability constant. If the α_{SA} of the added ligand is within an order of magnitude of the α_L of the natural ligands (van den Berg and Donat 1992), then the results are deemed to fall within the analytical window of the method. This was confirmed with all results that are presented in this study. This information has been added to the supplementary methods (section 3.2). A recent paper that details the use of SA as a competing ligand and its accuracy for measuring model organic ligands is also now available in Marine Chemistry (Mahieu et al. 2024).

- In the methodology section the authors state that "forward" and "reversed" titrations were performed but this is not shown in the results section neither discussed. Is that high K1 coming from reverse and low K3 from forward titrations? Results form both treatments cannot concur if more than one ligand (as it is 100% the case here) are present and a separate discussion and comparison are due. This must be added to a future version

  Response: Yes, both forward and reverse titrations were performed in this study. Forward titrations were performed initially for most samples, and if the samples lacked curvature in the

titration (a sign that there are either no ligands present, they are outcompeted, or they have already been saturated with iron) then a reverse titration was performed. The results of the L_1 ligands in the neutrally-buoyant plume, which is the focus of this manuscript, were all measured using forward titrations. There were a few samples that were not in the neutrally-buoyant plume, where dissolved iron was greater than the ligand concentrations, and these were measured using reverse titrations. These samples are presented in the supplement for completeness, but were not the focus of the main text. We have noted in the supplement table S2 which samples were analyzed by reverse titration via an asterisk in the "GT#" column. This has also been more clearly described in the table description.

- Self citation is excessive and the authors give as settled some particular arguments that are not accepted by the whole scientific community, example the referred prominence of siderophores as source of strong ligands in the ocean. I think that papers should reflect and discuss other visions in the field.

  Response: We are unsure if the reviewer is only referring to the citations pertaining to siderophores, which unfortunately have been competed by very few labs and authors. In terms of additional references for potential sources of strong ligands, we have added some discussion of humics and exopolysaccharides and have cited the appropriate studies for this discussion (Lines 52-53).

  I know that this is quite radical but I would suggest to mend the interpretation of the voltammetric data, commenting on inorganic complexes and discussing forward and reverse and focus on the interesting chromatographic finding. The paper must be put into the context of the ecological relevance of these finding more than in the relevance for iron cycling since the siderophore concentrations found here apparently only binds a very minor fraction of the iron concentration in solution.

  Response: We have edited some parts of the discussion to put into context the siderophore data with other studies that had previously focused on iron dynamics closer to the vent source, and the partitioning between inorganic and organic iron phases (e.g. Lines 221-226).

  As I was reading I took some note that should be of interest to the editor and authors. I attach them, part are a repetition of what I stated above:

  48 I find here that self-citation is a bit excessive, there are more people involved in this type of studies

- For being a L1 it must be a L2. This is based in results from a particular technique that do not match results from other CLE-AdCSV protocols. I mean that other analytical approaches do not always measure L1 and L2 using a different artificial ligand. Moreover, when log K is outside the analytical window, the limitation of the analytical window brings the value inside. The classification of ligands as a function of log K is not a sensible strategy.

  Response: Please see the response above regarding the analytical window. 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).

53 again self-citation. There are many more studies about transition of iron from estuarine waters to the sea that so not concur with this vision. The importance of humics (that I assume from the authors' previous publications that they consider weak ligands) has been well established in many studies (Laglera/van den Berg, Slagter, Yang and Muller studies by CLE-AdCSV and many other studies using fluorescence, coprecipitation). Other studies have found that transport is a function of the molecular weight of the ligand with prevalence of smaller fractions. The process and visions of different research groups are quite more diverse than simplified here.

Response: We agree, there is a rich field of iron dynamics in estuarine studies that unfortunately is not the focus of this manuscript. We meant only to provide an example of different high iron system where strong ligands have also been shown to play an important role in preventing dissolved iron precipitation and flocculation.

60-64 please revise grammar

Response: Grammar has been fixed in this section.

66 see my previous comment about organic ligands and hydrothermal fluids

Response: We were not sure what you were suggesting here, but the sentence now reads *"Although other unknown strong Fe-binding ligands have been observed in hydrothermal plumes and throughout the deep ocean (Buck et al., 2018), and siderophores have also been observed below the euphotic zone (Bundy et al., 2018), no previous studies have ever directly characterized siderophores in hydrothermal systems."*

71 word repetition

Response: The extra word was removed

72-76 impressive range of sampling sites with different physicochemical conditions. This gives relevance to the manuscrit

Response: We appreciate this feedback from the reviewer.

Appendix 226……. Methods

245 the concentration of buffer is possibly wrong. It should be millimolar and not micromolar. This concentration would not buffer at all against the bicarbonate natural buffer, let alone against the huge formation of hydroxides inevitably associated to the polarographic analysis of oxygen saturated solutions. If the buffer was settled at such concentration, the analysis was carried out at pH close to 9 (Laglera et al 2016)

Response: We thank the reviewer for your comment and for catching this error. We have confirmed that 7.5 mM borate buffer was added.

10 micromolar SA seems a compromise solution between the concentration suggested by Abualhaija and van den Berg (5 uM) and the concentration traditionally used by Buck and collaborators (25 uM). Since doubts about the use of SA increase (Gerringa et al BG paper) it is not clear that the effect of the Fe(SA)2 complex has been removed and not counted as L3. It would be good to show a linear titration of UV digested seawater in this condition to rule out such effect.

Response: The use of 10 µM SA was chosen in this environment both as a compromise from the findings of Abualhaija and van den Berg (2014) and also to ensure that we were using an appropriate analytical window to detect the range of ligands present in these samples from preliminary tests. This analytical window has been used previously and if indeed $Fe(SA)_2$ is not electroactive like Abualhaija and van den Berg (2014) and Gerringa et al. (2021) suggest, then the main impact on the analyses is a loss of sensitivity but not the detection of an artificial ligand class. Recent work by Mahieu et al. (2024) was able to determine accurate ligand parameters for DFO-B using 25 µM SA.

254 here the boric acid is at the same low concentration which makes me think that perhaps the buffer was not correctly implemented and the analysis was carried out at a very basic pH (at the surface of the electrode). For instance Hawkes et al (2013) 5 mM in each aliquot (50 mM in the paper is wrong).

Response: As noted above, the buffer was correctly implemented and this was simply a typo.

What was the pH of the solution here? NN is supposed to work only at low pH (8 or less) according to the intial Gledhlii/van den Berg papers. This pH is so far away from the pK of the buffer (close to 9) and the buffer concentration so low that its buffering effect would be null. This is usually detected by changes in the peak potential. Can the authors compare peaks for this work with peaks obtained in studies with higher concentrations of buffer?

Response: The samples were buffered as in previous studies, and the pH of the samples were appropriate for this method. The peaks obtained looked very similar to previous work.

260 I could not find in Hawkes et al (2013) any reference to $\chi min = 0.8$, $\chi max = 0.9$, and $c1high = 0.75$. What are these constants and what is the implication of fixing them at this value and not other? I found them in the R script and although I am not expert in R it seems that the authors of the R routine suggested to use 0.9 or 0.8 as maximum value reached during the RV if the shape of the curve was not that of a double michaelis-menten. I know the topic of this manuscript is not to criticize such but it all looks very arbitrary to me and not sure how much change in L and K would bring a change of value here. I do not suggest to recalculate anything but the method used is a bit arbitrary in the assignment of constants.

Response: The selection of these constants in the R code were not thoroughly explained in the original Hawkes et al. (2013) manuscript, but we have consulted with the authors about this and have chosen these values appropriately. We added them to the methods here for transparency for further studies.

265 onwards: I congratulate the authors for the effort to apply an internal quality criterion. This is randomly the case and improves greatly the relevance of this work.

Response: We appreciate the reviewer's feedback.

266 why the result is called L1? Is there L2 in samples?

Response: We changed this sentence to read, "In addition to measuring Fe-binding ligands by voltammetry, we also identified and quantified siderophores."

310 "siderophore concentrations reported here are estimates of siderophore concentrations in these environments based on ferrioxamine E." although this is obviously a strong limitation, possibly this is the only way to move forward. In cases like this applied to concentrations obtained by means of other techniques, cconcentrations of other siderohores are reported in DFOE equivalents and not simply as nM. Hopefully, at the time to evaluate total siderophore concentrations, overestimations and underestimation may compensate but it would be interesting to evaluate whether DFOE gives sensitivities around the average for all compounds commercially available. Because if DFOE is particularly more or less sensitive to the detector, the authors would incur in substantial over or under estimations of concentrations for other siderophores. Was this considered at the time to select DFOE? A comment should be added

Response: We thank the reviewer for this comment and recognize the limitations associated with this technique. Ferrioxamine E was chosen as it is one of the few commercially-available siderophores, and it has been used as the quantitative standard in past LC-MS studies (e.g., Boiteau et al., 2016). We agree with the reviewer that currently this is the way to move forward and that future work is needed to isolate/obtain more siderophores to test the recovery and ionization efficiencies of more compounds.

"Thus, the siderophore concentrations reported here are estimates of siderophore concentrations in these environments based on ferrioxamine E"

was amended to:

"Thus, the siderophore concentrations reported here are estimates of siderophore concentrations in these environments based on ferrioxamine E, chosen for its commercial availability and use in prior studies (e.g., Boiteau et al., 2016)."

and

"Limited sample volumes prevented analysis via LC-ICP-MS like previous studies, which, in addition to greater availability of commercial standard, would allow definitive identification in future work."

was amended to:

"Limited sample volumes prevented analysis via LC-ICP-MS like previous studies, which, in addition to greater availability of commercial standards and more analytical comparisons between ferrioxamine E with other siderophore types, would allow definitive characterization in future studies."

80 this sentence is 1) not based in a prior understanding of CLE-AdCSV in the case of 2018 Buck; as I referred before, the technique does not discriminate organic or inorganic ligands 2) not based in any experimental evidence in the case of the other two papers that are one a review and the other one a model where CLE-AdCSV ligand have been added.

Response: Please see discussion of this point above. 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.

83 onwards. Although the argument about complexation seems right and coherent, again a concentration of 10-90 nM ligands are 1 to two orders of magnitude higher than observed in very concentrated cultures or fertilization at any growth stage. Since hydrothermal plumes are not watermasses especially abundant in biomass, the biological release of tens of nanomols per litre of "organic" ligands is extremely unlikely. This would be energetically absurd, to release ligands for concentrations that are well over the iron requirement. That some aged/stabilized oxyhydroxides and/or iron sulphides are part of the sample is a more likely explanation.

Response: Elevated ligand concentrations have been found in several oceanic environments (e.g. Kleint et al. 2016), and we are not suggesting that these are all siderophores or discrete ligands secreted by bacteria in these systems. We, like other studies, suggest that there are ligands in these systems that are capable of binding iron and they could simply be natural organic matter present in the deep ocean containing functional groups capable of binding iron. As mentioned in your initial comments, we have tried to amend the manuscript to make it clear that we are not at all suggesting that all of the ligands we detected are siderophores.

Log K3 values around 8.8 are difficult to reconcile with what we know about analytical windows and CLE-AdCSV. This is especially true if the authors claim that can resolve ligands of log K 12-13 and 9-10 (separated 3 orders of magnitude) from the same titration. It would be a mathematical artifact

Response: Please see the response above regarding the analytical window.

94-96. Again there is only self citations about rivers where there is no consensus about and there are available results from other groups that differ substantially with the processes described here. In any case it is good that the adjective organic dropped in this paragraph.

Response: Please see the response above regarding estuarine iron cycling.

100-101 Again self citation. Recent evidence shows that a fraction of humics of riverine origin compete with siderophores for dFe (Slagter and Laglera papers in Arctic waters). Moreover, I insist that stabilized/growing inorganic fractions (of no biological origin) could be found in the L1 fraction and in the physicochemical conditions described here, constitute most of L1.

Response: We did not intend to say that humics are not important in riverine and estuarine systems. We merely included an example here of studies that found strong ligands to be important in another high iron environment.

101-103 all these processes are no doubt present, but very unlikely to produce L1 ligands in the order of tens of nM.

Response: Please see the response above regarding high concentrations of ligands.

107 None of the Cowen references include ligands measurements or even include the word ligand. The Lauderdale paper is a modelling paper and does not constitute empiric evidence. The bibliography does not support the argument

Response: The first few sentences were amended to,

"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)."

118-129 this section is very speculative and as such should be remarked. please remove significant since this term implies some statistics behind and this is not the case, it is just a speculation. The Hider and Kong reference is a review and only speculates about whether more products are expected. My problem here is that the paragraph is based in repeating a speculation. Other sources of L1 referred to in the bibliography do not deserve even a mention (humics, EPS, etc)

Response: We appreciate the reviewer's feedback and have edited this paragraph to include more evidence for this claim. Recent advances in genome mining are beginning to reveal more information about the potential for metallophore production in nature and predict a much higher diversity of siderophore-like products than is currently understood.

Reitz et al., 2022 (doi.org/10.1101/2022.12.14.519525) has been added as a reference, which predicts a high diversity of currently-uncharacterized siderophores with genome mining.

Significant has been replaced with substantial.

"That is, known siderophores represent a small fraction of what is expected to be produced in nature."

was amended to the following to add clarity to the claim:

"Recent work on siderophore biosynthesis pathways and advances in genome mining suggest that known siderophores represent a small fraction of what is expected to be produced in nature."

126 this calculation is addressed to increase the relevance of the paper but again is very speculative. A factor of 10 was found for overall ligands but the factor for siderophores following the evidence presented here should be 2.5. If the range in line 118 is increased ten fold, the range is 0.2-4% but it would be fairer to use a range about 0.03 to 0.1 %.

Response: We thank the reviewer for this comment. As the reviewer noted, Bundy et al., 2018 found 5-10% extraction efficiency for the bulk ligand pool and Waska et al., 2015 found 40% extraction efficiency for one siderophore. We believe these are fair upper and lower bounds for expected efficiencies. Bundy et al., 2018 was obtained with comparison to CSV results, as this study employs, while Waska et al., 2015, was obtained through analysis of one compound. We have adjusted the line to include ranges using both efficiencies for completeness.

The estimate now reads:

"Employing both corrections yields siderophore contributions to the total L1 pool of 0.1-4% and 0.025-1%, respectively."

132-133 I agree but a reference would be nice here.

Response: We added the reference Rizzi et al. (2019). The new line number is 183.

Rizzi, A., Roy, S., Bellenger, J.P. and Beauregard, P.B., 2019. Iron homeostasis in Bacillus subtilis requires siderophore production and biofilm formation. *Applied and Environmental Microbiology*, *85*(3), pp.e02439-18

138-139 apart of bringing back again the argument that the technique cannot measure "organic" L1, since the contribution of siderophores to L1 is estimated by authors as 4% tops. This is less than the CLEAdCSV error, that it is very difficult to bring down to ~5%, there are simulations at different error level of copper titrations in the literature. This uncertainty and low contribution would impede any statistically robust contribution of siderophores to the coupling of L1 and dFe. For that, siderophores should be a substantial contribution to L1 and their concentrations be well above the analytical error in the determination of both dFe and L..

Response: As mentioned above, we note in this work that we think we are measuring a lower bound on siderophores, but we do not state anywhere in the manuscript that we think all L_1 ligands are siderophores. We do, however, think that the patterns in the siderophores we were able to identify are likely representative of broader patterns in otherwise unknown siderophores,

so although we are likely missing some compounds, we believe that the siderophore concentrations would still likely scale with the L_1 ligands.

142-153 I like this paragraph and its finding, implying somehow more biodiversity in on-axis locations (assuming a wider variety would imply more bacterial species). The problem is that the relevance would be diminished if the last paragraph stands as it is. If the fraction of siderophores found is a minimum fraction of the total, these variabilities of small fractions would be irrelevant. I suggest to reduce the number of previous speculations and leave this paragraph as it is.

Response: As mentioned above, although we are missing some siderophores we still think it is interesting that we see siderophore diversity patterns that vary by site type. As the reviewer notes, we suspect this is driven by the microbial community diversity. We think it is an interesting finding and have left this observation in the manuscript.

160-161. Not so surprising if most of L1 is very refractory/low bioavailable inorganic iron released by the vent and stabilized in the oxic environment. Bacteria would need to solubilize a fraction of such iron and the likely mechanism would be siderophore release.

Response: Yes we agree.

169-170 this paragraph fits with the explanation that part of what is interpreted here as L1 is inorganic (<0.2 um) refractory iron.

Response: As discussed above, this L_1 pool could be a complex mixture of inorganic and organic phases, and both inorganic or organic pools of dissolved iron can potentially be refractory or inaccessible for microbial uptake (e.g. Manck et al. 2022). This has been explained in more detail in section 2.4.

175-182. I assume there were no bacteria counts in particles or free living. Particles in the ocean are hot spots of bacterial activity. It could be that this difference here in siderophore producers it is simply a matter of bacteria density.

Response: We agree, and unfortunately we do not have flow cytometry data of bacteria abundances.

202 In my opinion tis argument that concentrations of units to tens of nM of iron cannot be enough to suppress siderophore production. It is clearly a matter either of passive siderophore production (continuous production, and not a response to low iron concentrations) or that the bioavailability of iron is reduced which would make more sense if this is inorganic. Pleas rewrite this section

Response: As mentioned above, iron does not need to be inorganic to be inaccessible and lab studies have shown that a bacteria might produce a siderophore even if it can grow on a given iron source, but the source is slightly refractory (Manck et al. 2022). This is interpreted as siderophores being used by bacteria as a strategy for acquiring iron from iron sources and less

bioavailable, and is the argument we are using here. We did not imply that siderophores are being produced due to low iron concentrations, and instead suggest that hydrothermal vent systems are systems that favor the production of complex iron pools (e.g. a mix of organic and inorganic phases) that might be inaccessible to bacteria. We have clarified this in this section.

Buck, K. N., P. N. Sedwick, B. Sohst, and C. A. Carlson (2018), Organic complexation of iron in the eastern tropical South Pacific: Results from US GEOTRACES Eastern Pacific Zonal Transect (GEOTRACES cruise GP16), *Mar. Chem.*, *201*, 229-241.

Kleint, C., J. A. Hawkes, S. G. Sander, and A. Koschinsky (2016), Voltammetric investigation of hydrothermal iron speciation, *Front. Mar. Sci.*, *3*, 75.

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