Final author response: "Viable cold-tolerant iron-reducing microorganisms in geographically-isolated subglacial environments" by Nixon, Telling, Wadham and Cockell

Details of the changes made to the manuscript following provisional acceptance are included in the final author response below. In this version, only the comments from reviewers that have prompted changes are included below, along with reference to where in the amended manuscript the changes can be found.

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 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

We feel that the language used in the manuscript makes it clear enough that the methods used were enrichment- and not culture-based. The use of the term 'culture independent methods' (e.g. page 2, paragraph 3 of amended manuscript) has not been removed, since this is a widely used term in the literature.

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

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 microorganisms, and (2) whether these microorganisms are adapted to low temperatures. Both the hypothesis have been added to the Introduction section (page 2, lines 14-17).

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)

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 region of the bacterial 16S rRNA gene, which has been added to the amended manuscript (page 5, line 9), and so we feel are of equivalent appropriateness in this study.

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?

This second statement is incorrect and has been 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?

1/13 ditto

The title has been change 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

This has been corrected.

1/18 italicize Geobacter

This has been corrected.

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

This has been corrected.

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

This has been corrected.

4/30 centrifugation

This has been corrected.

7/15 environments

This has been corrected.

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

The reason for this statement is that Desulfosporosinus is conventionally thought of as a sulfate-reducing bacterium but out data suggest that it may be responsible 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 have changed our statement to "Our results suggest microbial iron reduction in subglacial environments is characterised by substantial metabolic and genetic diversity" (page 8, lines6-7).

7/25 Rhodoferax, Geobacter and Desulfosporosinus have been found in subglacial sediments expoerted by the river draining Leverett Glacier; Rhodoferax 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

The Cameron et al paper has been incorporated into the Discussion (page 8, lines 12-15).

8/4 define MIR at first use

This has been changed.

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

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 has been added to the Discussion (page 8, lines 27-28).

9/5 italicize Thiobacillus

This has been changed.

9/11-13 this statement needs some references

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 (Page 10, lines 1-2).

10/12 Mitchell

This has been corrected.

11/3 Schulze-Makuch

This has been corrected.

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

This has been added to the reference list.

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Reviewer 2 comments

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)

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 has been added to the Discussion section (Page 7 line 24 - page 8 line 2).

The posibility 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

The language of the manuscript has been toned down as appropriate.

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 sesequence 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

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 provide compelling evidence that such genera are responsible for the iron reduction observed (page 7, lines 20-23).

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

As suggested, we will expand this paragraph to address the possibility that iron reduction may in fact be indirect (page 7, lines 24-29, and page 8 lines 1-2).

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?

Figure 2 has been amended to include all genera representing 10% or more of the sample, and this made clear in the caption. Shannon diversity indices have also been included, and overall diversity of the samples discussed (page 6, lines 21-25).

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?

We believe this is a misunderstanding. All data representing greater than 1% of combined genus-assigned OTUs is included in the bar charts, 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 has been revised so that all taxa representing 10% or more of the sequences per sample are included in the legend. The caption to this figure has been amended to make it clear that which data was used.

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 s that they could be compared between sample types

The data reported in Figure 1 are replicates, and have been presented individually since it is not appropriate to calculate standard error or deviation for less than three replicates. Rates have been included in the new Table 2, and referred to in the results section (page 6, line 12).

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

Justification for the use of iron oxyhydroxide as the terminal electron acceptor has been added to the Discussion section (page 11, lines 4-5).

They argue that H2, 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 H2that 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 H2 (sulfate reduction, nitrate reduction etc). Third, and most importantly, they have not demonstrate that their enrichments can utilize H2. 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

These are valid points, and this part of the discussion has been toned down as recommended (page 10, lines 21, 22-23, and 26-28).

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

This has been corrected