Viable cold-tolerant iron-reducing microorganisms in geographically diverse subglacial environments

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Abstract. Subglacial environments are known to harbor metabolically-diverse microbial communities. These microbial
10 communities drive chemical weathering of underlying bedrock and influence the geochemistry of glacial meltwaters. Despite its importance in weathering reactions, the microbial cycling of iron in subglacial environments, in particular the role of microbial iron reduction, is poorly understood. In this study we address the prevalence of viable iron-reducing microorganisms in subglacial sediments from five geographically isolated glaciers. Iron-reducing enrichment cultures were established with sediment from beneath Engabreen (Norway), Finsterwalderbreen (Svalbard), Leverett and Russell Glaciers
15 (Greenland) and Lower Wright Glacier (Antarctica). Rates of iron reduction were higher at 4°C compared with 15°C in all but one duplicated second-generation enrichment culture, indicative of cold-tolerant and perhaps cold-adapted iron-reducers. Analysis of bacterial 16S rRNA genes indicate *Desulfosporosinus* were the dominant iron-reducing microorganisms in low-temperature Engabreen, Finsterwalderbreen and Lower Wright Glacier enrichments, and *Geobacter* dominated in Russell and Leverett enrichments. Results from this study suggest microbial iron reduction is widespread in subglacial environments,
20 and may have important implications for global biogeochemical iron cycling and export to marine ecosystems.

1 Introduction

Despite cold, dark and oligotrophic conditions subglacial environments worldwide harbour diverse microbial communities. Such communities have been documented at the ice-bed interface of valley glaciers (Sharp et al., 1999; Skidmore et al., 2005; Buzzini et al., 2005; Bhatia et al., 2006; Kaštovská et al., 2007; Mitchell et al., 2013; Hamilton et al., 2013), ice sheets
25 (Sheridan et al., 2003; Mikucki and Priscu, 2007; Lanoil et al., 2009), and in subglacial lakes (Priscu et al., 1999; Gaidos et al., 2004; Marteinsson et al., 2013; Christner et al., 2014). Given the absence of light in these environments, microbially-mediated redox reactions fuel heterotrophic and autotrophic microbial metabolism and drive chemical weathering reactions of the underlying bedrock (Sharp et al., 1999; Bottrell and Tranter, 2002; Tranter et al., 2005; Wynn et al., 2006; Hodson et al., 2008; Montross et al., 2012; Boyd et al., 2014). To date, subglacial microbiology research has focused on the microbial
30 cycling of carbon (Boyd et al., 2011; Stibal et al., 2012), nitrogen (Skidmore et al., 2000; Boyd et al., 2011) and sulfur (Boyd

et al., 2014; Harrold et al., 2016). The role of microbial iron cycling, and in particular iron(III) reduction, in subglacial biogeochemical cycling is poorly understood. This is surprising, given the importance of iron in a) catalysing the weathering of bedrock sulfides and promoting solute production (Bottrell and Tranter, 2002), b) influencing phosphorus availability to microorganisms via adsorption:desorption reactions on Fe(III) oxide surfaces (Gunnars et al., 2002) and c) the potential for subglacially-derived iron to impact global biogeochemical cycles by fertilising productivity in downstream ecosystems, including those in marine environments (Death et al., 2013).

Recent research has shown the flux of bioavailable nanoparticulate iron associated with glacial runoff from ice sheets to be an important source of nutrients to the surrounding oceans (Hawkings et al., 2014). These iron oxyhydroxide particles are thought to originate from the oxidation of dissolved ferrous iron from anoxic subglacial environments. The source of this Fe(II) remains unknown, though microbial iron reduction is a plausible but untested explanation (Hawkings et al., 2014).

Although a number of studies have demonstrated the presence of iron-reducing microorganisms in subglacial environments using culture independent methods (Skidmore et al., 2005; Mikucki and Priscu, 2007; Lanoil et al., 2009; Yde et al., 2010; Marteinsson et al., 2013; Mitchell et al., 2013), few studies have addressed their viability (Foght et al., 2004; Mikucki and Priscu, 2007) or their physiological characteristics. Our hypothesis is that subglacial sediments harbour active microorganisms that are capable of carrying out microbial iron reduction. Specifically, our aims in this study are 1) to assess whether subglacial sediments harbour active iron-reducing microorganisms, and 2) whether these microorgansims are adapted to low temperatures. We present data from enrichment cultures initiated from subglacial sediments entombed in basal ice, sampled at the glacier margins of five glaciers geographically isolated from one another: Engabreen, Norway; Russell and Leverett Glaciers, Greenland; Finsterwalderbreen, Svalbard; and Lower Wright Glacier, Antarctica. We demonstrate that viable iron-reducing microorganisms are present in each of these systems, that they are tolerant of, and may even be adapted to, low temperatures.

2 Materials and methods

2.1 Sample sites and subglacial sample acquisition

Engabreen (E; 66°41'N, 13°46'E) is a temperate glacier of the western Svartisen Icecap in northern Norway. The underlying bedrock is metamorphic, dominated by schists and gneisses with calcite-filled cracks (Jansson *et al*., 1996), and contains relatively little organic carbon (O'Donnell et al., 2016). The bedrock and basal ice is accessible through a system of underground tunnels leading to the glacier bed from the Svartisen Subglacial Laboratory. This basal ice was sampled by implementing hot water drilling, and sediment-laden ice removed from the resulting cavity 200 m below the glacier surface using a chain saw (Stibal *et al*., 2012).

Russell (R; 67°03'N, 50°10'W) and Leverett (L; 67°03'N, 50°07'W) are neighboring land-terminating outlet glaciers. Together they constitute a large discharge lobe emanating from the western Greenland Ice Sheet (GrIS); Leverett is the southern offshoot of the larger Russell glacier. Both are polythermal (cold and warm-based regions exist at the ice-bed interface), but warmer conditions dominate, as evidenced by accelerated glacial flow at the onset of the melt season (Sundal

5   *et al.*, 2011). The underlying bedrock is metamorphic, dominated by Archaean gneiss, which was subsequently reworked in the early Proterozoic (Henrikson *et al.*, 2000) and during numerous Holocene readvances, leading to the incorporation of organic matter (Ten Brink and Weidick, 1974). At Russell Glacier, samples were obtained from upthrusted subglacial sediment near the terminus. The outermost surface of the ice was removed using a chain saw before subsampling the remaining sediment-laden ice (Stibal *et al.*, 2012). Two subglacial samples were collected from Leverett; L was chain sawed

10   from a pressure ridge at the glacier margin, believe to be subglacial in origin, and FL ('Fresh' Leverett) was collected from the basal sediments at the ice-bed interface within an ice cave using a flame-sterilised spatula. At the time of collection, the latter sediment (FL) was assumed to be freshly melted.

Finsterwalderbreen (F; 77°28'N, 15°18'E) is a polythermal glacier on the southern side of Van Keulenfjorden, Spitsbergen,

15   Svalbard (Wadham and Nuttall, 2002). Similar to the Greenland glaciers, the thermal regime of this glacier is largely warm-based (Hodson and Ferguson, 1999). The major underlying geology is mainly sedimentary, comprising sandstone, siltstone, shale, limestone and dolomites (Dallmann *et al.*, 1990). Basal sediment was removed using a chain saw from a pressure ridge at the glacier terminus (O'Donnell et al., 2016).

20   Lower Wright (LW; 77°25'S, 163°0'E) is a cold-based glacier draining westwards from the Wilson Piedmont Glacier in the McMurdo Dry Valleys region of Antarctica. The glacier currently terminates in the permanently ice-covered Lake Brownworth (Stibal *et al.*, 2012). The underlying bedrock is dominated by granite-gneisses (Hall and Denton, 2002), however during the last few centuries numerous glacial advances have overridden and reworked lake sediments, evidence of which was found in the presence of algal matter in subglacial sediment (Wadham *et al.*, 2012). The sampling site featured

25   exposed frozen sediment sandwiched between layers of pure glacial ice at the interface of the ice-covered lake and the terminal moraines of the glacier. Blocks of this debris-laden ice were removed using a chain saw (Stibal *et al.*, 2012).

Samples were transported frozen from the field site to LOWTEX (University of Bristol) at stored at –20°C. Samples were prepared at the LOWTEX facility prior to their use in this study. Specifically, sediment-laden basal ice was placed in a

30   laminar-flow hood in pre-furnaced glass beakers and the outer layer of each sample was removed by washing with sterile deionised water. Samples were covered in furnaced aluminium foil and transferred to an anaerobic chamber, where they were thawed under 100% nitrogen atmosphere. The liberated sediment from basal ice samples was subsampled into sterile serum vials, crimp-sealed with thick butyl rubber stoppers and aluminium caps, and removed from the chamber. The headspace of each was immediately flushed with N_2 gas for 1 minute using sterile needles attached to sterile 0.2 µm syringe

filters, before being transferred to the University of Edinburgh where they were stored at 4°C for 24 hours before enrichments were initiated.

2.2 Microbial enrichment cultures

Strict anaerobic culturing techniques were used throughout (Miller and Wolin, 1974). Enrichment cultures were initiated by adding sterile anoxic freshwater basal medium at pH 6.8-7.0 to serum vials containing subglacial sediments. The basal medium contained (grams per L deionised water): $NaHCO_3$ (2.5), NH_4Cl (0.25), NaH_2PO_4 (0.06), KCl (0.1), 10 mL vitamin solution and 10 mL trace elements solution. The vitamin solution contained (mg L^{-1} deionised water) biotin (2.0), folic acid (2.0), pyridoxine-HCl (10.0), riboflavin (5.0), thiamine (5.0), nicotinic acid (5.0), pantothenic acid (5.0), vitamin B-12 (0.1), *p*-aminobenzoic acid (5.0), and thioctic acid (5.0). The trace elements solution contained (g L^{-1} deionised water): nitrilotriacetic acid (1.5), $MgSO_4$ (3.0), $MnSO_4.H_2O$ (0.5), NaCl (1.0), $FeSO_4.7H_2O$ (0.1), $CaCl_2.2H_2O$ (0.1), $CoCl_2.6H_2O$ (0.1), $ZnCl_2$ (0.13), $CuSO_4.5H_2O$ (0.01), $AlK(SO_4)_2.12H_2O$ (0.01), H_3BO_3 (0.01), $NaMoO_4$ (0.025), $NiCl_2.6H_2O$ (0.024) and $NaWO_4.2H_2O$ (0.025). Vitamin and trace element solutions were stored at 4°C in the dark. Enrichments were supplemented with acetate (10 mM) and lactate (10 mM) as the electron donors and poorly crystalline Fe(III) oxide (~100 mM) as the terminal electron acceptor. This combination of electron donors was chosen since the vast majority of iron-reducing microorganisms can use one or the other in the reduction of ferric iron, and indeed most can draw upon a much wider array of organic compounds (Lloyd, 2003). Iron oxide was synthesised as previously described (Lovley and Phillips, 1986b). $FeCl_2$ (1.3 mM) was added to the medium as a mild reducing agent. Enrichments were set up in triplicate and incubated at 4°C, 15°C and 30°C. Microbial iron reduction was determined by measuring the concentration of HCl-extractable ferrous iron with time, as previously described (Lovley and Phillips, 1986a, 1986b). Second-generation 4°C and 15°C enrichments were initiated in duplicate using a 10% v/v inoculum from initial enrichments. No 30°C initial enrichments were carried over to a second-generation since the focus of the study was on the presence of cold-adapted iron-reducing microorganisms. Therefore data presented here are from second-generation 4°C and 15°C enrichments and first-generation 30°C enrichments. A production of ≥5 mM Fe(II) over a period of 50 days was considered positive for microbial iron reduction. This concentration was chosen as a nominal figure that greatly exceeded the typical error of the ferrozine assay, as measured in calibration procedures where triplicate ferrozine measurements are conducted on calibration standards ranging in concentration from 1-50 mM. Blanks were not initiated in parallel to these enrichments, however in other experiments using the same medium and electron donor-acceptor couple no abiotic production of Fe(II) was observed over a longer period of time (see Figure S1).

2.3 Bacterial community analysis

DNA was extracted from 5.0 ml of one replicate of each second-generation 4°C enrichment culture using the FastDNA(R) SPIN kit for Soil (MP Biomedicals, Illkirch, France). Each 5 ml subsample was concentrated by centrifugation at 4570 x *g*

for 15 minutes (Heraeus Multifuge 3SR+ with swung-out rotor, Thermoscientific, UK) and resuspended in 500 µl sterile basal medium. DNA extraction was carried out according to manufacturer's protocol. Early attempts to extract DNA from 4°C LW and F enrichments failed. Subsequent attempts were successful after incorporating the protocol of Direito and others (2012) to counteract any adsorption of DNA onto clay mineral matrices. Specifically, the FastDNA SPIN kit phosphate buffer was substituted for an equal volume of 1 M sodium phosphate buffer in 15% molecular-grade ethanol, pH 8.0. Samples were incubated in a heating block at 80°C for 40 min following the lysis step in the FastDNA SPIN kit protocol. All subsequent processing was as instructed in the manufacturer's protocol.

Extracted DNA from E, L, FL and R enrichments was amplified for the V3 hypervariable region of the 16S rRNA gene using primers 27F (5'-AGA GTT TGA TCC TGG CTC AG-3') and UN1492R (5'-TAC GGT TAC CTT GTT ACG ACT T-3') (Lane, 1991). Each 50 µl PCR reaction mixture contained 1 µl DNA template, primers (0.4 µM each), deoxynucleoside triphosphates (dNTPs; 200 µM), 1.5 mM MgCl$_2$, 5 µl 10 X PCR buffer, 5 µl bovine serum albumin (BSA), and 0.5 µl *Taq* polymerase. Template DNA was initially denatured at 94°C for 4 minutes, followed by 30 cycles of denaturing (30 seconds at 94°C), annealing (30 seconds at 55°C), and extension (60 seconds at 72°C), and a final extension at 72°C for 5 minutes.

DNA extracted from F and LW was amplified for the V3 hypervariable region of the 16S rRNA gene using primers 357F (5'-CCT ACG GGG AGG CAG CAG-3') and 518R (5'-ATT ACC GCG GCT GCT GG-3') (Muyzer *et al.*, 1993). The PCR reaction mix was kept as described above. For this primer set, template DNA was initially denatured at 94°C for 4 minutes, followed by 35 cycles of denaturing (30 seconds at 94°C), annealing (30 seconds at 54°C) and extension (30 seconds at 72°C), and a final extension at 72°C for 5 minutes.

Amplified DNA was sequenced for Bacterial 16S ribosomal RNA using the Roche 454 Pyrosequencing platform (Research and Testing Laboratories, Austin, TX, USA). Resulting sequence reads were quality checked and analyzed via the Research and Testing Laboratories pipeline. This analysis is split into two stages. Firstly, sequences were quality trimmed, denoised, and checked for chimeras (Edgar, 2010, 2011; Edgar et al., 2011). Secondly, phylogenetic analysis was carried out, involving clustering sequences into operational taxonomic units and using the distributed .NET algorithm BLASTN+ (KrakenBLAST) to query the seed sequence of each cluster against a database of high-quality sequences compiled from the National Center for Biotechnology Information (NCBI). Sequences were resolved to taxonomic levels based on the BLASTN + derived sequence identity percentage. Species-level identification was assigned to sequences with greater than 97% identity, genus level is assigned to 95%-97% identity, family level to 90%-95% identity, order level to 85%-90% identity, class level to 80%-85% identity, and phylum level to 77%-80% identity. All sequences characterized by a match of less than 77% were discarded. Data reported in this study were taken from the Counts file of genus-level identification. The Shannon's index of diversity (H) was calculated for genus-assigned sequence datasets for each enrichment.

3 Results

3.1 Occurrence of microbial iron reduction

Results for positive or negative detection of microbial iron reduction in 4°C, 15°C and 30°C enrichment cultures after 50 days are summarised in Table 1. All 4°C enrichments tested positive for microbial iron reduction. In contrast, half of enrichments incubated at 15°C and only one of six incubated at 30°C were positive for microbial Fe(III) reduction. Particularly high Fe(II) concentrations were detected in 15°C Leverett (L), in which 37.75 ± 0.002 mM was measured after 58 days.

[Table 1 here]

The relative rates of microbial iron reduction in 4°C compared with 15°C duplicated enrichments are shown in Figure 1 and given in Table 2. With the exception of L, significantly greater microbial iron reduction is evident at T=58 in 4°C compared to 15°C enrichments (Student's t-test, 2-tailed, type 2: p-values < 0.005), indicative of cold-tolerant iron-reducing microorganisms. In the case of Leverett, iron reduction in the 15°C enrichment was significantly higher (p-value < 0.05).

[Table 2 here]

[Figure 1 here]

3.2 Phylogenetic diversity of cold-tolerant enrichment cultures

Genus-level taxonomic affiliations of 16S rRNA genes amplified from DNA extracted from second-generation 4°C enrichments are shown in Figure 2, along with corresponding Shannon's diversity indices (H). Overall genus-assigned diversity is highest in the second-generation enrichment initiated with L (Shannon's H of 1.92), followed by LW (H of 1.39), whilst the enrichment initiated from F is characterised by the lowest diversity (Shannon's H of 0.26), followed by E (H of 0.74). Enrichments fro FL and R exhibit similar overall diversity (H indices of 1.00 and 0.97, respectively). Sequences affiliated with *Desulfosporosinus* (95-95% sequence match) dominate the second-generation enrichment cultures originating from samples E (60.3%), F (94.3%) and LW (54.6%), and is present but not dominant in R (23.0%) and FL (3.4%). In contrast, sequences affiliated with

Geobacter (95-95% sequence match) dominate enrichments from FL (78.2%) and R (66.0%). Sequences affiliated with Geobacter are also present in L (14.0%) but are absent in E, F and LW. Other identified genera of relevance to microbial iron reduction are *Desulfitobacterium* (23.5% of sequence reads from LW), and *Rhodoferax* (18.30% of sequence reads from L).

A number of the 16S rRNA gene sequences from each enrichment could be resolved to species-level taxonomic identification. Of relevance for microbial iron reduction are *Geobacter psychrophilus* (>97% sequence match) which accounted for 46.3% of the *Geobacter* reads in FL; *Rhodoferax ferrireducens* (>97% sequence match) in enrichments L (16.2%) and FL (0.08%); and *Desulfosporosinus lacus*(>97% sequence match) in enrichments E (0.08%) and R (0.01%).

[Figure 2 here]

4 Discussion

4.1 Distribution and characteristics of iron-reducing microorganisms in subglacial sediments

Very limited information exists on the distribution and activity of microbial iron reduction beneath glaciers and ice sheets, despite the potential for this process to contribute iron to downstream ecosystems through glacial runoff. The iron reducing enrichment cultures initiated with subglacial sediments in the present study indicate that viable iron-reducing microorganisms are widespread beneath glaciers, and based on activity data, are likely to be cold-adapted. Members of the *Geobacter* genus dominate two of the cold temperature enrichments (FL and R) while *Desulfosporosinus* dominates three of the environments (E, F, LW; see Figure 2). These two genera appear to be responsible for most of the iron reduction observed, though species of *Desulfitobacterium* and *Rhodoferax* may also contribute. Further work is warranted to demonstrate that micoorganisms present in these sediments that belong to these genera are indeed capable of MIR, and to attempt to quantify their contribution to iron and carbon cycling *in situ*.

The possibility that iron reduction detected in the enrichments was indirect cannot be ruled out. Given the prevalence of sulfate-reducing genera such as *Desulfosporosinus* in these positive enrichments, the most plausible indirect mechanism of reduction would be via the abiotic reduction of ferric iron by sulfide following microbial sulfate reduction. However no sulfide production (characterised by non-magnetic black precipitate or the

recognisable rotten egg smell) was observed in these enrichments. Furthermore, the data presented in Figure 1 is for second-generation enrichments initiated with a 10% (v/v) inoculum from initial enrichments. Since no sulfate was added to enrichment medium, and no sulfide was observed in either stage of enrichments, we believe our data provides evidence of direct iron reduction.

Compared to other, less comprehensive studies, our results suggest microbial iron reduction in subglacial environments is characterised by substantial metabolic and genetic diversity. The vast difference in phylogenetic diversity between two low temperature enrichments initiated with sediments from different locations beneath the Leverett glacier (Figure 2) further highlight this diversity. The presence of iron-reducing microorganisms in subglacial systems have been identified using culture-independent methods in a number of studies (Skidmore *et al.*, 2005; Mikucki and Priscu, 2007; Lanoil *et al.*, 2009; Yde *et al.*, 2010; Marteinsson *et al.*, 2013; Mitchell *et al.*, 2013), yet only two studies investigated subglacial sediments proximal to those used here. Yde and others (2010) identified 16S rRNA gene sequences affiliated with the genera *Rhodoferax* and *Geobacter* in basal ice from the margin of Russell Glacier. More recently, Cameron and others (2016) also found evidence for the presence of *Rhodoferax*, *Geobacter*, and *Desulfosporosinus* in 16S rRNA gene sequence libraries from subglacial sediments draining Leverett Glacier; of these *Rhodoferax* was particularly abundant, representing more than 20% of reads in some samples. Similarly, Mitchell et al., 2013 detected 16S rRNA gene sequences affiliated with *Rhodoferax* in sediments sampled from beneath Robertson Glacier, Alberta, Canada; sequences affiliated with *Geobacter* were not detected. In this study we detected sequences closely related to *Rhodoferax* as the dominate sequence type in enrichments from Leverett Glacier (Figure 2), though sequences affiliated with *Geobacter* were also detected in the enrichment.

To date, only two studies have enriched for microbial iron reduction (MIR) in subglacial sediments. The first, by Foght and others (2004), enriched for iron-reducing microorganisms in sediments from beneath two temperature glaciers in New Zealand using ferric-citrate, yeast extract and tryptone in a semisolid medium. The enrichments were positive for microbial iron reduction, but the study found the microorganisms responsible to be few in number. This is consistent with the expectation that iron-reducing microorganisms should not be dominant in subglacial communities, since they depend on a supply of organic carbon from primary producing chemolithoautotrophs (e.g. Boyd *et al.*, 2014). It is possible that organic carbon of pre-glacial origin (legacy organic carbon) could serve as a carbon source, but this has yet to be demonstrated. The second study to enrich for microbial iron reduction was conducted by Mikucki and Priscu (2007), using outflow sediments from Blood

Falls in Antarctica as their inoculum and medium containing amorphous iron oxide and yeast extract, with a pressurised H_2 headspace. The 16S rRNA analysis of the sediment identified sequences closely related to the iron-reducer *Geopsychrobacter electrodiphilus*, although an isolate from a positive microbial iron reduction enrichment was a close relative of *Shewanella frigidimarina*. In all other studies *Rhodoferax* has been reported and implicated in subglacial MIR (Skidmore *et al*., 2005; Lanoil *et al*., 2009; Mitchell *et al*., 2013).

Although members of the genera *Desulfosporosinus* are conventionally thought of as sulfate-reducing bacteria, results from this study serve as compelling evidence that they are capable of low temperature iron reduction, though visually undetectable levels of sulfate reduction in the enrichment cultures cannot be ruled out. Only one characterised strain belonging to this genus is known to carry out dissimilatory iron reduction; *Desulfosporosinus lacus* (Ramamoorthy *et al*., 2006). This species was identified in two of the enrichments in which *Desulfosporosinus* was abundant, namely Engabreen and Russell. This genus has only been reported in one other study of cold glacial ecosystems (Marteinsson *et* al., 2013), in which a number of bacterial 16S rRNA gene clones from DNA extracted from subglacial lake sediments from beneath Vatnajökull ice cap in Iceland were closely associated to *Desulfosporosinus*. The authors hypothesised that *Desulfosporosinus* is carrying out the reduction of sulfate and other oxidised sulfur species in this environment, whereas *Geobacter*, also detected in their bacterial clone library, carries out ferric iron reduction. The results from our study suggest that *Desulfosporosinus* may be able to reduce iron in this subglacial lake. Indeed a number of sulfate-reducing taxa are capable of conserving energy from iron reduction (e.g. *Desulfosporomusa polytropa*, Sass *et al*., 2014; *Desulfotalea psychrophilia*, Knoblauch *et al*., 1999), which is consistent with the higher energy yield associated with iron reduction when compared to sulfate reduction (Neal *et al* ., 2003).

Whilst a true determination of psychrophily requires metabolic and growth data, on the basis of activity data alone, the iron-reducing microorganisms enriched in our current study appear to be cold-adapted, and at least cold tolerant (Figure 1). While numerous studies have provided evidence for cold-adapted micoorganisms (e.g., *Thiobacillus* sp. RG5 (Harrold *et al*., 2016) and their activities (e.g., nitrification and nitrate reduction (Boyd *et al*., 2011) and methanogenesis (Telling *et al*., 2015)), only a handful of iron-reducing microorganisms have been characterised as psychrophilic to date, namely *Desulfofrigus oceanense* (opt. T 10°C, min -1.8°C; Knoblauch *et al*., 1999), *Desulfuromonas svalbardensis* and *D. ferrireducens* (both opt. 14°C, min. -2°C; Vandieken *et al*., 2006). A larger number of iron-reducing microorganisms can tolerate temperatures near or below freezing (e.g., *Pelobacter propionicus*, Schink, 1984; Lonergan *et al*., 1996), and the sediment samples used in this study had

been frozen to -20°C for months to years prior to use in enrichments. However, the vast majority of characterised iron-reducing strains are psychrotolerant, capable of growth at low temperatures but have much higher optimal growth temperatures, such as strains of *Geobacter* (Nevin *et al*., 2005; Sung *et al*., 2006; Shelobolina *et al*., 2007, 2008), *Desulfuromusa* (Liesack and Finster, 1994) and *Pelobacter* (Schink, 1984). The results from our study
5   suggest that iron reducing microorganisms in a variety of subglacial ecosystems are adapted to the cold conditions that characterize these environments.

4.2 Potential controls on activity of iron-reducing microorganisms beneath glaciers

A key determinant of the *in situ* activity of iron-reducing microorganisms is the availability of electron donors
10   and ferric iron, as well as the absence of oxidants with higher reduction potentials such as oxygen (O_2) or manganese oxide (MnO_2) which should be preferentially utilized for energetic reasons. In anoxic environments depleted in MnO_2, understanding the supply of both electron donors and ferric iron is important in constraining the activity of iron-reducing microorganisms *in situ*. We note that the type and availability of organic matter will vary greatly between subglacial catchments, depending on factors such as hydrological isolation from the surface,
15   timings of advance and retreat over soils and vegetation, and underlying lithology. Although some glaciers have overridden soils, lacustrine or marine deposits rich in organic carbon of different bioavailabilities (e.g., Russell and Leverett glaciers are underlain by paleosols, Ten Brink and Weidick, 1974; Lower Wright glacier is underlain by former lacustrine deposits, Wadham *et al*., 2012), and this legacy organic carbon may represent a source of carbon an electron donors, others are underlain by metamorphic (e.g., Engabreen, Jansson *et al*., 1996)
20   or carbon-poor igneous bedrock (e.g. Vatnajökull; Marteinsson *et al*., 2013). In situations where organic carbon is in limited supply, iron reducing bacteria could augment their supply of reductant using H_2 gas which is supplied to subglacial communities by crushing of the underlying bedrock (Telling *et al*., 2015), though the concentrations are likely to be low and other microbial metabolisms would compete for it. Although a source of cellular carbon is still required in hydrogen-driven microbial iron reduction, demand for organic compounds would be lower.
25   Therefore the presence of iron-reducing microorganisms in subglacial environments may not be constrained by the amount of bioavailable organic carbon. Future work should incorporate hydrogen utilisation tests to demonstrate whether subglacial microbial communities are able to draw upon molecular hydrogen as an electron donor supply.

The supply of ferric iron electron acceptors is also important. Although some iron reducers have been shown to use crystalline iron oxides (e.g., *Shewanella oneidensis*, Kosta and Nealson, 1995; *S. alga,* Roden and Zachara, 1996; *Geobacter sulfurreducens*, Cutting *et al* ., 2009), the vast majority of characterised strains prefer poorly-crystalline iron oxides and oxyhydroxides, and it is for this reason that poorly crystalline oxyhydroxide was chosen as the terminal electron acceptor in the enrichments reported on here. Ferrihydrite has been widely reported in glacial sediments and meltwaters (Raiswell *et al*., 2009; Hawkings *et al*., 2014), and is generated as a reaction product of pyrite oxidation (Mitchell *et al*., 2001) or oxidation of other iron bearing minerals. Interestingly, incubation of the crystalline iron oxides hematite and magnetite in the subglacial meltwater stream at Robertson Glacier resulted in higher biomass loadings than other silicate or carbonate minerals, suggesting that these minerals were being used in energy metabolism (Mitchell et al., 2013). While magnetite has not been detected in sediments from Robertson, Hematite has been detected albeit in low amounts (Skidmore, unpublished data). Thus, the range of electron donors and types of ferric iron electron acceptors available in subglacial environments is therefore likely to be more varied than those used in this study. If true, then the true diversity of iron-reducing microorganisms in the glacial systems studied here is likely to be under-represented.

4.3 Implications for iron export from glacial systems

Recent research has shown the flux of bioavailable nanoparticulate iron associated with glacial runoff from ice sheets to be an important source of nutrients to the surrounding oceans (Hawkings *et al*., 2014). Importantly, these iron oxyhydroxide particles are thought to originate from the oxidation of dissolved ferrous iron from anoxic subglacial environments. The source of this Fe(II) remains unknown, though microbial iron reduction is a plausible, albeit undemonstrated, explanation (Hawkings *et al*., 2014). Results from our study indicate that microbial Fe(III) reduction is a possible source of the Fe(II) emanating from glaciers worldwide and may be responsible for a portion of the Fe(III) flux to marine ecosystems from glacial systems. Since Fe(III) availability often limits the primary production of marine phototrophs, the activity of iron-reducing bacteria in subglacial environments could have an indirect but significant effect on global carbon budgets (Statham *et al*., 2008; Death *et al*., 2013). Iron cycling can also have an important impact on the availability of phosphorus to microorganisms, due to the adsorption of phosphorus to solid phase Fe oxyhydroxide particles (Gunnars *et al*., 2002). Further studies are required to characterise *in situ* Fe(III) reducing metabolic activity, and to quantify its impact on the export of bioavailable iron to past and present marine ecosystems. Subglacial environments are considered analogous to potential habitats for life on Mars (Skidmore *et al*., 2000; Christner *et al*., 2008; Fisher and Schulze-

Makuch, 2013), and microbial iron reduction has been identified as a plausible metabolism to fuel microbial life (Nixon *et al.*, 2012). This study lends weight to this hypothesis, and suggests similar past or present perennially cold and dark environments are within the bounds of habitability.

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Sample	4°C	15°C	30°C
Engabreen (E)	+	-	-
Russell (R)	+	-	-
Leverett (L)	+	+	+
Fresh Leverett (FL)	+	+	-
Finsterwalderbreen (F)	+	-	-
Lower Wright (LW)	+	+	-

Table 1: Occurrence of microbial iron reduction in enrichment cultures. Positive (+) and negative (-) indications refer to second-generation duplicated (from single initial) 4°C and 15°C, and initial 30°C enrichment cultures. Initial enrichments were innoculated with thawed subglacial sediment in anoxic bicarbonate-buffered medium amended with ~100 mM poorly-crystalline ferric iron oxide and 10 mM each of lactate and acetate. Second-generation enrichments were established using a 10% v/v inoculum from initial enrichments. Microbial iron reduction was monitored by measuring the production of HCl-extractable Fe(II) over time using the Ferrozine assay (Stookey, 1970). Enrichments were considered positive where an increase in Fe2+ concentration ≥5 mM (compared with blanks) was measured within 50 days of initiating the enrichment. This concentration was chosen as a nominal figure that significantly exceeded the typical error of the ferrozine assay, as measured in calibration procedures where triplicate ferrozine measurements are conducted on each of five calibration standards ranging from 1-50 mM.

	Average Fe(II) production (µM ml^{-1} h^{-1})	
	4°C	**15°C**
Engabreen (E)	0.8 ± 0.0	0.1 ± 0.0
Finsterwalderbreen (F)	0.6 ± 0.0	0.0 ± 0.0
Leverett (L)	1.0 ± 0.2	1.9 ± 0.0
Fresh Leverett (FL)	2.2 ± 0.0	0.6 ± 0.0
Russell (R)	1.1 ± 0.1	0.0 ± 0.1
Lower Wright (LW)	1.6 ± 0.0	0.8 ± 0.0

Table 2: Rates of microbial iron reduction in second-generation enrichment cultures. Data are expressed as average (and standard deviation) Fe2+ production (µM) per ml of duplicated enrichment per hour.
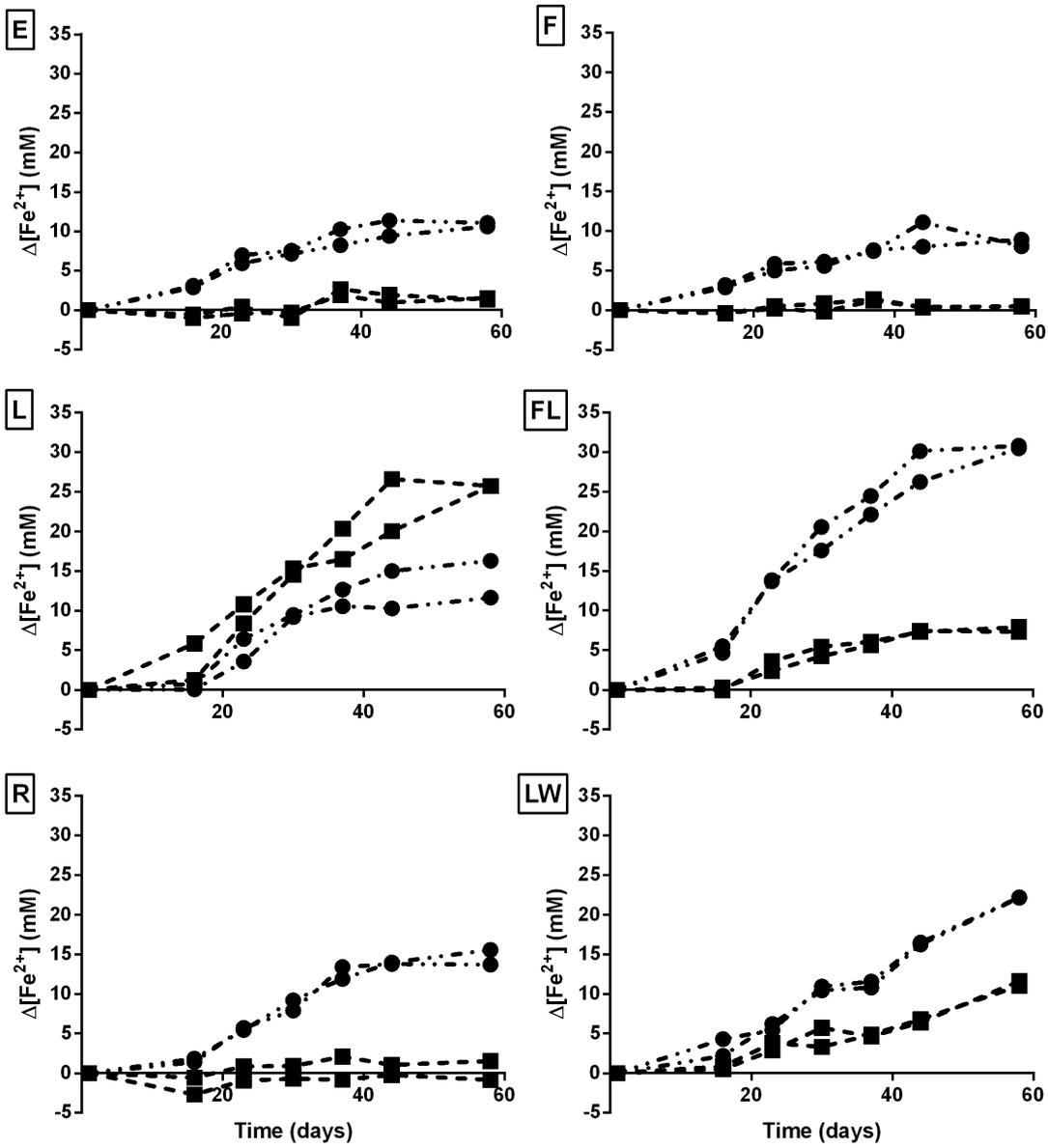
Figure 1: Microbial iron reduction in subglacial enrichments over time. Data, expressed as change in Fe^{2+} concentration (mM) over time, correspond with second-generation 4°C (circles, dot-dashed line) and 15°C (squares, dashed line) Engabreen (E), Finsterwalderbreen (F), Leverett (L), Fresh Leverett (FL), Russell (R) and Lower Wright (LW) enrichments.
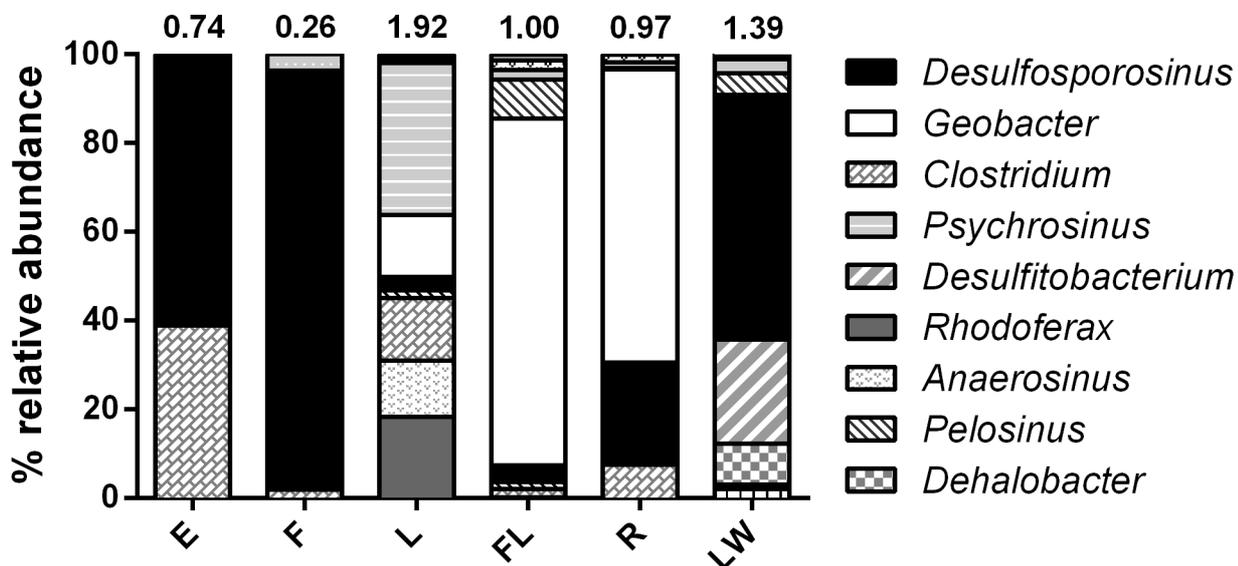
Figure 2: Genus-level taxonomic identification of 16S rRNA genes amplified from DNA extracted from second generation Fe-reducing enrichments incubated at 4°C and originated from Engabreen (E), Finsterwalderbreen (F), Leverett (L), 'fresh' Leverett (FL), Russell (R) and Lower Wright (LW) glaciers. Data show genera that represent more than 1% of combined data sets. All genera known to include strains capable of microbial iron reduction, and genera that are assigned to 5% or more of sequences for each sample, are included in the legend. Shannon's diversity indices calculated using the complete datasets for each sample are given above the corresponding bar in the chart.