

Interactive comment on “Cryptic role of tetrathionate in the sulfur cycle: A study from Arabian Sea oxygen minimum zone sediments” by Subhrangshu Mandal et al.

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Anonymous Referee #2

Referee's Comment: The authors investigate the capacity of microorganisms in sediments from the Arabian Sea to metabolize redox-intermediate sulfur species. This is an important question with significance for understanding carbon turnover in sediments as well as for interpreting sedimentary records, in which these rapid processes are often invisible. The study relies primarily on the successful culturing of organisms with at least the facultative capacity to metabolize tetrathionate, and on metagenomic and metatranscriptomics datasets for a series of depths in two cores. The data are

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interesting and would fill an important gap in the literature, if the authors can address possible methodological concerns below. A more serious issue, however, appears in the argument regarding tetrathionate production from pyrite. This idea is not present in any of the three papers cited, which evidences a serious misunderstanding about this process and causes me to recommend rejection of the paper in its current form. Additional comments and suggestions follow.

{Note – I am not able to speak to the appropriateness of the metagenomic methods described here, since this is outside my area of expertise.}

Authors' Response: We thank the Reviewer for these comments, and believe he appreciated the underlying science of this study. We also agree with most of the concerns pointed out subsequently, so have now dealt with each one of them in course of this extensive revision.

So far as the citation oversight is concerned we have already tendered our apologies and explanations for the same during the open review process Furthermore, it has also been conveyed through those series of communications that we have now removed all those portions of the manuscript which discussed the potential pyrite-derived and MnO₂-driven tetrathionate production in marine sediment (as the data in hand were not fully adept in handling that issue) and based all the discourse on origin of tetrathionate on the sound microbiological data. The relevant Specific Comments of the Referee (with respect to Line 492, and then Line 498), Authors' Initial Responses, Reviewer's Subsequent Comments, and finally Authors' Subsequent Response have all been appended below for your kind perusal.

Referee's Comment: General comment – for reading clarity, there are many places where codes could be simplified. You only have two cores – they could be referenced as A and B, or by collection depth, much more readably than SSK42/5 and SSK42/6. The names of slurry media types are similarly difficult to read (e.g. was 'T' tetrathionate or thiosulfate?).

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Authors' Response: We agree that simpler nomenclatural codes could have been formulated for the sediment-cores. However, the purpose of adhering to the present nomenclature based on the cruise identity (RV Sindhu Sankalp, SSK42) is to maintain referral consistency across the series of papers we are currently publishing based on various biogeochemical aspects of the ten different cores collected on board SSK42, and named SSK42/1 through SSK42/10. For instance, the same SSK42/5 and SSK42/6 have been analyzed (alongside SSK42/1 through 4, SSK42/7 and SSK42/8) from separate perspective in Fernandes et al., 2018; so we believe that the readers should be allowed to clearly identify that these are the same 5th and the 6th cores of the SSK42 cruise that were also investigated and reported in Fernandes et al., 2018.

As for the nomenclature of the media types, please note that throughout the manuscript, codes for thiosulfate-containing media ended with 'T' while those for tetrathionate-containing media ended with 'Tr' (and not 'T'). However, in the revised manuscript, we have now incorporated further clarification for the basis of this nomenclature by stating as follows. "For each experiment testing the formation or oxidation of tetrathionate, 10% (w/v) sediment-sample was suspended in artificial sea water (ASW) supplemented with thiosulfate (T) or tetrathionate (Tr), i.e. ASWT or ASWTr broth medium (Alam et al., 2013), respectively; the culture flask was incubated aerobically at 15°C on a rotary shaker (150 rpm)."

Referee's Comment: Bulk sediment porewater geochemistry – please consider converting information in Table 1 to a pair of depth profile figures, one for each site. Please provide an appropriate number of significant figures for your data (you are currently reporting sub-nM precision).

Authors' Response: We agree, and have now included depth profile figures in the revised manuscript and also provided an appropriate number of significant figures for the data (notably, the current manuscript does not report any concentration in sub-nM precision). As for significance of values, in the revised manuscript, we have now mentioned (i) the different concentrations of sulfate, sulfide and thiosulfate that were

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measured for the construction of the corresponding calibration curves, and (ii) what maximum percentage of deviation was found from actual concentrations for sulfate, sulfide or thiosulfate based on triplicate analyses of the respective sets of standards.

4. Thiosulfate and sulfide concentrations – No details are provided about how porewater sampling was conducted, or how porewater samples were chemically preserved. (Were zinc acetate, bromobimane, or other preservatives used? How much time elapsed between collection and IC analysis? What transformations might have occurred among your redox-sensitive dissolved species?)

Authors' Response: We have now overhauled the portion dealing with sampling details (including those for pore-waters) in such a way that all confusions emanating from the previous text are now resolved. This said, it is noteworthy that answers to these questions were already there in our previous publication [Fernandes et al., 2018, Enhanced carbon-sulfur cycling in the sediments of Arabian Sea oxygen minimum zone center. Sci. Rep. 8: 8665] which also dealt with these SSK42 cores (albeit from other perspectives) and was cited a number of times within the sampling-related section of the present manuscript. We had therefore thought that further repetition of the details would be redundant. However, now we understand that as an independent paper this manuscript should carry its own sampling details and have therefore brought back the necessary details taking sufficient care of literary repetitiveness; for instance, we have now mentioned in this revised manuscript also that (i) sodium azide was used to arrest further microbial activity within the samples for chemistry, (ii) all IC analyses were completed within one week of retrieval of the samples to the laboratory on land, and (iii) all the pore-water vials were crimped immediately after N2 flushing, and stored at 4 °C until further analysis.

Referee's Comment: Depth trends - Figure 1 seems to show that all five categories of relevant genera increase in their relative proportion smoothly with depth, which seems like it could be to first order a change in dilution of the microbial population with organisms that prefer the shallower sediments (aerobes or those that subsist on fresh,

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relatively shallow organic matter). The significance of this first-order depth trend needs to be discussed separately in the discussion. Standard depth profiles of the five genera groups would be much easier for the reader to interpret.

Authors' Response: We completely agree with your appreciation of the depth trends, so in the revised version of the manuscript we have now discussed all these issues under a new section titled "Trends of geomicrobial parameters down the sediment-depths corroborated sulfur cycle functions centered on tetrathionate".

Referee's Comment: A more thorough description of how cores 42/5 and 42/6 differ sedimentologically would improve the analysis, especially since the key correlations from Figs 1 and 2 are only strongly significant in one of the cases.

Authors' Response: In the revised manuscript we have now appended a thorough explanation as to why the key correlation coefficients such as those indicated in Figs 1 and 2 are only strongly significant in case of SSK42/5 and slightly weaker for SSK42/6. As you would see discussed in the revised manuscript, relative abundances of metagenomic reads ascribed to the genera of tetrathionate-forming, oxidizing, and respiring bacteria also fluctuate more or less synchronously along SSK42/6, excepting the region between 250 and 275 cmbsf (Fig. 2), which is the sulfate-methane transition zone (SMTZ) of this sediment-horizon (notably, SMTZ in the SSK42/5 sediment-horizon laid below the 280 cmbsf sediment-depth explored in this core; see Fernandes et al., 2018 for the methane profiles of the SSK42 cores). Whilst lack of synchrony in the lower end of this core resulted in relatively weaker correlation values as compared to those obtained for SSK42/5 (Table S7), we hypothesize that the changes in geochemistry and community architecture associated with the advent of SMTZ potentially impact the in situ population ecology of the tetrathionate-metabolizing bacterial types also. Sedimentation rate, age-depth profile and other geochemical features of the two cores separated by a distance of only one kilometer are otherwise largely comparable.

Referee's Comment: Line 267 – I read this statement to mean that some represen-

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tatives of each of these groups have been observed to cycle tetrathionate. Clearly, though, that does not apply to all members of these broad taxonomic groups, which makes it very difficult to tell whether the organisms might generally or facultatively cycle tetrathionate or something else entirely. Are statistics available on what fraction of, say, *Salmonella* the genetic machinery for tetrathionate conversion has?

Authors' Response: Line 267 did not deal with the kind of issues that you have mentioned here as it actually encompassed the Materials and Methods section. However we presume that your concerns are centered on the articulations that were made in lines 372-411. There, it has been clearly distinguished (already in the initial manuscript) that for one category of genera each and every member strain in the literature is known to possess tetrathionate-forming/oxidizing property, so the presence of such genera is more definitely indicative of the concerned processes *in situ*, whereas for another category of genera only some (and not all) member strains are known to possess tetrathionate-forming/oxidizing/reducing property, so their presence indicates further additional possibilities of such processes *in situ*. Furthermore, it may be noted that to keep this discrimination explicit, trends of relative abundance for the first category were depicted in Figs. 1 and 2 (these data are clearly free from diversity/abundance over-estimation), while those for the second category were all presented separately in Supplementary Tables S8-S13 (these data are likely to involve unknown proportions of diversity/abundance over-estimation, and so have been kept in isolation from the definitive estimates given in Figs. 1 and 2).

We have now edited the text in such a way as to make these issues more clearly comprehensible.

Referee's Comment: Line 423 –Unless the data is included here, your own unpublished conclusions can't be cited like this.

Authors' Response: For these data illustrating the feasibility of aerobic metabolism in these sediment horizons, please note that the same constitute a completely separate

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paper of our group, under consideration elsewhere, and those data are too voluminous to be incorporated here. Anyway, we have added in this revised manuscript that genes for aerobic respiration by aa3-/cbb3-type cytochrome-c oxidases (coxABCD / ccoNOPQ) and cytochrome-bd ubiquinol oxidase (cydABX / appX) were identified in the assembled metatranscriptome from 275 cmts of SSK42/6 in general, and the portions of the metatranscriptomic dataset in particular which matched with sequences from the tetrathionate-oxidizing isolates, thereby suggesting that potential activity of this aerobic metabolic process is possible in this environment (the relevant gene and transcript Tables have also been incorporated in the revised manuscript as Supplementary Materials).

Referee's Comment: Much of section 3.3 is a reporting of results without context or discussion, which is challenging to parse for key points – consider separating out your results. Figures would be very helpful. How do these rates compare with other culture studies or with the size of the porewater pools? What are these depth patterns? What do you want your reader to gain from this information?

Authors' Response: We agree that discussion of the slurry incubation data in the context of the rest of the results would enrich the manuscript, and so have done the same under a new Discussion section 4.1 titled "Trends of geomicrobial parameters down the sediment-depths corroborated sulfur cycle functions centered on tetrathionate". Furthermore, as per your suggestions, we have now incorporated new figures to depict the depth trends of the slurry incubation data. This said, it may be noted that the main purpose of the slurry incubation experiments were to check whether the tetrathionate-metabolizing bacteria are alive *in situ*. Accordingly, the two Result sections 3.3 and 3.4 were (and are still) titled as - The tetrathionate-forming/oxidizing microorganisms of the ASOMZ sediments are alive and active *in situ*, and - Active tetrathionate-reducing microorganisms in ASOMZ sediment, respectively. Notably further, within 3.3 and 3.4, the "live and active" issue was addressed by pure-culture isolations and metatranscriptomics in addition to the slurry incubation experiments.

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Referee's Comment: The title of section 3.3 isn't quite true – these experiments show the presence of living organisms that can at least facultatively do these metabolism; it does not show that they are actively conducting these metabolism *in-situ*. Can you integrate this conclusion with your RNA results or show actual *in-situ* abundances of your cultured organisms?

Authors' Response: We thank you for this nice suggestion and believe the kind of data recommended would go a long way in making the conclusions robust. Accordingly, we have carried out whole genome shotgun sequencing and annotation for the three tetrathionate-forming isolates *Halomonas* sp. MCC 3301, *Methylophaga* sp. MTCC 12599 and *Pseudomonas bauzanensis* MTCC 12600; the two tetrathionate-oxidizing isolates *Halothiobacillus* sp. SB14A, and *Pusillimonas ginsengisoli* MTCC12558; and the tetrathionate-reducing isolate *Enterobacter* sp. RVSM5a. Subsequently we have mapped the metagenomic sequence data from the 25 distinct sample-sites of SSK42/5 and SSK42/6 separately onto each of above mentioned *de novo* sequenced genomes – remarkably, significant percentages of the metagenomic read-sets were found in this way to match sequences from the individual genomes. The data, which clearly give a picture of the relative abundances of the strains in each of the 25 distinct sediment-samples have been presented in the form of a new heat map figure.

Referee's Comment: Line 385 – intracellular vs extracellular tetrathionate. Most of these examples of tetrathionate producers generate tetrathionate as an intracellular intermediate species during metabolism. Please discuss how you envision other members of the microbial community accessing these species.

Authors' Response: We agree that literature is very scant and obscure (limited to only two papers of 1989 and 1992) in relation to tetrathionate formation during sulfate/sulfite reduction by members of the genera *Desulfovibrio* and *Desulfobulbus* (which were mentioned in Line 385 of the previous manuscript). Whilst in the two papers available there is no clear-cut indication of whether the tetrathionate formed during sulfate/sulfite reduction by these organisms appear in the spent-media or not, the amount

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of tetrathionate produced in those cases is said to be in the micro molar range, so intracellular accumulation, if that is at all the case, would not pose major physiological problem for the cells. In view of these uncertainties in the knowledge base we have now removed these two instances from the list of bacteria cited as additional and likely sources of tetrathionate, both from the text as well as Tables S8 and S9.

Notably, however, unlike for the two sulfate-reducers mentioned above, there are definite reports from sulfur-oxidizing chemo-/photo-lithotrophic bacteria that polythionates (including tetrathionate) – whether provided in the media as substrates or formed during the oxidation of thiosulfate - do not accumulate intracellularly (only a few of the several relevant references are given below). All polythionates have high biological reactivity, and the concentrations of polythionates involved in lithotrophic growth of bacteria are in the milli molar range, so most of the chemolithotrophic bacteria are known to uptake and use polythionates as and when required but never accumulate the same intracellularly. Moreover, the papers cited below have amply demonstrated in pure culture experiments that polythionate species formed from thiosulfate, whether subsequently oxidized to sulfate or not, are released into the extracellular milieu (spent media) as detectable (by cyanolysis method) solutes.

Given this physiological aspect of lithotrophic tetrathionate production, any other tetrathionate-metabolizing bacteria present in the system, whether a natural environment or a mixed culture, would get equal opportunity to utilize the polythionate formed as the former organism itself gets.

1. Ghosh, W., Bagchi, A., Mandal, S., Dam, B. and Roy, P., 2005. *Tetrathiobacter kashmirensis* gen. nov., sp. nov., a novel mesophilic, neutrophilic, tetrathionate-oxidizing, facultatively chemolithotrophic betaproteobacterium isolated from soil from a temperate orchard in Jammu and Kashmir, India. *International Journal of Systematic and Evolutionary Microbiology* 55: 1779–1787.
2. Wood, A.P. and Kelly, D.P., 1985. Physiological characteristics of a new thermophilic obligately chemolithotrophic *Thiobacillus* species, *Thiobacillus tepidarius*. *International Journal of Systematic and Evolutionary Microbiology* 35: 434-437.
3. Wood, A.P., Woodall, C.A. and Kelly, D.P., 2005. *Halothiobacillus neapolitanus* strain OSWA isolated from “The old sulphur well” at Harrogate (Yorkshire, England). *Systematic and Applied Microbiology* 28: 746-748.
4. Hensen D., Sperling D., Truper H.G., Brune D.C. and Dahl C., 2006. Thiosulphate oxidation in the phototrophic sulphur bacterium *Allochromatium vinosum*. *Molecular Microbiology* 62: 794–810.
5. Alam, M., Pyne, P., Mazumdar, A., Peketi, A. and Ghosh, W., 2013. Kinetic enrichment of 34S during proteobacterial thiosulfate oxidation and the conserved role of SoxB in S-S bond breaking. *Applied and Environmental Microbiology* 79: 4455-4464.
6. Pyne, P., Alam, M., Rameez, M.J., Mandal, S., Sar, A., Mondal, N., Debnath, U., Mathew, B., Misra, A.K., Mandal, A.K. and Ghosh, W., 2018. Homologs from sulfur oxidation (Sox) and methanol dehydrogenation (Xox) enzyme systems collaborate to give rise to a novel pathway of chemolithotrophic tetrathionate oxidation. *Molecular Microbiology* 109: 169-191.

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Microbiology 35: 434-437. 3. Wood, A.P., Woodall, C.A. and Kelly, D.P., 2005. *Halothiobacillus neapolitanus* strain OSWA isolated from “The old sulphur well” at Harrogate (Yorkshire, England). *Systematic and Applied Microbiology* 28: 746-748. 4. Hensen D., Sperling D., Truper H.G., Brune D.C. and Dahl C., 2006. Thiosulphate oxidation in the phototrophic sulphur bacterium *Allochromatium vinosum*. *Molecular Microbiology* 62: 794–810. 5. Alam, M., Pyne, P., Mazumdar, A., Peketi, A. and Ghosh, W., 2013. Kinetic enrichment of 34S during proteobacterial thiosulfate oxidation and the conserved role of SoxB in S-S bond breaking. *Applied and Environmental Microbiology* 79: 4455-4464. 6. Pyne, P., Alam, M., Rameez, M.J., Mandal, S., Sar, A., Mondal, N., Debnath, U., Mathew, B., Misra, A.K., Mandal, A.K. and Ghosh, W., 2018. Homologs from sulfur oxidation (Sox) and methanol dehydrogenation (Xox) enzyme systems collaborate to give rise to a novel pathway of chemolithotrophic tetrathionate oxidation. *Molecular Microbiology* 109: 169-191.

Referee's Comment: Line 445-453 - How do you possibly get as many as six separate samples that have identical observed rates (e.g., of 141⁷⁷ Cs' 1 μ mol S/d/g)?

Authors' Response: In the above experiments it was remarkable that the individual communities present within the sediment-depth-zones spanning 2-90 cmbsf, 120-175 cmbsf, or 220-275 cmbsf, exhibited mutually identical rates of tetrathionate oxidation despite having dissimilar composition/prevalence of chemolithotrophic taxa. This could be explained as follows. When a natural sample is incubated in selective culture media (such as ASWTr) certain specific microbial species present in the sample often outgrow all metabolic competitors by virtue of higher substrate affinity or culture-condition suitability. Consequently, the growth/substrate-utilization phenotype(s) manifested by such enriched consortium cultures are contributed to by the selected few rather than the entire community of metabolic equivalents present in the sample (Roy et al., 2016). In the light of these issues it seems quite plausible that specific sets of chemolithotrophic taxa, more adept to ASWTr-growth than others, are present across the sediment-samples within the 2-90 / 120-175 / 220-275 cmbsf zones, and it was only their typical rates of

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tetrathionate oxidation in vitro which we incidentally recorded as the in vitro tetrathionate oxidation rates of the communities. Whatever may be the actual tetrathionate formation/oxidation rate of the SSK42 sediment-samples in vitro or in situ, results of the slurry culture experiments illustrated that tetrathionate-forming and oxidizing bacteria of SSK42/5 and SSK42/6 were alive in situ.

Referee's Initial Comment: Line 492 – The authors cite three papers to support a link between MnO₂ cycling and pyrite oxidation to tetrathionate and other dissolved species. I have no idea what the authors are referencing, which is troubling and forces me to recommend rejection. The Berner and Petsch paper from 1998 does not include the words manganese, thiosulfate, or tetrathionate. There is similarly no mention of MnO₂ in the Luther 1991 paper. And, although the Jørgensen and Bak paper discusses manganese, it is in the context of “manganese or iron oxides” which could similarly be used as electron acceptors, not anything about pyrite oxidation.

Authors' Initial Response: WE ARE EXTREMELY SORRY for this "copy-paste" goof-up committed in the haste of submitting multiple manuscripts within the same time-window! The actual reference that should have been used is Schippers, A., and Jørgensen, B. B.: Oxidation of pyrite and iron sulfide by manganese dioxide in marine sediments, *Geochim. Cosmochim. Acta.*, 65, 915-922, [https://doi.org/10.1016/S0016-7037\(00\)00589-5](https://doi.org/10.1016/S0016-7037(00)00589-5), 2001. (Please note that this was already included in the Reference list and cited in another context of Discussion). This paper should have also been cited in this particular context of tetrathionate production from pyrite, instead of the three irrelevant references that mistakenly got inserted. Please see Line numbers 4-8 of the Abstract itself of Schippers and Jørgensen, 2001, which clearly states that “FeS₂ and iron sulfide (FeS) were oxidized chemically at pH 8 by MnO₂ but not by nitrate or amorphous Fe(III) oxide. Elemental sulfur and sulfate were the only products of FeS oxidation, whereas FeS₂ was oxidized to a variety of sulfur compounds, mainly sulfate plus intermediates such as thiosulfate, trithionate, tetrathionate, and pentathionate. Thiosulfate was oxidized by MnO₂ to tetrathionate while other intermediates were oxidized to sul-

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fate.”

Referee's Subsequent Comment: Thank you for clarifying the correct references here, your thinking is far clearer now. Although this reference does describe pyrite oxidation via MnO₂, I am still unconvinced that it is relevant to the sediments in the current study. If one reads beyond the abstract of that paper, one also finds that “Below 7.5 cm, where the content of Mn did not exceed 0.2% (w/w), a dissolution of FeS₂ was not detectable.” One also finds that the abiotic incubations produced intermediately only for days to weeks and not longer, decreasing rapidly with depth. Manganese concentrations in the current paper (71-172 ppm) are orders of magnitude lower than the threshold for activity reported before, and there are no depth trends in either pyrite or MnO₂ discussed, or porewater metal ion data, that might support this mechanism as active. Purported Mn driven oxidation also appears to increase, rather than decrease, with depth, in contrast with the prior report. I do not think pyrite is a source of dissolved S species in this system; stronger evidence is required.

Authors' Subsequent Response: We agree with your observation that the MnO₂-FeS₂ interaction as a source of tetrathionate has been overstretched and indeed needs more experimentation to substantiate. We agree to curtail this particular discussion component and limit only to the observation and conclusion drawn from the microbial studies.

Referee's Initial Comment: Line 498 – The entire argument for pyrite oxidation by MnO₂ appears to be that there is detectable Mn in the sediments. (Basically all sediments have this??) There must be more one could say on this topic : : : depth trends? Differences between the two cores? Comparison with typical sediment Mn concentrations? Otherwise I'd leave the Mn discussion out. I would certainly not use this discussion to conclude that “Pyrites (via abiotic reaction with MnO₂) and thiosulfate (via chemolithotrophic oxidation by members of the bacterial group designated as A in Fig. 4) are apparently the main sources of tetrathionate”. This has not been demonstrated.

Authors' Initial Response: Chemolithotrophic conversion of thiosulfate to tetrathionate

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by members of the bacterial genera *Pseudomonas* and *Halomonas* (designated as A in Fig. 4) has been experimentally demonstrated - we have shown such isolates of both *Pseudomonas* and *Halomonas* which are capable of chemolithotrophically converting thiosulfate to tetrathionate in vitro. Furthermore, when metagenomic sequence data obtained for each of the 25 distinct sediment-samples of SSK42/5 and 6 were assembled and annotated individually, 23 out of the 25 contig-collections obtained were found to contain genes for tetrathionate formation (namely, genes encoding subunits of the thiosulfate dehydrogenases *TsdA* that converts thiosulfate to tetrathionate; see Denkmann et al., 2012; Pyne et al., 2018) [Table S3]. Whole metatranscriptome sequencing and analysis for the 275 cmbsf sediment-sample of SSK42/6 also revealed the gene-catalog obtained via annotation of the assembled contigs to encompass homologs of the thiosulfate dehydrogenase gene *tsdA* [Table S19]. These data clearly supported the potential in situ functionality (metabolically active state) of thiosulfate to tetrathionate converting bacteria.

Referee's Subsequent Comment: I do not dispute that thiosulfate-to-tetrathionate conversion was demonstrated and is quite intriguing; the piece of your claim that has not been demonstrated is related to pyrite. Without showing any depth trends, porewater metal ion data, or pyrite-specific (tracer) incubations, there is no data evidencing the involvement of pyrite.

Authors' Subsequent Response: We agree with your observation that the MnO₂-FeS₂ interaction as a source of tetrathionate has been overstretched and indeed needs more experimentation to substantiate. We agree to curtail this particular discussion component and limit only to the observation and conclusion drawn from the microbial studies.

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