April 2019

“Evidence for microbial iron reduction in the methanogenic sediments of the oligotrophic SE Mediterranean continental shelf“ by Vigderovich et al.

Response to comments from anonymous reviewer #2 (our response in blue):

This manuscript presents pore-water data (S, CH_4, Fe^{2+}, H_2, d^{13}C-DIC), results of incubation experiments as well as data on the abundance and diversity of bacteria and archaea in sediments of the South Eastern Mediterranean continental shelf. Besides a typical zone of organoclastic iron reduction observed close to the sediment surface the authors report a second zone of enhanced Fe^{2+} pore-water concentrations within the methanic sediments below the sulfate/methane transition. Evidence for iron reduction in methanic subsurface sediments is commonly found in high accumulation continental shelf and margin sediments and a strong research interest currently exists in elucidating which (bio)geochemical pathways and potential microbial organisms mediate this “deep” iron reduction.

In this respect, the paper focusses on an important and topical research question and is in principle suitable for Biogeosciences. However, I regret to say that the manuscript has numerous flaws and appears as if it has not been prepared with the required care. The manuscript thus needs a major overhaul before I can recommend publication. The English also requires quite some polishing and I would suggest to ask an English native speaker to proofread the manuscript. There are numerous typos (which I have not all corrected in detail) and the wording is imprecise in many places – all this need careful checking and correction.

We thank the reviewer for the thorough and constructive review. We addressed and accepted all comments (see below) and revised the manuscript accordingly. In addition, we have edited and proofread the English.

Several issues that need to be considered when preparing a revised version:

1) The most important point is that the discussion is not adequate as it stands, several assumptions are not supported by the data and many key publications have not been cited. Often statements occur in the form of single sentences without “really” discussing the data obtained in the framework this study.

We accept this comment. In the revised version we have extended and strengthened the discussion (see below in the specific comments the clarifications and added calculations). We carefully analyzed the data, clarifying what is indicated directly and is supported by other publications (also listed below), and what is speculative.

2) It is not clear to me which novel findings your study contributes to the topic of deep iron reduction. This needs to be outlined precisely.

In the revised version we present and clarify the novel aspects of this paper:

a. **Combining** geochemical profiles, microbial profiles and incubation experiments to show evidence for microbial iron reduction in the deep methanic zone and the potential microbial population performing this reduction.
b. Showing that this deep iron reduction can occur even in sediments of oligotrophic seas, such as the **oligotrophic** SE Mediterranean. We suggest that the availability of iron minerals for reduction is linked to an intensive methane cycle (see below, addressing the comment on L. 111).

In the revised version we emphasize the connection between the deep iron reduction and the methane cycle more clearly.

3) Please provide a map that shows the study area and the three sampling locations and a table that summarizes the dates, exact positions, precise names etc. of the samples used in this study.

A map and a table were added to the revised manuscript.

4) Please, also add a table that gives the details of the sequential extractions performed in this study.

A table with the sequential extractions details was added to the revised manuscript.

5) Referencing is not adequate – i.e. several relevant papers are missing. I have listed some publications below but a careful literature search should be performed.

We thank the reviewer for this list. We added those references and several more. The following references were added (see below the specific places):

Boetius et al., 2000; Egger et al., 2017; Emerson et al., 1980; Hinrichs et al., 1999; Hoehler et al., 1994; Iversen and Jorgensen, 1985; Knittel and Boetius, 2009; Li et al., 2012; Lovley 1991; März et al., 2018; Milkov and Sassen, 2002; Milkov, 2004; Moutin and Raimbault, 2002; Niewöhner et al., 1998; Oni et al., 2015; Orphan et al., 2001; Paull et al., 2008; Riedinger et al., 2017; Wurgaft et al., 2019; Zhang and Lanoil, 2004.

6) Please, precisely distinguish between and separate Results and Discussion. The Results chapter already contains a lot of interpretation/discussion and several references, which is formally incorrect.

We accept this comment, and the results and discussion sections were properly separated, the data was presented first and then discussed. We also moved the references to the discussion section.

Specific comments:

Line 2 and throughout the manuscript: I do not like the term “methanogenic” very much because it implies that methane formation occurs in the respective sediment layer/interval. Based on your considerations on page 3 (lines 102 ff. and lines 112 ff.) concerning the current oligotrophic conditions in the study area as well as the deeper gas front detected based on seismic profiling, I suggest that it is likely that methane is diffusing/migrating up from deeper layers into the sediment depths investigated in this study. I would thus propose to speak of “methanic” sediments, which is more neutral.

We completely agree with the reviewer that some of the methane in the pore-water originates from deeper sediments. This is indeed an important factor in this system that was discussed in our previous studies (which did not focus on iron - Sela-Adler et al., 2015; Wurgaft et al., 2019). We clarified this point in the revised version and included flux calculations (see below, addressing the comment on L. 111). As suggested, we rephrased the term to "methanic".

L. 20: What exactly do you mean by “mechanistic” nature?

The microbial link between the iron and the methane cycles in marine sediments, either by competition between methanogens and iron reducing bacteria due to environmental conditions, methanogens switching from methanogenesis to iron reduction metabolism or iron driven AOM. We explained this in the revised version.

L. 25: delete “cores”; in the deeper methanic zone

Deleted.

L. 27: Do you mean Fe^{2+} concentrations in pore water?

Yes. Specified in the revised manuscript.

L. 37: Li et al. (2012) is only one of a vast amount of literature on this topic – you may add a few other papers. So change to (e.g. Li et al., 2012; Riedinger et al., 2017 (Frontiers in Earth Science); März et al., 2018 (Mar. Geol.).

We added all the relevant references. In addition to the ones above, we cited Egger et al., 2016; Ettwig et al., 2016; Sivan et al., 2014; Slomp et al., 2013. These references support the fact that Fe(III) minerals have a key role in the biogeochemical cycles of carbon, sulfur, phosphorous and nitrogen.

L. 45: What exactly do you mean with “outward” diffusing methane? This is not clear to me. Please, specify.

The term infers that the methane diffuses away from the methanic zone to the SMTZ or deep layers. In the revised manuscript this was clarified.

L. 47: Key papers on sulfate-mediated AOM are missing here: please add at least Hinrichs et al. (1999) and Boetius et al. (2000). . . . it should then read: (e.g. Hoehler et al., 1994; Hinrichs et al., 1999; Boetius et al., 2000) . . .. and you may of course add further papers.

As we do not focus on sulfate-mediated AOM, we did not include most works on this topic. However, we agree with the reviewer that at least the key works should be included. We added thus these references, as well as Orphan et al., 2001; Knittle and Boetius 2009.

L. 49: Also here Valentine (2002) is only one example of a vast amount of literature on this topic. You may also wish to cite Niewöhner et al. (1998), GCA, here.

Niewöhner et al. (1998) work from the west African margin was added.

L. 51: Has to be iron “reduction” (instead of oxidation)

Corrected.

Ls. 58/59: Please, give the respective references.

The reference was added (Lovley 1991)

L. 60: Please rephrase to: . . . incubation of marine seep sediment . . ..

The sentence was rephrased as suggested.

Ls. 61 ff.: Please also cite the following papers in this context: März et al. (2008), Oni et al. (2015), Egger et al. (2018), who have also presented evidence for Fe-coupled AOM in marine, coastal, and brackish sediments.

We accept this comment and have added these references (Marz et al. 2008; Oni et al., 2015; Egger et al., 2017) here as evidence for deep iron reduction.

Ls. 68 ff.: Please, also cite Oni et al. (2015) here who have presented microbial studies for the methanic zone of North Sea sediments.

Added, but in line 62 (in the original version), since the original line 68 is about freshwater sediments and Oni et al. (2015) studied the North Sea sediments. The original line 62 was rephrased to: "It was suggested through the modeling of geochemical profiles in deep sea sediments (Sivan et al., 2007; Marz et al., 2008; Riedinger et al., 2014), in microbial studies of marine sediments (Oni et al., 2015)…"

Ls. 74 ff.: This sentence is hard to follow and sounds a bit odd. Please, rephrase.

The sentence was rephrased to: "Whereas Fe(II) is highly soluble, Fe(III) that is the most abundant specie of iron under natural conditions, appears as low solubility minerals."

L. 79: I would not speak of "inactive" in this context but rather of "of low reactivity". Furthermore, I do not find it surprising that reactive iron oxides are preserved and present below the SMT. This finding has already been explained by several studies/papers – amongst others by Riedinger et al. (2005), GCA, März et al. (2008), Mar. Geol., and März et al. (2018), Mar. Geol.

The term "inactive" was changed to "of low reactivity" as suggested. We accept the comment and removed this word.

L. 87 ff.: You may also wish to cite Oni et al. (2015) here.

This sentence focuses on methanogenesis inhibition by iron reduction, and thus this reference was not added here. It can be found in other places in the revised manuscript (see above).

L. 92: What exactly do you mean with "reactivate" in this context? This is not clear to me – please specify. Were the Fe oxides "unreactive" before? By which process/condition have they been "reactivated"?

We infer that the iron oxides, which were not reduced in the upper sedimentary column by bacteria or archaea, are reduced in the deeper sediments, even though there is less energy for redox reactions. This suggests that there is some advantage at these depths that allows their reduction. Several processes may explain this reactivation: 1) Iron reducing bacteria succeed in outcompeting methanogens due to environmental changes, 2) the methanogens themselves switch to iron reduction due to some advantages (electron shuttling such methanophenazines?), or/and 3) the methane that is produced is more available for reduction than other organic substrates (Fe-mediated AOM). We clarified this point better in the revised version.

L. 97: What precisely is a "basic" incubation experiment?

We infer a fundamental incubation experiment. We removed the word basic and rephrased it in the revised version to: " We show both geochemical pore-water profiles and microbial investigation at three different stations combined with a simple incubation experiment with slurry…"

L. 99: Please, rephrase to: . . . possible links between the cycling of iron and methane".

Changed as suggested.

L. 102: I find it hard to imagine that the Levantine Basin is really one of the most oligotrophic marine settings in the world. I thought that globally the most oligotrophic ocean area is the South Pacific Gyre?! Please, check carefully and rephrase accordingly.

To the best of our knowledge, the Levantine basin is considered an ultra-oligotrophic marine system. For example, Thingstad et al. (Science, 2005) discussed the phosphorus imitation in the "Ultraoligotrophic Eastern Mediterranean", as well as several other studies, which ranked the Mediterranean basin as oligotrophic to ultraoligotrophic based on nutrients, chlorophyll a and PP pools (Krom et al., 1991; Antoine et al., 1995; Siokou-Frangou et al., 2010; Kress et al., 2014; references in Herut et al., 2016 and more). However, we rephrased the sentence to: "The Levantine Basin of the SE Mediterranean Sea is an oligotrophic nutrient-poor marine system (Kress and Herut, 2001)."

L. 109: I do not believe that the TOC contents are/were really "zero". I think this is an issue of the detection limit of the specific analytical method used. Please check.

Indeed, a typo mistake. We corrected the sentence: "… the Levantine Basin have low TOC levels of ~1% (~0.5 – 1.4%; Sela-Adler et al., 2015; Astrahan et al., 2017)."

Ls. 111 ff.: I do not understand the argumentation in this sentence. How can you conclude that methane found in shallow sediments is of biogenic origin if a deep gas front has been detected by seismics? Are you sure that the methane found in the shallow sediments investigated here really formed in situ. I guess it is much more plausible – I particular given the current oligotrophic conditions and low TOC contents discussed above – that methane has migrated up from deeper sources.

As written above, we agree that some of the methane has migrated from deeper sources, at least in Station SG-1. However, our results indicate that part of the methane is also produced *in-situ* in the methanic zone (zone 3) based on our geochemical profiles mainly of $\delta^{13}C_{CH4}$ and $\delta^{13}C_{DIC}$ (Sela-Adler et al., 2015), and the mcrA profile (presented here). The geochemical profiles show the transition from sulfate reduction to methanogenesis, a clear SMTZ, very low carbon isotopic value of the methane (between -80 and -100‰) and classical "*in-situ*" diffusive $\delta^{13}C_{DIC}$ profiles with the significant increase in the isotopic values below the SMTZ in the methanogenic zone. The microbial profile shows that the mcrA gene copy number increases with depth and peaks below the SMTZ. All fits to *in-situ* biogenic methane production in zone 3, in addition to some migration. We clarified this in the text, writing clearly the two sources of methane and their supporting evidence.

As mentioned above, we discussed the migration of methane in our previous studies. In Wurgaft et al. (2019) that focused on sulfate reduction rates in the SMTZ based on alkalinity and DIC profiles, we wrote: "The similarity between sulfate reduction rates in the ultra-oligotrophic Southeastern Mediterranean and these eutrophic regions suggests that "external" methane, which is not the product of degradation of organic material originating in the water column but rather derives from deeper deposits, provides an important source of reducing power to the SMTZ. Such deep methane deposits and upward fluxes are common in many continental margins (e.g. (Milkov and Sassen, 2002; Milkov, 2004; Paull et al., 2008; Zhang and Lanoil, 2004))".

We agree with the reviewer that this source may explain our results of the low TOC. In the revised manuscript we suggested and clarified that this source of methane leads to intensive sulfate-mediated AOM in the SMTZ, and that this intensive process and biomass may serve as additional substrate that “fuels” the deeper zone, activating the iron-oxides. We added to the text the following part with the calculation of the biomass that is produced from this source: “The importance of methane flux as a carbon source that supports the deep microbial community in the sediments of the SE Mediterranean can be inferred by comparing the organic carbon flux from the photic zone, with the flux of organic carbon that is oxidized by sulfate in the pore water. Using sediment traps, Moutin and Raimbault (2002) estimated an export flux of 7.4±6.3 mg C m^{-2} d^{-1}, which leaves the photic zone there. However, Wurgaft et al. (2019) estimated that the flux of DIC entering the SMTZ from sulfate reduction is equivalent to 8±3 mg C m^{-2} d^{-1}. While the difference between the two fluxes is statistically insignificant, it should be noted that the flux of organic material that survives aerobic oxidation in the water column and the upper part of the sediment column, as well as anaerobic oxidation by other electron acceptors with higher energy yield (Emerson et al., 1980; Froelich et al., 1979), is likely to be substantially smaller than the flux measured by Moutin and Raimbault (2002). Therefore, it is unlikely that export flux from the photic zone constitutes the sole source of carbon to the SMTZ. Wurgaft et al. (2019) suggested that methane originating from deep sediments and migrating upwards in the pore-fluids provides an important source of carbon to the SMTZ in SG-1. Methane sources of such are common along continental margins sediments (e.g. Milkov and Sasson, 2002; Milkov, 2994; Paull et al., 2008; Zhang and Lanoil, 2004). Here, we suggest that the supply of methane leads to intensive sulfate-mediated AOM in the SMTZ, and that this process produces(??) biomass which may serve as additional substrate. (New sentence) that “fuels” the deeper zone, activating the iron-oxides.”

Ls. 114 ff.: Also the argumentation in this sentence is odd. Even if waters are anoxic they almost always have the typical marine sulfate concentration of 28-30 mmol/l. Thus, anoxia does not necessarily lead to sulfate reduction.

We agree with the reviewer and clarified the sentence at the beginning of the study site section: " The bottom seawater across the continental shelf is well oxygenated and sulfate concentration in the water-sediment interface is ~30 mmol L^{-1} (Sela-Adler et al., 2015)."

Ls. 120 ff: The cores were sampled during cruises of R.V. Shikmona ...

Corrected as suggested.

Ls. 122 ff.; This sentence sounds odd. Please, rephrase.

We rephrased this sentence to: "These stations were previously investigated for other purposes…"

L. 132: . . . the “stable carbon” isotopic composition . . . explain the abbreviation DIC

DIC- dissolved inorganic carbon. This abbreviation is explained in L 39.

L. 134: “at” -20◦C

Corrected.

L. 136: The wording in this sentence is a bit odd. Do you mean that the surface sediment has been lost during sampling (which is usually the case during gravity or piston coring)?

We refer to the uppermost sediment of the piston core, which is indeed usually mixed with the top seawater entrapped between the surface sediment and the piston. To avoid any disorder in the surface sediment, we have used a box corer sub-sampled by Perspex push cores for the top ~30 cm sediments. We revised therefore the sentence to: “The uppermost sediments were collected using a 0.0625 m^2 box corer (Ocean Instruments BX 700 Al). Two ~30 cm sediment cores were sub-sampled using Perspex tubes during the September 2015 and January 2017 cruises."

L. 137: Does it mean that you have sub-sampled the box corer by means of push cores? If yes, please say so.

Revised, see above.

L. 139: Does it mean that you have determined methane both in pore-water as well as sediment samples? How precisely and how have the pore water and solid phase been separated?

We have measured the methane from the total wet sediment, by transferring the sediment sample immediately to a crimped bottle with 5 mL of NaOH and flushed with nitrogen. Then measured the methane in the headspace. We explained it in the revised version.

Ls. 141 and 289: Some details of how precisely these incubation experiments have been performed are missing. How were the respective experiments/bottles killed? Did you use molybdate to inhibit sulfate reduction?

We agree with the reviewer that additional details regarding the incubation experimental design were needed. The revised manuscript includes the specific information regarding the "killed" bottles (sediment killing via autoclave). Molybdate was not used in the experiment.

L. 143: Refer to the respective figure with pore-water profiles here.

We agree with the comment and the figure (Fig 1) was referred in the revised MS.

L. 145: anoxic instead of anaerobic

Changed.

L. 147: anoxically instead of anaerobically

Changed.

L. 151: You are talking about mineral contents here – so the unit (mmol L-1) is not correct.

We changed the units of the mineral content to grams in the revised MS and the final Fe(III) concentrations in mmol L^{-1} units in brackets.

L. 152: In line 146 you have stated that incubations lasted for 3 months. Here you speak about 14 days?!

The sediment was incubated only with synthetic sea water without sulfate (in a 1:1 sediment:water ratio) for three months prior to the experiment. The experiment then began with the division of the slurry to the 60 mL bottles, the addition of more synthetic water (final sediment:water ratio of 1:3) and some manipulations (addition of iron oxides and H_2 to some treatments). In the revised MS we clarified this point better.

L. 161: It has to be “total sulfur” instead of sulfate. Sulfate can’t be measured by ICP-AES.

Correct, ICP-AES measures total sulfur. Since sulfide was not detected in the samples (by Cline method) and these are marine samples, we assume that "total sulfur" here is actually sulfate. However, we changed the title to total sulfur and clarify its meaning in the revised version.

L. 162: has to be “inductively” and Perkin “Elmer” At this point I stopped to correct typos and odd wording – there are just too many.

Corrected.

Ls. 166 ff.: a pore-water profile can’t be “performed”; please also state which parameters have been analysed and in which figures they are shown; what do you mean with “and not their average”? This is absolutely unclear to me.

The word "performed" was changed to "produced" in this context throughout the text. We agree with the reviewer that the other term is unclear, and it was removed.

Ls. 170 ff.: I would suggest to insert a table, which gives the details of the extraction used – including reagents, solid-phase/reagent ratios, shaking times, etc.; please, also state whether the extractions has been performed on dry or wet sediment samples; if you used wet samples, how has porosity been determined? By the way, carbonate associated Fe is not an “iron oxide” as stated at the beginning of this sentence.

We thank the reviewer for the suggestion, in the revised version a detailed table was added with the specifics of the extractions. The extractions were conducted on dry sediment. In addition, the word "oxides" was changed to "minerals".

Ls. 202 ff.: Again: pore-water profiles can’t be performed. Please, rephrase.

Rephrased to "produced".

Ls. 204 ff.: As also stated above you have not determined sulfate but total sulfur. So, rephrase accordingly and also correct this in Fig. 1 and throughout the manuscript.

The reviewer is correct, ICP-AES measures total sulfur. We clarify this in the revised version as mentioned above.

L. 207: increase “with depth”

Corrected.

Ls. 207 ff.: I do not fully understand this sentence. Moreover, part of this sentence is interpretation/discussion and should thus not be part of the Results chapter.

We agree with the reviewer that the sentence is not clear, we also agree with the other comment and moved it to the discussion chapter. The sentence was rephrased to: "The maximum methane concentration was approximately 10 mmol L^{-1} at ~140 cm depth…"

Ls. 215 ff.: Large parts of this is discussion/interpretation.

We agree and moved it to the Discussion chapter.

Ls. 229: I found this sentence confusing because from the chapter 2.2 “Sampling” it was not clear to me that the sites have been sampled three times. Please clarify and give a table summarizing the dates, exact positions, precise names etc. of the samples used in this study. What is the “Aug-13 core”? Where is it shown in Fig. 1? A legend and/or respective explanations in the figure caption are missing.

We agree with the reviewer that the study sites sampling time was not clear in the previous version. The stations SG-1 and PC-3 were sampled three times each during different cruises and station PC-5 was sampled once. The text was clarified and a table with the specifics was added.

L. 238: Why are deviating points not discussed?

The few deviations are of only one data point each, and are probably due to an analytical error during the measurement/sampling process. We clarify it in the revised version.

Ls. 248 ff.: I can't find Fig. S1; solid-phase values are "contents" (not concentrations)

Figure S1 can be found in the supplementary material, perhaps there was an error and the reviewer did not receive the file?

The word "concentrations" was changed to "content".

Ls. 257 ff.: A lot of this is already interpretation/discussion. Moreover, papers should not be cited in the Results chapter.

We agree, and moved part of it to the discussion, as well as the references.

L. 303: Which station precisely do you refer to here? "at this station"? How do you know that intensive methanogenesis occurs in the respective sediment layer? Due to the fact that TOC contents in the shallow sediments are low and free gas is detected in deeper layers, I would rather suggest that methane is migrating up from the deeper subsurface. Please discuss and consider this carefully.

We are referring to station SG-1. This is clarified in the revised version. We agree that some of the methane migrated up from deeper subsurface (see above). We rephrased the sentence to: "At station SG-1 methane reaches higher concentrations, which leads to intensive methane oxidation by sulfate at the SMTZ…"

Ls. 305 ff.; This sentence needs to be rephrased.

We agree and rephrased it to: "…causing it to occur at shallower depth and produce lower $\delta^{13}C_{DIC}$ values than the other two stations, as observed in previous studies (e.g. Sivan et al., 2007)."

Ls. 314 ff. and 331 ff.: As already stated above I do not agree that methanogenesis necessarily occurs in the respective sediment zone. To me it seems more likely that methane has migrated up from deeper layers.

As mentioned above, we now refer to the two sources.

Ls. 317, 351 and throughout the manuscript: What do you mean with iron oxide "reactivation"? This is odd.

Please see above.

Ls. 334 ff.: I do not understand at all how the findings link or relate to the Last Glacial Maximum?! How can the current environmental conditions be attributed to the Last Glacial Maximum or Mid-Pleistocene? You need to much more carefully discuss this.

We removed this sentence. The hypothetical environmental conditions are discussed by Sela-Adler et al., 2015 and Schattner et al., 2012, while not directly linked to this study.

L. 339: anoxic instead of anaerobic

Corrected.

Ls. 346 ff.: This has not been described in the respective methods chapter.

The reviewer is correct, the matter is elaborated in the revised version in the methods chapter: "One mL of H_2 was added by gas tight syringe to two bottles with addition of hematite and two bottles with addition of magnetite (to final concentration of ~4% of the Head space volume)."

Ls. 351 ff.: And how does all of this relate to your data?

Cryptic sulfur cycle is observed more and more in marine sediments (e.g. Holmkvist et al., 2011; Brunner et al., 2016). It seems that this cycle is possible here based on the microbial populations that contain those that may be involved in sulfur cycling (from 16S analysis). Also, pyrite was found in the methanogenic zone (Wurgaft et al., 2019). We clarify this point in the revised version.

Ls. 358 ff.: Numerous papers that have discussed and presented evidence for Fe mediated AOM in natural aquatic sediments have not been cited here.

As mentioned above, in the revised version we include the main literature on Fe-AOM.

Ls. 363 ff.: I would not overinterpret methane concentrations, which have been determined ex situ because methane typically suffers from strong degassing during core retrieval.

We agree with the reviewer and rephrased this sentence which now emphasizes just the general trend: " In our profiles AOM could be a valid option. As can be inferred from figure 5, some association was observed between the dissolved Fe(II) concentrations in zone 3 and the methane concentrations. It seems that at high concentrations of Fe(II), methane concentrations are low and vice versa."

Ls. 412-415: These two sentences more or less say the same.

We agree with the reviewer that the two sentences sound similar, however the first sentence is the key sentence of the paragraph, and the following three sentences are listing the main results of the study.

From the discussion, as it is presented, it is not clear to me at all which novel findings your study and data contribute to the discussion on and research topic of potential drivers of deep iron reduction.

Please see above.