

## ***Interactive comment on “Viable cold-tolerant iron-reducing microorganisms in geographically-isolated subglacial environments” by Sophie L. Nixon et al.***

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We thank the reviewers for their comprehensive and helpful remarks, and we are pleased to offer the final responses below. We feel that making the proposed minor changes and addition of requested data analysis to the manuscript will strengthen the paper, and we look forward to implementing them if the manuscript is accepted.

Reviewer 1 comments

The methods were adequate and are well described. My only reservation is about calling this approach ‘culturing’ as environmental samples were used and no attempts to isolate single species/strains were made. Working with real cultures would allow

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for direct attribution of processes (in this case iron reduction) to specific organisms, which is not quite possible here as the incubations still contain a mixture of microbes. It is known that non-dominant species can significantly contribute to biogeochemical processes (see e.g. Pester et al 2010 ISME J). This should be acknowledged and discussed in the ms in my opinion

Response: The manuscript will be revised to ensure it is clear to the reader that these were enrichment-, and not culture-based methods. Indeed the potential for syntrophic relationships between different microorganisms within a mixed community is the reason for working with enrichments as opposed to attempting to isolate individuals. In our view, studying mixed communities has more relevance to the occurrence of a process in situ

Also, using 16S rRNA instead of DNA would have been useful in terms of providing information on the active part of the community as opposed to “potentially active” DNA-based data

Response: We agree with the reviewer that sequencing extracted RNA would shed more light on the active part of the community. However, in our study DNA was extracted from active enrichments, and whilst it is likely that some extracted DNA derives from non-active or even dead members of the enrichment community, the fact that these were second-generation enrichment cultures which show enrichment of sequences closely related to taxa that are known to carry out iron reduction provides compelling evidence that these are the most plausible members of the communities conducting this metabolism

The hypothesis and aims of the study should be explicitly stated in the introduction (before “Here we present data. . .” on page 1/line 14)

Response: The hypothesis of the study is that subglacial sediments harbour active microorganisms that are capable of carrying out microbial iron reduction. The aims are (1) to assess whether subglacial sediments harbour active iron-reducing microorgan-

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isms, and (2) whether these microorganisms are adapted to low temperatures. Both the hypothesis and aims will be added as advised.

Why were different primer sets used for different samples? (27-1492 for E, L, FL, R and 357-518 for F and LW; 5/6-15)

Response: Different primer sets were used since the set 27-1492 was found to be contaminated after processing the samples reported on here (note a negative PCR control was run at the same time the subglacial samples were amplified, and no background DNA was found, hence we are in no doubt that the reagents were not contaminated at the time of use in this study). The primer set was replaced with a different universal set, 357-518, which was applied to the remaining samples. We would like to highlight that both primer sets target the V3 hypervariable region of the bacterial 16S rRNA gene, and so we feel are of equivalent appropriateness in this study.

Are these really bacteria-specific (as is suggested on 5/20) or do they pick up archaea as well? It would have been useful to look at archaea in these samples - potential syntrophies of iron reducers with some archaea could be quite interesting.

Response: The samples were assayed by Research and Testing Laboratories for bacterial 16S only, and as such we do not have any data on the presence and identification of Archaea in these samples.

There seems to be a discrepancy between what you say on 6/4-6 ("All 4C enrichments tested positive for microbial iron reduction. . .") and on 6/15 ("The only instance i which statistically significant production of Fe(II) was evident. . ."). Isn't Fe(II) production what you used as a sig of iron reduction?

Response: This second statement is incorrect and will be removed from the manuscript. We thank the reviewer for drawing our attention to this.

1/2 "geographically-isolated" is a bit confusing. Is geography really the main factor you want to emphasize in the title?

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1/13 ditto

Response: We will change the wording to 'geographically diverse'. We feel that referring to the dispersed geographical origin of the samples used in our study highlights the potential ubiquity of this process in these environments.

1/17 italicize *Desulfosporosinus*

Response: This will be corrected.

1/18 italicize *Geobacter*

Response: This will be corrected.

1/30, 2/26 the correct citation for this is Stibal et al 2012 Global Change Biology 18: 3332-3345

Response: We are grateful for the reviewer for pointing this out and will correct this in the manuscript.

2/23, 3/15 the correct citation is O'Donnell et al 2016 Biogeosciences 13: 3833-3846

Response: This will be corrected in the manuscript.

4/30 centrifugation

Response: This will be corrected in the manuscript.

7/15 environments

Response: This will be corrected in the manuscript.

7/19-22 this section doesn't quite make sense. MIR is characterised by a greater metabolic and genetic diversity compared to what exactly? How do the differences between the two temperatures highlight this diversity? Please clarify/rewrite

Response: The reason for this statement is that *Desulfosporosinus* is conventionally thought of as a sulfate-reducing bacterium but our data suggest that it may be respon-

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sible for the observed iron reduction in some of the subglacial sediments studied. Also the vast majority of characterised iron-reducing microorganisms are mesophilic, and our data suggests that the iron reducers in our enrichments may be cold-adapted. In light of this comment, we will change our statement to “Our results suggest microbial iron reduction in subglacial environments is characterised by substantial metabolic and genetic diversity”.

7/25 Rhodoferrax, Geobacter and Desulfosporosinus have been found in subglacial sediments exported by the river draining Leverett Glacier; Rhodoferrax in high abundances (>20% of reads in some samples). The results have recently been published (Cameron et al 2016, Environmental Microbiology doi: 10.1111/1462-2920.13483) although the iron reducers are not specifically mentioned in the ms

Response: The Cameron et al paper will be incorporated into the Discussion. We are grateful to the reviewer for bringing this paper to our attention.

8/4 define MIR at first use

Response: This will be changed.

8/9 can't iron reducers use legacy OC?

Response: It is possible that iron reducers could use overridden organic carbon originating to pre-glacial times, though to the best of our knowledge this has yet to be demonstrated. The reviewer is right to bring this to our attention, and this will be included in the Discussion.

9/1-14 this section is unnecessary as it doesn't add much to the story

Response: We feel this section should not be removed since it is important to place our work in the context of what else is known about iron reduction in cold environments, and specifically cold-adaptation. Furthermore, to remove this section would mean removing the statement that our data suggests iron reducers in subglacial sediments are cold-adapted, which we also do not wish to do.

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9/5 italicize Thiobacillus

Response: This will be changed.

9/11-13 this statement needs some references

Response: The existing statement in the manuscript is an observation based on the lead author's own substantial literature review of characterised iron-reducing microorganisms and their temperature adaptations, however this has yet to be published. As such it is difficult to amend this statement with supporting references. Instead, this statement will be modified to include specific examples of prolific mesophilic iron-reducing microorganisms and the appropriate references.

10/12 Mitchell

Response: This will be corrected.

11/3 Schulze-Makuch

Response: This will be corrected.

11/4 Nixon et al 2012 is not in the reference list

Response: This will be added to the reference list.

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Reviewer 2 comments

First, I am quite concerned that there were no killed controls included in their analyses; thus they have no way of telling whether the iron reduction that they observed during their enrichments was biological in nature...

Response: We agree with the reviewer that the absence of killed controls makes it difficult to rule out abiotic iron reduction. However, the fact that some samples gave rise to iron reduction at certain temperatures and not at others serves as compelling evidence that the production of ferrous iron observed in these experiments derives from bio-

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logical processes. For example, enrichments with Engabreen and Finsterwalderbreen subglacial sediments gave rise to iron reduction at 4C but not at 15C or 30C. In these cases it is difficult to imagine an abiotic process occurring at the colder of the three temperatures but not at 15C or 30C. Furthermore, we provide data in Supplementary Information of blank controls in which no, or insignificant, iron reduction was detected in the absence of an inoculum.

It also appears that the only chemical species that they followed was iron; thus there is no way to tell from this data whether the iron reduction observed was direct (i.e. microbial iron reduction) or indirect (i.e. microbial sulfate reduction, which produces sulfide, which could then secondarily reduce iron oxides to produce iron (II), or other means)

Response: First, no sulfide production (characterised by non-magnetic black precipitate), nor the recognisable smell that accompanies it, was observed in these enrichments. Second, the data presented in Fig 1 is for second-generation enrichments initiated with a 10% (v/v) inoculum from initial enrichments. No sulfate was added to the enrichment medium, and since no sulfide was observed in initial enrichments data, we believe our data provides evidence of direct iron reduction. We do however acknowledge that indirect iron reduction via sulfate reduction cannot be ruled out, and this point will be added to the Discussion section.

The possibility of indirect iron reduction is particularly problematic because both iron and sulfate reducing bacteria (amongst others) utilize the organic carbon substrates provided in the enrichment cultures. Whilst this concern does not invalidate their observation that iron reduction occurs and thus could have downstream implications, much of their discussion relies on the assumptions that the iron reduction is biological and direct. Thus, the language needs to be seriously toned down throughout the manuscript regarding how confident they are in their results

Response: We will amend the Discussion to make it clear that in using widely used

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carbon substrates in our enrichment approach, it is likely that we also stimulated non-iron-reducing microorganisms, such as *Clostridium* (see Figure 2). The choice of acetate and lactate as electron donors in these enrichment cultures was made since the vast majority of characterised iron-reducing microorganisms can draw upon one or the other for microbial iron reduction, and as such a lactate + acetate electron donor mix is widely used to enrich for iron-reducing microorganisms. This will be highlighted in the Discussion section, and the language will be toned down as suggested.

Secondly, they identify their bacteria in their enrichment cultures by 16S rRNA gene analysis and then proceed to discuss their potential role as iron reducing bacteria. This approach is based on two unwarranted assumptions: 1) because a sequence is abundant, it is carrying out the metabolism of interest - this is not necessarily true, even in an enrichment culture, 2) taxonomy is equivalent to physiology - just because a sequence is related to a known iron-reducing species does not necessarily mean that the sequence originates from an iron-reducing bacterium. . . . Thus, the data presented do not directly demonstrate which bacteria may be carrying out iron reduction in subglacial sediments - they only provide indirect evidence that would need to be confirmed more directly. This holds for the previous papers that they cite as observing iron-reducing bacteria in non-culture based approaches - these papers are quite careful not to claim that they have identified iron-reducing bacteria in subglacial environments (unless they have actually done culture work); only that they have found relatives of iron-reducing species in subglacial environments. I would strongly recommend modification of the discussion to reflect these concerns

Response: The reviewer is correct in pointing out these issues with 16S rRNA gene analysis, and we will amend the Discussion to reflect the limitations in this type of analysis, and the need for future research to attempt to confirm inferred physiology through further culture-based approaches. We do feel, however, that given the significant enrichment of genera with iron-reducing representatives (e.g. *Geobacter* and *Desulfosporosinus*) from these second-generation enrichment cultures, our data pro-

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vide compelling evidence that such genera are responsible for the iron reduction observed.

Third, it is difficult to determine whether this process might be cold-adapted in all systems as there are no intermediate temperatures between 15C and 30C. This is a big jump and a large transition zone for many bacteria. Furthermore, there was no work beyond the first round of enrichments for the 30C samples. Thus, it is unclear whether other conditions may have produced different outcomes. In at least one case reported here, the final iron concentrations are much higher at 15C than 4C; thus more work is needed to determine whether this process is cold adapted or not. Generally their language is fairly circumspect with regard to this issue but they should be cautious in their interpretation

Response: We have taken care not to claim that the evidence demonstrates cold-adaptation. Rather, the claim of cold-tolerance is backed up by the occurrence of greater MIR at 15C or lower compared with 30C. Given that one of the aims of this work was to establish whether any viable iron-reducing microorganisms present are cold-adapted/-tolerant, their activity above 15C was of limited interest. The generally accepted definition of psychrophily is optimal growth at or below 15C (Morita, R.Y. (1975) Psychrophilic bacteria. *Bacteriological Reviews*, 39(2): 144-167). Whilst we cannot and do not claim to have demonstrated cold-adapted iron-reducing microorganisms (such a claim would need to be backed up by direct measurements of growth, as acknowledged in the Discussion section), the temperatures adopted in this study were highly appropriate to our goals. Furthermore, that we detected positive iron reduction in all 4C enrichments, but only half at 15C, highlights the greatest potential biogeochemical impact at the colder temperature.

The major concerns above lead to further questions. The section of the discussion addressing the possible role of *Desulfosporosinus* in iron reduction relies on the assumptions that the iron reduction was direct, biological, and carried out by *Desulfosporosinus*, none of which can be confirmed for the reasons outlined above. I would

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suggest removing this paragraph, or expanding it to address the possibility that they are acting as SRB and indirectly reducing the iron

Response: As suggested, we will expand this paragraph to address the possibility that iron reduction may in fact be indirect.

There was no discussion on the overall biodiversity or comparison between treatments (i.e temperatures) of the enrichments. Since they have this data (from the high throughput sequencing), why is it not included?

Response: This data will be included, and Figure 2 will be amended to include all genera representing 10% or more of the sample. Shannon diversity indices will also be included, and overall diversity of the samples discussed.

For Figure 2, it is unclear why they are only reporting a subset of the data ("only genera known to include strains capable of microbial iron reduction, and other major taxa, are included in the legend"). Why not report all data And what do relative abundances mean if not all data is reported?

Response: We believe this is a misunderstanding. All data are included in the bar charts, and thus relative abundances are true to the full dataset, however not all taxa are listed in the legend. This was done to make the figure easier to understand, and to avoid cluttering the legend with taxa that represent a very small proportion of each sample. As above, this will be revised so that all taxa representing 10% or more of the sequences per sample are included in the legend. A clarifying sentence will be added to the caption to make it clear that all data is included in the bar charts.

For figure 1: why are there two lines for each treatment? Are these replicates? If so, why are they showing both replicates rather than a mean and standard error? Also, it would be useful if they would calculate the rate of iron reduction so that they could be compared between sample types

Response: The data reported in Figure 1 are replicates, and have been presented

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individually since it is not appropriate to calculate standard error or deviation for less than three replicates. Rates of iron reduction will be included, as suggested.

They use amorphous iron oxyhydroxide as the source of Fe(III) for their enrichments. Does the source of the iron matter? They don't discuss crystalline or chelated iron or whether that might make a difference in the outcomes, except to discuss crystalline iron as a possible source in the subglacial environment

Response: This part of the discussion will be expanded to include a justification for the use of iron oxyhydroxide, namely that it is the most thermodynamically favourable, and most widely used, iron oxide acceptor for microbial iron reduction (Nixon et al 2012. *Planetary and Space Science*, 72: 116-128). We will also comment on the differences we might have seen in terms of rates, extent of iron reduction and diversity of bacteria in the enrichment cultures had we used more crystalline iron oxides as terminal electron acceptors.

As mentioned above, they don't report data for tracking other chemical species. Did they measure degradation of the C species? It would be useful to know if the iron reduction is stoichiometric with the C utilization. If that data is available, please report it. Not required, but useful.

Response: This data is not available.

They argue that H<sub>2</sub>, derived from chemical reactions with rocks, could be a source of reductant in subglacial environments with low organic C levels. This is a possibility, but there are several issues with this argument. First, the concentrations of H<sub>2</sub> that would be produced in this way are likely to be quite low. Second, a lot of other biological and chemical pathways would be competing for that H<sub>2</sub> (sulfate reduction, nitrate reduction etc). Third, and most importantly, they have not demonstrate that their enrichments can utilize H<sub>2</sub>. So, I would like to see that part of the discussion toned down - electron source may well be a limiting factor for iron reduction in the subglacial

C11

Response: These are valid points, and this part of the discussion will be toned down as recommended.

It is unclear to me what the relative abundance of their proposed iron reducers is in unenriched samples. How many fold enrichment do they see? Are these abundant in "normal" subglacial environments, or did they grow "weeds" in their enrichment culture? Is there any direct indication that these microbes are important in these environments? If not, they need to be careful in their interpretations of how significant their findings are to the actual identity of iron reducers in subglacial systems

Response: Since we do not have 16S rRNA sequencing data from the sediments prior to initiating enrichments, it is not possible to address this with data, but we feel we have acknowledged this in the Discussion section. We will better emphasise the need for further research to characterise the extent and importance of microbial iron reduction in situ in the Discussion section.

Phosphate adsorbs to iron oxides, it is not "coupled" (p. 10, line 29)

Response: This will be corrected.

In sum, this is an interesting initial attempt at examining iron reduction in subglacial sediments. The enrichment culture approach is appropriate; however there are important missing pieces of the puzzle here. I would strongly recommend assessing the confidence they have in their data and its relevance to the real world systems they are discussing. The language of the manuscript needs to be toned way down and other data should be included, if available. Other analyses of the sequence data and further analysis of the iron reduction data would be helpful.

Response: We thank the reviewer for raising these concerns, however we are confident that we can make the necessary changes to the language of the manuscript to alleviate these issues. We will better emphasise that the purpose of the study was to assess the presence of viable iron-reducing microorganisms (and their activity at

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different temperatures) in these subglacial sediments. We did not attempt to simulate in situ conditions, and we will ensure the manuscript makes clear the differences between ours and real world systems. We believe that our study represents an important first step in better characterising the process of microbially-mediated iron reduction in subglacial sediments, and paves the way for more in depth studies on the subject.

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