

Discussion



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Author for correspondence:

J. A. Mikucki

e-mail: jmikucki@middlebury.edu

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Subglacial Lake Whillans microbial biogeochemistry: a synthesis of current knowledge

J. A. Mikucki^{1,2}, P. A. Lee³, D. Ghosh¹, A. M. Purcell¹,
A. C. Mitchell⁴, K. D. Mankoff⁵, A. T. Fisher⁶,
S. Tulaczyk⁶, S. Carter⁷, M. R. Siegfried⁷, H. A. Fricker⁷,
T. Hodson⁸, J. Coenen⁸, R. Powell⁸, R. Scherer⁸,
T. Vick-Majors⁹, A. A. Achberger¹⁰, B. C. Christner¹⁰,
M. Tranter¹¹ and the WISSARD Science Team

¹Department of Microbiology, University of Tennessee, Knoxville, TN 37996, USA

²Department of Biology, Middlebury College, Middlebury, VT 05753, USA

³Hollings Marine Lab, College of Charleston, Charleston, SC 29412, USA

⁴Department of Geography and Earth Sciences, Aberystwyth University, Aberystwyth, UK

⁵Department of Geosciences, The Pennsylvania State University, University Park, PA 16802, USA

⁶Earth and Planetary Sciences, University of California, Santa Cruz, CA, USA

⁷Institute for Geophysics and Planetary Physics, Scripps Institution of Oceanography, University of California, San Diego, CA 92093, USA

⁸Department of Geology and Environmental Geosciences Northern, Illinois University, DeKalb, IL 60115, USA

⁹Department of Land Resources and Environmental Sciences, Montana State University, Bozeman, MT 59717, USA

¹⁰Department of Biological Sciences, Louisiana State University, Baton Rouge, LA 70803, USA

¹¹Bristol Glaciology Centre, Geographical Sciences, University of Bristol, Bristol BS8 1SS, UK

PAL, 0000-0001-5523-1429; MT, 0000-0003-2071-3094

Liquid water occurs below glaciers and ice sheets globally, enabling the existence of an array of aquatic microbial ecosystems. In Antarctica, large subglacial

lakes are present beneath hundreds to thousands of metres of ice, and scientific interest in exploring these environments has escalated over the past decade. After years of planning, the first team of scientists and engineers cleanly accessed and retrieved pristine samples from a West Antarctic subglacial lake ecosystem in January 2013. This paper reviews the findings to date on Subglacial Lake Whillans and presents new supporting data on the carbon and energy metabolism of resident microbes. The analysis of water and sediments from the lake revealed a diverse microbial community composed of bacteria and archaea that are close relatives of species known to use reduced N, S or Fe and CH₄ as energy sources. The water chemistry of Subglacial Lake Whillans was dominated by weathering products from silicate minerals with a minor influence from seawater. Contributions to water chemistry from microbial sulfide oxidation and carbonation reactions were supported by genomic data. Collectively, these results provide unequivocal evidence that subglacial environments in this region of West Antarctica host active microbial ecosystems that participate in subglacial biogeochemical cycling.

1. Antarctic subglacial lakes: an underexplored microbial habitat

Antarctica has often been called ‘a frozen wasteland’ [1]. Yet as scientists have explored the continent with advanced geophysical, glaciological and biogeochemical tools, data have revealed a sub-ice water world that may be inhabited by microbes. Evidence for the existence of 17 subglacial lakes in the East Antarctic was first reported decades ago in 1973 [2]. The findings of Oswald & Robin [2] substantiated the theory of persistent subglacial water in Antarctica and also led to speculation that these lakes might provide refuge for microorganisms. It is now accepted that there are a variety of aqueous features beneath ice sheets including ‘wetlands’ or saturated sediments, streams, rivers and lakes [3,4]. As of this writing, 379 perennial subglacial lakes have been identified below the Antarctic ice sheets [5] and these lakes likely represent a variety of chemistries ranging from fresh [6,7] to highly saline [8,9]. Subglacial hydrological environments require meltwater, which forms from pressure-induced melting of basal ice or heating from geothermal flux at the bed [10]. The presence of liquid water beneath the Antarctic ice sheet is predicted once it reaches its pressure melting point with appropriate thermal conditions in the ice and subglacial rocks. Localized heating, such as in the case of volcanic subglacial systems in Iceland, can also generate lakes [11]. Subglacial volcanic systems have not yet been detected in West Antarctica, but their presence has been inferred based on aerogeophysical data and high geothermal flux measurements in the upper Bindshadler and Kamb Ice Streams [12] and upper Thwaites Glacier [13].

The few samples that have been collected from below the Antarctic ice sheets yielded data that suggested viable ecosystems were present, but there has been considerable debate upon the validity of these claims [14]. Surface water from Subglacial Lake Vostok, East Antarctica, which had accreted to the base of the ice sheet, was recovered during drilling of the Vostok ice core [15]. Samples of this material contained microbial cells and offered the first evidence for deep subsurface life in Antarctica [6,16,17]. A sediment core collected from under the Kamb Ice Stream (KIS), West Antarctica, which is due north of Whillans Ice Stream (WIS), was also shown to contain viable microbial cells [18]. These authors concluded that the KIS sample was most likely enriched in cell numbers relative to expected *in situ* abundances due to prolonged storage at 4°C. However, the organisms identified using nucleic acid sequencing and cultivation were unlikely to have resulted from contamination given the low porosity and hydraulic conductivity of the sediments [19]. Many of the phylotypes found in KIS sediments were related to groups from other freshwater subglacial environments. Indeed, it was eventually shown that samples collected at Subglacial Lake Whillans (SLW) harboured several taxonomic groups that were closely related to the KIS taxa [7], hinting at their potential hydrological connectivity and/or presence of similar ecological conditions.

Subglacial water travels along the underlying landmass, influenced by both underlying topography and ice surface characteristics, exchanging material with the glacier base along its way through regelation, at times accumulating in depressions of the hydraulic potential. Eventually, subglacial water, with entrained subglacial sediment, flows across the glacier's grounding line into the ocean where marine organisms can use any associated nutrients. In some regions of the Antarctic ice sheet, especially near its margins, hydrological flow rates can be faster, and numerous 'active' lakes that drain and refill as water moves through the catchment have been detected [20,21]. In some regions, water accumulates in deep geologically controlled depressions forming large, closed basin lakes, such as Subglacial Lake Vostok [22]. Saturated sediments and groundwater aquifers are thought to be more widespread beneath Antarctic ice than lake features, particularly at the coastal margins of ice streams [23,24], and may also underlie many lakes and streams (reviewed in [25])

Subglacial lakes are of interest to many disciplines within the scientific community. From the microbiological perspective, there is interest in understanding how microbial communities isolated from the rest of Earth's surficial environments and ecosystems, for perhaps tens of millions of years, have evolved and function [26]. Lake sediments, in particular, contain important data such as sediment types, their geochemical signatures, and possible biomarkers and fossils, all of which are important for interpreting past Antarctic environments, ice-sheet dynamics and climates. Properties of these lakes and sediments can provide insights regarding fundamental processes in palaeoglaciology, palaeoclimatology, landscape evolution, lake formation and palaeohydrology. Lessons learned from sampling these difficult-to-access systems on the Earth can also guide and improve future explorations of extraterrestrial targets such as Jupiter's moon Europa (e.g. [27]) or Saturn's moon Enceladus (e.g. [28]), and increase our knowledge of microbial survival and growth under extreme conditions of cold, dark isolation.

2. The WISSARD project

The Whillans Ice Stream Subglacial Access Research Drilling (WISSARD) project was a collaborative, interdisciplinary project funded by the United States National Science Foundation from 2009 to 2015. The goal of WISSARD was to examine SLW and the WIS subglacial hydrological and sedimentary system of which SLW is a part, to better understand its glaciological, geological, microbiological, geochemical and palaeolimnological properties [29]. WISSARD's two main science aims were: (i) to understand the role of shallow subglacial lakes and wet sediments in ice-stream dynamics and their potential effect on the stability of the West Antarctic Ice Sheet [30,31] and (ii) to understand the microbial metabolic activity and phylogenetic diversity in SLW (this article).

In January 2013, SLW became the first subglacial Antarctic lake to be drilled into and sampled directly, a major milestone of the WISSARD project [32]. Although refrozen lake water has recently been collected from Subglacial Lake Vostok [33], the extent of contamination due to mixing of lake water with the borehole drilling fluid was extensive, preventing an unequivocal analysis [34].

3. Ecological setting of Subglacial Lake Whillans

SLW is a shallow lake under the Whillans Ice Plain that is part of the greater Siple–Gould Coast system of ice streams, which are responsible for draining about one-third of the West Antarctic Ice-Sheet [35]. Until 2005, most lakes discovered in Antarctica were found in deep troughs and detected using classic radar techniques. However, improvements in measuring ice surface elevation both spatially and temporally (e.g. [20,21]), along with refinements to ice-stream modelling, have allowed for detection of smaller, dynamic lakes such as SLW that manifest as depressions or bumps in the ice surface in areas of fast flow, akin to standing waves in a river.

Like many subglacial lakes beneath the Siple–Gould Coast Ice Streams, SLW appears to be a dynamic feature, formed by a coincidence of geology, glacier flow and subglacial hydrology [36]. It is considered an 'active' lake in that it has demonstrated drainage and refilling events that

have occurred since its discovery [20]. The observation of draining and filling events indicate that the residence time of water in SLW is likely to be of the order of years to decades, whereas larger closed basin lakes such as Subglacial Lake Vostok have estimated residence times of at least 10 000 years [37].

Subglacial water entering SLW is sourced from various portions of the KIS and WIS. Analysis of water flowpaths along with the observed volume changes of SLW and neighbouring lakes has shown that water diverted from KIS can constitute up to 80% of the water in lower WIS, depending on whether the subglacial lakes in the upper parts of these ice streams are filling or draining [38]. Water piracy within the catchment supplying SLW is not limited to KIS, but also occurs further downstream and plays an essential role in supplying water to SLW. In 2005, a region just upstream of the point where WIS widens and flattens substantially began slowly rising in response to uneven slowdown of the ice stream [39], which manifested as a small, localized ice-surface elevation change. This event caused water from the upper KIS and WIS, which was flowing into a larger subglacial lake to the south, to be diverted towards SLW, and increased the filling rate of SLW by a factor of 20 [38]. As the ICESat observations of ice-sheet dynamics only covered a single 6 year period from 2003 to 2009, only one instance of water flow switching has been captured by this dataset, though a second activation of SLW in 2013 [30] probably indicates another instance of water piracy. There is evidence for multiple episodes of switching on and off between both WIS and KIS over the past 1000 years [40]. Thus, it is likely that hydrological systems supplying water to both SLW and the grounding zone downstream have switched on and off multiple times, much like a subaerial braided stream channel, and periodically deliver water, solutes and particulates mixed from across the catchment into the ocean.

It was initially believed that most subglacial lakes, including SLW, originated as ‘overland flow’ or meltwater that travelled directly along the ice-bed interface to the lake. However, recent modelling work by Christoffersen *et al.* [24] suggests that storage of water in subglacial sediments also plays a significant role in the catchment supplying WIS, with an estimated 45% of the water entering SLW possibly having spent significant time in the till pore spaces. Water-saturated till allows for extended rock–water interactions which release solutes and nutrients from the rock into porewaters. This interaction has important consequences for resident microbial communities and the availability of released nutrients for their growth. Solute-rich porewaters would either diffuse or be flushed from the system by continued flow through of basal meltwaters [41]. Alternatively, over time solutes could accumulate downstream in a lake setting. Given the short residence time of water in SLW, the former scenario of continued flushing of solutes seems more likely.

4. Sampling Subglacial Lake Whillans

(a) Clean access approach

Accessing the bed through a thick layer of glacier ice is a significant engineering challenge, and, as a result, there have been very few opportunities to make direct observations of and to sample subglacial environments [42]. A critical requirement for microbiological sampling is collecting subglacial materials without introducing surface microbial contamination via sampling equipment or introducing chemicals that would compromise scientific results. Additionally, the international scientific community has emphasized the importance of maintaining the pristine nature of subglacial environments by practising environmental stewardship of the ecosystem [43]. Several previous ice-drilling projects have employed techniques such as UV irradiation of drilling equipment [44,45] and instrumentation disinfection with solutions of hydrogen peroxide, bleach or organic solvents. The approach of the WISSARD project was to clean all instruments entering the lake with a 3% hydrogen peroxide solution, UV-irradiate all cables and hoses deployed down the borehole and reduce microorganisms in the drilling water using a combination of filtration and UV exposure [46]. To minimize the potential for water exchange, the borehole water level was lowered prior to penetration into the lake cavity, which allowed lake water to enter the borehole and prevented drilling water from entering the lake proper [32].

A hot water drill and water treatment unit were designed specifically for the WISSARD project [47,48]. The drilling water was circulated through the treatment system, which consisted of two filtration modules (2.0 and 0.2 μm pore size) to remove particulates and two germicidal UV lamps that delivered dosages of 40 000 $\mu\text{W s}^{-1} \text{cm}^{-1}$ at 185 nm and 175 000 $\mu\text{W s}^{-1} \text{cm}^{-1}$ at 245 nm to destroy organics [46,47]. After passing through the filtration and UV treatment system, the drilling fluid was heated to 90°C and pressurized for drilling operations within the boiler units [49], which also served to ‘flash pasteurize’ the water. The effectiveness of the water treatment unit was verified in the USA prior to deployment and when mated to the hot water drill during a test that penetrated the McMurdo Ice Shelf [50]. Drilling fluid was initially generated by melting snow collected at the surface near the drill location, but upwind of airfield and power generation operations. Water within the borehole was continually recirculated through the water treatment system during drill operations.

The efficacy of the water treatment system and results from samples collected using the WISSARD drill in full operation have been published elsewhere [7,46]. Large-scale laboratory testing of the filtration unit demonstrated that 99% of microbial cells were removed or killed as measured by microbial cell counts and adenosine triphosphate (ATP) concentrations [46]. Biogeochemical parameters measured during full-scale operations at the SLW drilling site showed that water in the borehole was distinct from SLW lake water samples. Cellular ATP was approximately 100 times lower and microbial cell counts were approximately 200-fold lower in the borehole than in the SLW water column [7]. Comparison of the 16S rRNA genes amplified from DNA extracted from borehole and water column samples showed that the community composition was statistically distinct. For example, phylotypes of the genera *Janthinobacterium* and *Tumebacillus* were abundant in the borehole but not observed in lake water samples. Average conductivity and temperature (see §5a) were also distinct between the borehole (5.3 $\mu\text{S cm}^{-1}$; -0.17°C) and the water column (720 $\mu\text{S cm}^{-1}$; -0.49°C), which confirmed lake water intrusion into the borehole, rather than borehole water entering the lake, following penetration [32].

(b) Microbiological sampling

Borehole instrumentation and deployment are described in detail elsewhere [32] and are the focus of other articles in this issue [31]; we include a brief description of the tools used to collect samples for biological and geochemical analyses. Bulk water samples were collected using a 101 Niskin sampler (General Oceanics, Inc.) from the approximate centre of the water column (approx. 1 m off the lake bottom). Water samples for genomic analyses were collected using an *in situ* water filtration unit designed for borehole deployment (McLane Research Laboratories; [7]). Sediments were collected using a gravity multi-corer customized for borehole deployment (UWITEC) that contained three 6 cm (diameter) by 70 cm (length) plastic core tubes. The multi-corer enabled the collection of the sediment–water interface and typically recovered approximately 30–40 cm of sediment and approximately 500–1000 ml of overlying lake water [31].

(c) Methods

(i) Oxidation reduction (redox) profiles

Redox and pH profiles were measured in two intact sediment cores. The sediment cores were stored at 4°C until processing was possible (approx. 24 h later). Cores were mounted onto a core stand and extruder (UWITEC) and the stand was secured within a class 100 clean hood for analysis. Microelectrodes, controlled by a micromanipulator (Warner Instruments), were used to profile pH and oxidation reduction potential (ORP) at 5 mm intervals throughout the top 40 cm of the cores. Measurements were made using a Ag/AgCl₂ reference electrode with a pH electrode (Microelectrodes Inc.) or platinum wire electrode, with a pH/ORP multimeter. The platinum

microelectrode was manufactured for ORP measurements by placing a platinum wire in glass tubing and heating the tube to embed the platinum. The tip was then fine sanded to expose the tip of the wire.

(ii) ^{14}C -bicarbonate incorporation

Water from the multi-core tubes (approx. 500 ml) was collected into sterile 50 ml glass serum vials (three live and three killed with 2% paraformaldehyde) with no headspace and immediately sealed with butyl rubber stoppers for ^{14}C -bicarbonate incorporation experiments (following the methods in [51]). Samples were amended with ^{14}C -labelled bicarbonate to a final concentration of $2\ \mu\text{Ci ml}^{-1}$ (stock concentration $0.1144\ \text{mCi ml}^{-1}$) and incubated in the dark at 4°C for 8 days. Time-course experiments of water collected from the lake proper showed rates were linear for 10 days [7]. Incubations were terminated by collecting biomass on a $0.2\ \mu\text{m}$ polycarbonate filter. Filters were acidified with 0.5 ml of 3N HCl and dried at 60°C . Radioactivity retained in the biomass on the filters was determined using a calibrated scintillation counter after addition of 10 ml of Cytoscint ES scintillation cocktail (MP Biomedical). Background radioactivity was determined with paraformaldehyde-killed control samples and subtracted out.

(iii) Microscopy

Cells were stained with SYBR Gold nucleic acid stain (Invitrogen) $25\times$ concentration and visualized with epifluorescence microscopy. Images obtained using scanning electron microscopy were prepared from 6 ml of sample that was filtered onto a $0.2\ \mu\text{m}$ black polycarbonate filter (Millipore), gold sputter coated, and visualized on a Zeiss Auriga. For transmission electron microscopy, $10\ \mu\text{l}$ of sample was placed onto a carbon-coated copper grid and negative stained with uranyl acetate prior to visualization on a Zeiss Libra 200 MC microscope.

(iv) Molecular gene analyses

Bulk sediment samples from the multi-core were collected for molecular analyses (see [52] for sampling and storage protocols). Nucleic acids were extracted from sediment samples as previously described [52] in a class II type A2 clean hood (LabConco model no. 3460001) using the FastDNATM SPIN Kit (MP Biomedicals) according to the manufacturer's protocol. DNA retrieved from SLW sediment samples was amplified with primers specific for functional genes of key steps in three distinct carbon fixation pathways in prokaryotes (see the electronic supplementary material, tables S1 and S2). Amplicons were cloned into pCR4 vector (Life Technology) and sequenced using vector-specific primers. A total of 96 *cbbm*, 10 *niff* and 6 *acc* clones from each library were analysed for this study. Sequences were identified for close relatives using the BLASTx server [53]. Operational taxonomic units (OTUs) were determined by grouping sequences that are more than or equal to 95% identical. Amino acid sequences of closest relatives were selected for phylogenetic analyses. The corresponding nucleotide sequences of each OTU were translated into amino acid sequences using the same reading frame determined with their corresponding relatives from the BLASTx alignment results. Sequences were aligned with ClustalW; phylogenetic trees were produced by the neighbour-joining calculation of amino acid substitution rates in the MEGA v. 5.0 [54] software suite using the p-distance model and pairwise gap deletion. 16S ribosomal RNA (rRNA) gene data discussed were previously published [7].

5. Biogeochemical results from Subglacial Lake Whillans

(a) Water column structure

SLW was penetrated after drilling through 802 m of glacier ice [32]. The drill site was placed over the deepest portion of the lake as inferred by the area with the lowest hydropotential, largest surface elevation change and deepest measured surface depression [55]. At the time of

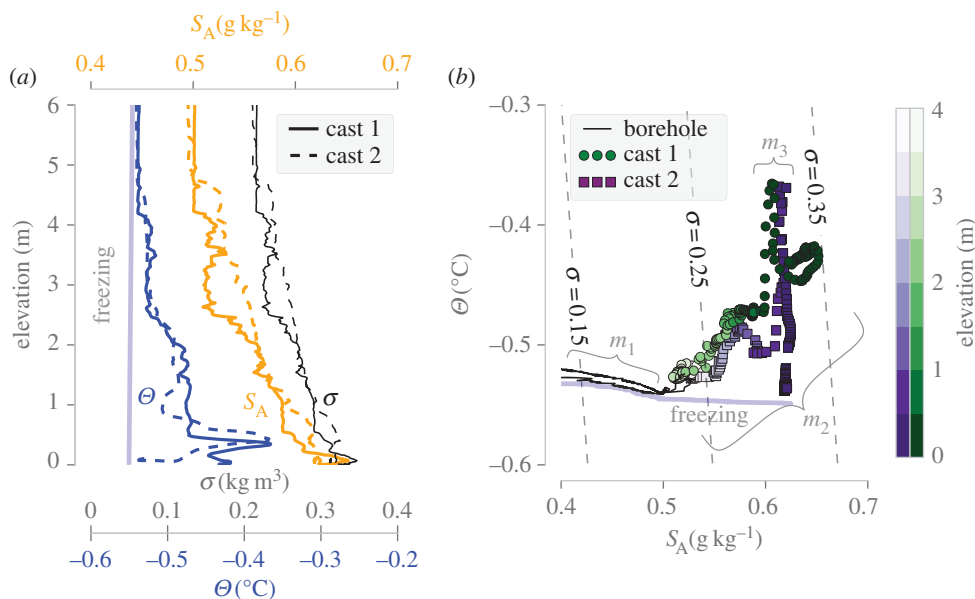


Figure 1. Temperature, salinity and density from two CTD casts in Subglacial Lake Whillans. (a) Conservative temperature (Θ °C, blue, left, bottom axis, with freezing temperature as thick line), absolute salinity (S_A g kg $^{-1}$, orange, centre, top axis) and potential density ($\sigma = (\rho_{S_A}, \Theta, \text{depth} = 0) - 1000$ kg m $^{-3}$, black, right, lower axis) for 6 m above the lake bed, including both the lake and borehole waters. (b) Θ - S_A plot with σ as background contour, and casts differentiated by both symbol and colour for the bottom 4 m (lake and borehole). Three water masses are highlighted with braces: m_1 is in the borehole and follows the freezing line, m_2 includes the lower portion of the borehole and is a roughly linear trend from the borehole to the bottom water, and m_3 is a thermal intrusion.

drilling, the lake was in the process of refilling, following a low stand in 2010 [32]. Using a borehole camera and a conductivity, temperature and depth system (CTD; SBE 19plus V2 SeaCAT Profiler; Seabird Electronics), the thickness of the liquid water column at the time of sampling was estimated to be between 1.6 and 2.2 m [32]. Each of the two CTD casts touched the lake bottom, and during the second cast the sensor was laid on its side. Thus a greater maximum pressure was recorded during the second cast, equivalent to approximately 37 cm in additional water depth. The casts were aligned at their lowest point (figure 1a), based on the assumption of a flat lake bottom and no change in water elevation in the borehole. Properties including absolute salinity, conservative temperature and density were derived using the TEOS-10 equations ([56,57], for a limnological setting [58]), resulting in salinity being approximately 50% higher than inferred initially [32].

Data collected with the CTD indicate that SLW is a cold, freshwater lake (figure 1a). While both CTD casts indicate a largely monotonic and stable density structure, the temperature and salinity profiles are more complex. Borehole water is relatively fresh and close to the freezing temperature, whereas water in SLW tends to be more saline and warmer (figure 1b). The overall agreement between casts, near stable density stratification and borehole waters near the pressure melting point suggest that borehole water and intrusion of drilling fluid during lake entry did not significantly impact water column observations.

Within the lake, the coldest bottom temperatures recorded by the CTD in cast 2 (approx. -0.55°C) were found adjacent to the lake bottom, and agree with bottom temperatures from repeated measurements made with several independent thermistors from a geothermal probe [10]. The thermal profile varies slightly between casts (figure 1a). In cast 1, the warmest lake water (approx. -0.39°C) peaks at approximately 62 cm off the bottom, whereas in cast 2 the thermal maximum is located 38 cm off the bottom. Differences in lake thermal structure

between casts are attributed to natural and induced mixing of water masses. The deepest part of the SLW water column does not appear to be thermally stable, because there is an increase in temperature with elevation above the lake bottom. However, the deepest water is the most saline, resulting in relative density stability, and may indicate the inflow of cold, saline water along the lake bottom.

A plot of conservative temperature (Θ) versus absolute salinity (S_A) (figure 1b) helps to resolve the presence and mixing of water masses. The borehole water mass (m_1 brace) is at the freezing point and has low conductivity. A second water mass (m_2) shows a positive, roughly linear increase in salinity and temperature, which may indicate the mixing of colder, fresher water (from the melting of the ice roof) and warmer, saltier water (from SLW). The final water mass (figure 1b, m_3) shows a range of temperatures with a narrow range of salinity and is interpreted to represent a thermally unstable anomaly. Changes in Θ and S_A between casts could result from a dynamic subglacial hydrological regime, or disturbances from other instruments and borehole water circulation.

(b) Sediment properties

SLW sediment was homogeneous fine-grained diamicton with no evidence of particle size sorting and is interpreted as till [59]. The overall diamicton texture and the absence of silt or sand lags indicated slow basal water flow, even during the episodic lake filling and draining events documented [36,60]. Porosity and water content were notably higher in the upper 40 cm than in the more consolidated till below [59]. Fossil diatoms and other microfossils and particulates derived from older marine sediment were rare and fragmented in all SLW sediments, despite the fact that the source rocks that make up the till include a significant marine component. When compared with tills collected further upstream from beneath the WIS [61,62], the relative lack of diatoms in the SLW sediments was interpreted largely as a result of mechanical degradation, from both subglacial shear strain in deforming till and long-distance transport from their upstream source rocks [63,64].

In the SLW sediments, many of the fossil siliceous sponge spicules are of the class Hexactinellida. These spicules, which are much denser than diatoms and more resistant to dissolution, have evidence of dissolution pitting while the diatom fragments do not (J Coenen 2015, unpublished data). The observed pitting is analogous to microbially mediated pits observed on spicules recovered from other marine sediments ([65]; reviewed in [66]). While the observed dissolution (or etch) pits could result from dissolution of sponge spicules in silica-limited waters (silica concentrations in SLW have not been measured), it is surprising that the opaline silica of diatom fragments did not manifest pits. As diatoms fragment, their surface area increases, which would favour the dissolution of diatom fragments over sponge spicules in silica-limited waters. However, diatom fragments have a fresh appearance despite mechanical breakage, whereas sponge spicules have etch pits. It is possible that the sponge spicules have been selectively colonized to access an as yet unknown nutrient.

(c) Microbial community diversity: morphological diversity

The WISSARD project was the first study to confirm the presence of a metabolically active microbial community in a lake beneath the Antarctic ice sheet [7]. A unique attribute of the project was 'state-of-the-art' science laboratories constructed within 12.2 m (i.e. 40 ft.) containers and attached to the drilling platform. The laboratories allowed for sophisticated microbiological, molecular and biogeochemical analyses to be conducted on site. First, on-site measurements of the water column revealed a cellular ATP concentration of 3.7 pmol l^{-1} and an average microbial abundance of 1.3×10^5 microbial cells ml^{-1} of SLW water [7]. These values are similar to or higher than other subglacial ecosystems and groundwater studies elsewhere in the world. For example, cell abundances of 2.1×10^4 cells ml^{-1} were detected in Grímsvötn lake water in Iceland, and 3.8×10^7 cells g^{-1} of lake sediment [67], whereas abundances that are comparable with SLW

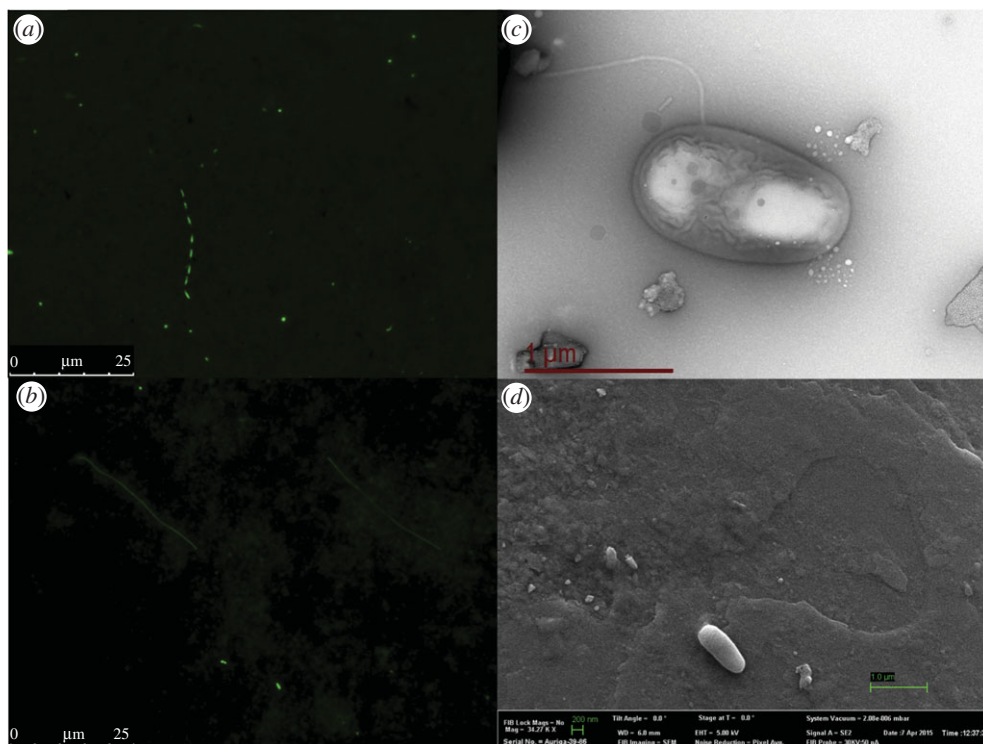


Figure 2. Epifluorescence microscopy images of SYBR Gold nucleic acid-stained microbial cells from the Subglacial Lake Whillans water column (*a*) and (*b*). Transmission (*c*) and scanning (*d*) electron microscopy images of microbial cells from the Subglacial Lake Whillans water column.

were observed in a different subglacial volcanic lake in Iceland, West Skaftá ($4.7\text{--}5.7 \times 10^5$ cells ml^{-1} ; [68]). Blood Falls, a subglacial outflow in the McMurdo Dry Valleys of Antarctica, contained an average 6×10^4 cells ml^{-1} during an outflow event in 2004 [8].

Microorganisms visualized in SLW samples had high morphological diversity [7]. Water samples stained with a DNA-binding stain revealed both long and short filaments, thin and thick rods (up to $750\text{--}1500\text{ nm}$ wide), spirals, vibrio, cocci and diplococci (e.g. figure 2*a,b,d*). While the morphology of microbial cells does not confer information on taxonomy, it can provide insight on the ecological niche of the microorganism. For example, morphology can influence nutrient acquisition, motility, surface attachment, cell division and passive dispersal [69,70]. These selective traits can impact the evolutionary path of an organism ([70] and references therein). In general, smaller cells (as small as 200 nm) are suggestive of an energy-limited or nutrient-deprived environment [70]; in SLW, the cells were generally large with cocci up to $1\text{ }\mu\text{m}$ in diameter, rods up to $3\text{ }\mu\text{m}$ long and filaments up to $62\text{ }\mu\text{m}$ long. The long thin filaments observed in SLW water samples ranged from 7 to $62\text{ }\mu\text{m}$ in length and were $300\text{--}400\text{ nm}$ in width. It is possible that the microbes elongate into rods or filaments to increase the ratio of cell surface area to volume as a response to nutrient limitation ([71] and references therein). While the identity and function of the long filaments in SLW remains unknown, long, filamentous microbes (up to 1.5 cm in length) of the family Desulfobulbaceae have been shown to shuttle electrons from sulfide to oxygen across spatially segregated redox zones in marine sediments [72]. Taxa closely related to the Desulfobulbaceae were present in SLW water samples, albeit a rather minor ($0.001\text{--}0.003\%$) component. A flagellum was visualized on an SLW cell using transmission electron microscopy (figure 2*c*). If these organisms were motile, it may assist with the acquisition of nutrients or chemotaxis throughout the water column.

(d) A chemosynthetic ecosystem below ice

Sunlight cannot penetrate through the thick ice overlying subglacial environments, thus microbes in environments such as SLW must derive energy from nutrients available within the underlying bedrock and sediment or supplied via meltwater from the overlying ice [73,74]. Efficient nutrient recycling between members of the community would also be important without fresh inputs of fixed carbon from photosynthetic primary production. Geochemical data collected from SLW suggest that upward diffusion from the sediments is the major source of ions to the water column as well as reduced N and S compounds [7]. A flux of nutrients from the sediments appears to support increased metabolic activity near the sediment–water interface (see §5e).

There is growing evidence that chemosynthesis forms the base of the subglacial foodweb. Christner *et al.* [7] reported average rates of dark ^{14}C -bicarbonate incorporation of $32.9 \text{ ng C l}^{-1} \text{ d}^{-1}$ in samples collected from the SLW water column, fuelled by nitrification. These rates exceed average measured rates of heterotrophic production, although decomposition of organic matter must be the ultimate source of ammonium for nitrification [7]. SLW carbon fixation rates are comparable to other subglacial ecosystems including Blood Falls (approx. $14 \text{ ng C l}^{-1} \text{ d}^{-1}$; [51]) and Grímsvötn in Iceland ($40 \text{ ng C l}^{-1} \text{ d}^{-1}$; [67]). Dark carbon fixation measured in water directly overlying SLW sediments (collected from the multi-corer) were approximately three times higher ($100 \text{ ng C l}^{-1} \text{ d}^{-1}$) than rates in the water column (approx. 1 m above the sediment–water interface). These higher rates may be a result of chemoautotrophic organisms living at the sediment–water interface with access to reduced electron acceptors (such as S or possibly CH_4) diffusing from the sediments. Dark carbon fixation rates at the sediment–water interface are comparable to dark carbon fixation rates reported for the deep North Atlantic Ocean. Herndl *et al.* [75] found average rates of approximately $75 \text{ ng C l}^{-1} \text{ d}^{-1}$ (range $6.96\text{--}400 \text{ ng C l}^{-1} \text{ d}^{-1}$) for prokaryotic fixation and approximately $4 \text{ ng C l}^{-1} \text{ d}^{-1}$ (range $0.96\text{--}14.8 \text{ ng C l}^{-1} \text{ d}^{-1}$) for archaeal C fixation, with Reinthaler *et al.* [76] reporting an average of approximately $31 \text{ ng C l}^{-1} \text{ d}^{-1}$ (range $1.44\text{--}264 \text{ ng C l}^{-1} \text{ d}^{-1}$) for microbial fixation.

Analysis of 16S rRNA gene sequences, a robust phylogenetic marker molecule, revealed a diverse community in SLW samples dominated by taxa related to chemolithoautotrophic species that used reduced N, Fe and S species in energy-generating metabolic pathways [7]. The water column of SLW was more species rich than the surficial sediments (0–2 cm depth) containing 3931 and 2424 groups or OTUs. Cluster analysis indicated that the top 2 cm of sediments and water column were similar [7] but community structure at sediment depths below 2 cm were statistically different ([52]; A Achberger 2015, unpublished data). The Betaproteobacteria were the most abundant bacterial division in both the water (more than 38% of sequences) and sediments (more than 59% of sequences). Betaproteobacteria appear to dominate other freshwater subglacial aquatic ecosystems [77]. For example, 47% of the OTUs from samples collected below the Robertson Glacier in the Arctic were Betaproteobacteria [78].

The analysis of nucleic acids from SLW samples revealed three betaproteobacterial OTUs that were closely related to sequences retrieved from sediments below the KIS [18]. The closest cultured relatives to these environmental sequences included *Polaromonas glacialis* (5% of OTUs in SLW water and 1.8% in sediments), an aerobic heterotroph, which was also the second most abundant OTU in SLW waters [7]. The OTUs with highest sequence identity to chemolithoautotrophic organisms *Sideroxydans lithotrophicus* (4.8% of OTUs in SLW water and 12% in 0–2 cm sediments) and *Thiobacillus denitrificans* (6% of OTUs in 0–2 cm SLW sediments) were also detected in KIS sediments and could reflect the fact that both KIS and SLW sediments originate from a similar source further upstream.

The most abundant OTU in the water column (13% of all sequences) was related to ‘*Candidatus Nitrotoga arctica*’ a nitrite-oxidizing betaproteobacterium [7]. These sequences were also abundant in the surficial sediments (7.8%). The most abundant archaeon detected (2.5% in the water column) grouped within the Thaumarchaeota [7]. All members of this lineage currently in culture are ammonia-oxidizing chemolithoautotrophs [79]. Nitrification is likely to be an important function in SLW based on both a high abundance of 16S rRNA gene sequences related to known

ammonia- and nitrite-oxidizing taxa and isotopic evidence ($\Delta^{17}\text{O}$ values of nitrate) indicative of microbial oxidation rather than atmospheric sources [7].

While evidence suggests ammonia and nitrite oxidation dominate in the SLW water column, geochemical and functional gene data indicate that sulfur oxidation may be important in the surficial sediments. Sulfate is present in excess of seawater in the top 15 cm of the SLW core, suggesting contributions from sulfur oxidation [7]. Purcell *et al.* [52] analysed sediment samples to a depth of 34 cm and detected genes involved in S-oxidation including the adenosine-5'-phosphosulfate (APS) reductase and reverse-acting dissimilatory sulfite reductase (rDSR). Using quantitative PCR, these authors determined that *aprA*-containing prokaryotes were a significant portion of the community (i.e. up to 14.5% of 16S rRNA gene abundance in the surficial sediments).

Although very few subglacial environments have been sampled and even less have been analysed using next-generation sequencing techniques, the *Sideroxydans*-like phylotypes appear to be abundant in freshwater subglacial communities. Approximately 80% of the *aprA* sequences found in sediments from the uppermost depths (0–4 cm) of SLW were related to a *Sideroxydans* species. The abundances of 16S rRNA genes (as described above) also support *Sideroxydans* dominance in SLW surficial sediments [7]. Analysis of 16S rRNA gene sequences related to the *Sideroxydans* group retrieved from SLW were 99% identical to those from KIS (clone B77; [18]). The distribution of this organism extends beyond the Siple Coast subglacial environments; a *Sideroxydans* sp. constituted approximately 12% of a bacterial cDNA library from sediments beneath Robertson Glacier in the Canadian Rockies [78]. The only known cultured representative of the *Sideroxydans* are obligate chemoautotrophic iron and sulfur oxidizers [80], suggesting that these are important processes in subglacial ecosystems.

The community structure in SLW sediments changes with depth; below 4 cm, 36–68% of the *aprA* sequences were too distantly related to known sequences to determine whether these genes putatively operate in a sulfur-oxidizing or reducing capacity [52]. It is possible that these genes are related to uncharacterized sulfur-oxidizing prokaryotes. Similar sequences are found in other environmental samples including lake sediments [81,82] and in Blood Falls [8] but the organisms containing them have not been isolated or characterized. Below 8 cm, key genes involved in sulfate reduction were observed [52]. The appearance of dissimilatory sulfite reductase (*dsrA*) and *aprA* related to known sulfate-reducing prokaryotes within the genera *Desulfobacterium* and *Desulfotomaculum* indicate the potential for sulfate reduction [52]. However, members of *Desulfotomaculum* are known to form endospores [83] and therefore may not be active *in situ*.

Rates of sulfate reduction were measureable in SLW sediments [52] but were extremely low (average = $1.4 \text{ pmol cm}^{-3} \text{ d}^{-1} \pm 0.60$). Similarly, low rates have been measured in marine sediments [84] where it has been dubbed 'the cryptic sulfur cycle' [85,86]. A cycling of sulfur compounds with intermediate oxidation states (i.e. elemental sulfur or sulfur oxyanions) has been described previously in Blood Falls [8]. Purcell *et al.* [52] concluded that this finding was possible evidence for a 'cryptic sulfur cycle' operating in SLW; a notion supported with the limited recovery of dissimilatory sulfite reductase (DSR) genes, which encode for a key step in microbial sulfate reduction.

Based on the two published reports on the SLW microbial ecosystem [7,52], chemosynthesis appears to be an important process in both the water column and surficial sediments. However, rates of dark carbon fixation activity in SLW sediments were not determined. Sediment samples were surveyed for genetic evidence of dark carbon fixation. Ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO), which codes for the key carboxylation enzyme in the Calvin cycle and was the first metabolic pathway for carbon fixation discovered, is broadly distributed across all three domains of life [87]. Thus, it is not surprising that genes encoding for RuBisCO have recently been detected in Arctic subglacial environments; Boyd *et al.* [88] detected *cbbL* transcripts related to *Sideroxydans lithotrophicus* in sediments from below Robertson Glacier. However, numerous other pathways for carbon fixation are available to chemosynthetic organisms (reviewed in [89–91]) and may be important in subglacial ecosystems. Other pathways

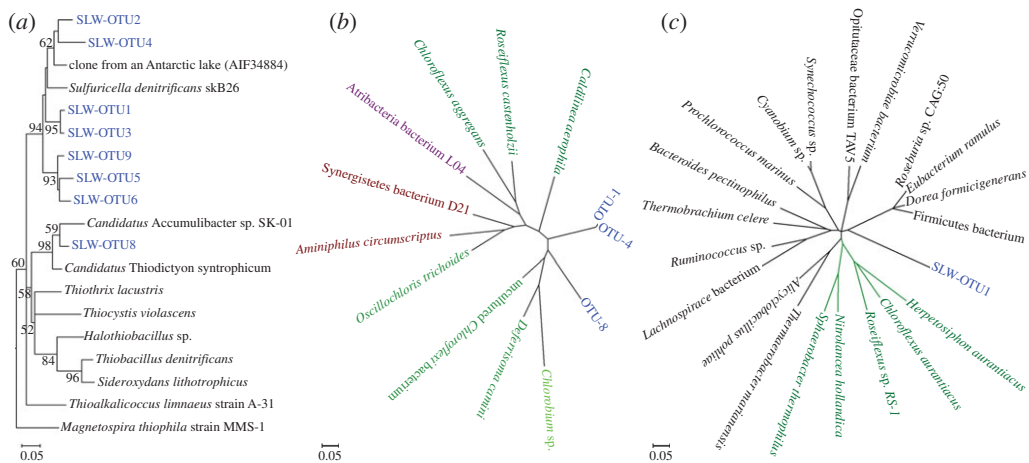


Figure 3. Phylogenetic trees of carbon fixation functional gene OTUs from Subglacial Lake Whillans (SLW) sediments. Neighbour-joining reconstructions of (a) *cbbM*, (b) *nifH* and (c) *acc* sequences from SLW sediments and the most identical cultured organisms that contain these genes and environmental sequences. Representative nucleotide sequences of each OTU were translated into amino acid sequences using the same frame determined by their corresponding relatives in the BLASTx alignment results. Values at nodes indicate bootstrap support from 1000 replicates. Scale bar indicates the branch length corresponding to 0.05 substitutions per amino acid.

of carbon fixation might confer a competitive advantage to microbes by operating more efficiently in a nutrient- or energy-limited system. There have been a limited number of functional gene surveys for key steps in the diverse carbon fixation and, to date, most have been conducted in hydrothermal vent ecosystems (reviewed in [91]).

Analyses of several functional genes indicate that at least three carbon fixation pathways may operate in SLW. Seven distinct groups or OTUs of form II RuBisCO (*cbbM*) (figure 3a) were detected in the SLW surficial sediments. The SLW *cbbM* OTUs are related to betaproteobacterial sulfur oxidizers (figure 3a), a result that adds support to the 16S rRNA gene [7] and *aprA* gene [52] data. Pyruvate ferredoxin oxidoreductase (*nifH*) is one of the four CO₂-fixing enzymes in the reverse tricarboxylic acid (rTCA) cycle—a pathway discovered in anoxygenic phototrophs, and primers for this gene have been previously described [92]. The *nifH* sequences retrieved from SLW sediment clustered into six OTUs. These groups were only 62–70% identical at the amino acid level to their closest relatives and distantly related (deeply branched) with the divisional clusters of Chloroflexi, OP9 and Chlorobi sequences (figure 3b); members of these phylogenetic groups are known to operate the rTCA cycle [87]. Several SLW *nifH* OTUs cluster with a recently described candidate division, Synergistetes [93], the function of which is not known at present. Two OTUs related to the Chlorobi were detected in SLW sediments by 16S rRNA analysis, although the relative abundance was low (approx. 0.07%). Attempts to amplify other genes of the rTCA cycle have not yet been successful. Lastly, the possible presence of genes from the 3-hydroxypropionate bicycle (3-HP bicycle) pathway was investigated. To date, this pathway has only been detected in members of the Chloroflexi group [87]. To our knowledge, there have been no surveys assessing the abundance of 3-HP bicycle functional genes in environmental samples. Primers designed for genes of two key carboxylating steps including acetyl CoA carboxylase (*acc*) and the propionyl CoA carboxylase (*pcc*) were successfully amplified from SLW sediments. Chloroflexi-like OTUs were also detected in SLW surficial sediments by 16S rRNA gene analysis [7] and two of these OTUs were most closely related to the Caldilineaceae family with 0.25% and 0.08% relative abundance. While only one OTU from the *acc* clone library was phylogenetically analysed, it revealed the presence of a deeply branched acetyl CoA carboxylase that aligns between the Actinobacteria and Chloroflexi lineages (figure 3c).

These results indicate that several carbon fixation pathways exist in SLW sediments, and may indicate a variety of niche spaces for subglacial microbes to occupy and contribute to organic matter production. This diversity might also reflect redox conditions within microzones of the SLW sediments. Some enzymes such as form II of the *cbm* gene and enzymes in the anaerobic pathways such as rTCA are oxygen sensitive and are used by microaerophilic and anaerobic species [91]. There is also the potential for an energetic advantage in organisms that use anaerobic carbon fixation pathways, such as the rTCA cycle. Under aerobic conditions, the Calvin cycle uses 7–9 ATPs per mole of pyruvate fixed, versus 1–5 ATPs in the rTCA cycle [89,90]. Several of the sequences detected in this survey are deeply branched, which could be a reflection of the fact that these genes have not been robustly examined in environmental samples or, alternatively, the lineages detected in SLW may represent novel groups.

(e) Redox chemistry and thermodynamics

Thermodynamic modelling based on redox chemistry and Gibbs free-energy estimates provides a link between the aquatic chemistry of an ecosystem and the metabolic structure of the microbial community present. The classical view in aquatic geochemistry is that the order of oxidants used in the remineralization of organic carbon follows a predictable sequence based on decreasing free energy returns (i.e. the electron tower or thermodynamic ladder; [94]), which is paralleled by an ecological succession of organisms [95]. This view probably holds true in many marine environments that contain a near-continuous excess of organic carbon raining down from the surface as a result of photosynthesis. However, subglacial microbial communities are devoid of sunlight and deprived of photosynthetically derived energy. These sub-ice communities are therefore reliant on chemolithotrophy for ATP production. While the remineralization of reduced organic compounds (e.g. via ammonification or the oxidation of methane to carbon dioxide or bicarbonate) can still take place in a chemolithotrophic system, the importance of inorganic substrates to drive ATP production is considerably higher. In these circumstances, the taxonomic composition and any potential ecological succession of the microbial community is more likely to be determined by the spatial and temporal availability of the inorganic substrates, whether dissolved species or a solid phase [96].

The nature of any chemolithotrophic processes present is dependent on the nature of electron donor/acceptor couples available for respiration, which in turn are dependent on the chemical properties of the subglacial environment [97]. A number of factors have been implicated in controlling subglacial chemistry. Early findings suggested that meltwater inputs controlled the amount of oxygen entering some subglacial systems and, consequently, their redox state [74]. Other studies pointed to bedrock composition as being influential in governing microbial communities by providing mineral and carbon substrates [96,97]. As the fourth most abundant element in the lithosphere, iron is likely to have a critical role in the redox chemistry of many subglacial water masses. In general, the reduction potentials for reactions involving iron are lower than those for oxygen and nitrate, but higher than those for sulfate or carbon dioxide reduction. As a result, iron could act as an electron donor in environments where there is a supply of oxygen or nitrate or it could act as an electron acceptor once oxygen and nitrate are depleted. A recent study has shown pyrite to be the dominant mineralogical control on subglacial sediment-associated biofilm communities [96]. Nonetheless, these differences in community structure may result in part from the hydrology of each environment and residence time of water in the system. Rapid turnover of the water in the lake system (years to decades as in the case of SLW) probably provides more frequent replenishment of ‘high-energy’ electron acceptors such as oxygen and nitrate from basal ice melt while slow turnover (millennia or longer as in the case of Blood Falls; [8]) results in the depletion of these compounds.

Many aquatic environments contain multiple redox pairs in disequilibrium with each other resulting in mixed observed potentials. Still, redox potentials (E_h measurements) can provide a qualitative assessment of the state of that environment [95] and, by extension, estimates of the thermodynamic favourableness of microbially mediated processes. The E_h level for the water

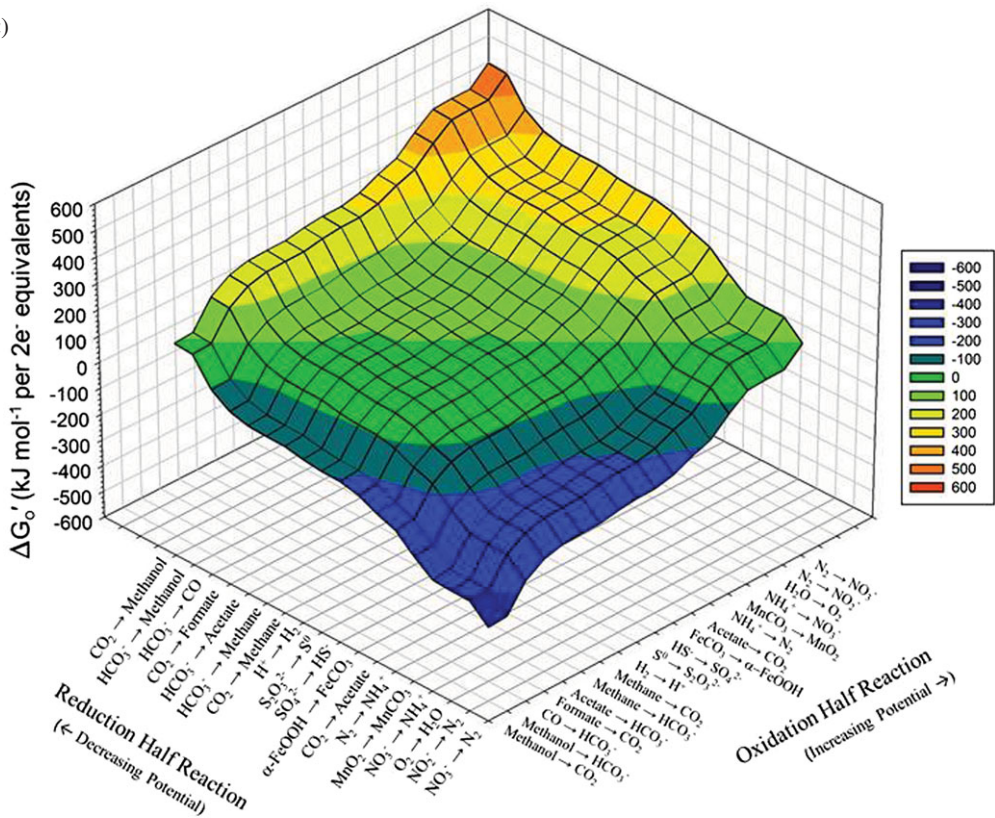
column of SLW has been reported as 382 mV [7] or equivalent to $p\varepsilon = 7.06$ at *in situ* temperatures. In Blood Falls, the outflow of subglacial water from beneath the Taylor Glacier in the McMurdo Dry Valleys, E_h was determined to be 90 mV ([8]; or $p\varepsilon = 1.69$). These results are consistent with the low but measureable levels of oxygen and nitrate reported for SLW, and the lack of oxygen and oxidized forms of nitrogen in Blood Falls outflow [7,8], respectively. By comparison, E_h levels in the perennially ice-covered lakes of the McMurdo Dry Valleys, Antarctica, range from greater than 750 mV ($p\varepsilon > 13.54$) in the well-oxygenated surface waters of Lakes Bonney and Fryxell to less than -250 mV ($p\varepsilon < -4.30$) in the anoxic/euxinic bottom waters of Lakes Fryxell and Vanda [98]. Simple estimates of the Gibbs free energy (i.e. the thermodynamic favourableness) from the coupling of a relatively small number of plausible redox reactions (electronic supplementary material, table S4) shows the wide variety of redox couples that could be used to derive energy for metabolic processes in the SLW water column (figure 4a). However, the E_h level measured in the water column suggests that the redox conditions may not be sufficiently reducing for some reactions (such as sulfate reduction or methanogenesis from carbon dioxide) and sequences related to known phylotypes that mediate these reactions were not detected in samples of SLW water.

The redox characteristics of the SLW sediments reveal both interesting and unexpected patterns (figure 4b). At the sediment–water interface, E_h levels are close to those of the water column. Below the interface at a depth of 2–3 cm, E_h values steadily decreased to less than -100 mV, indicative of reducing conditions. Between 3 and 9 cm, E_h began to increase to positive values. E_h and pH measurements were made at depths below 9 cm and while the general increase in E_h continued with depth, the data are not considered further due to unstable readings and poor electrode performance in this region (the data are shown in the electronic supplementary material, figure S1a, for completeness). The readings in the lower part of the core took longer to stabilize than those in the upper sediments, which could indicate electrode poisoning due to the presence of sulfide species (see next paragraph). As SLW is the first subglacial environment in which these measurements have been made, it is not known whether the observed redox profile is a general feature of subglacial environments or unique to SLW. While the origin of the redox minimum remains to be determined, it could result from a complex interplay of microbial and geochemical processes involving the depletion and replenishment of electron acceptors. Nonetheless, these observations suggest that the redox conditions in the sediments are not in equilibrium with the overlying water column.

The redox minimum was also coincident with a dark band, characteristic of black iron sulfide precipitates, that was observed in the sediment cores when initially retrieved (electronic supplementary material, figure S1b). Iron sulfides are common in marine sediments and can include pyrite (FeS_2) and/or greigite (Fe_3S_4) framboids [99–101] or less thermodynamically stable iron sulfide precipitates [102]. Although initially present, the dark bands were not visible in cores returned to the USA (transported and stored at 4°C until examination, approx. four to five months after collection), suggesting a less stable precipitate. A possible alternative explanation is that a pulse of sediments rich in reduced iron and/or sulfur was delivered to the lake and settled at the sediment–water interface. However, a scenario involving significant sediment delivery is unlikely to be based upon mineralogical examination of the sediment cores [59]. The higher, more oxidizing redox conditions seen in the water column might be due to a relatively recent flushing event that brought ‘new’ water with higher levels of oxygen and nitrate and consequently a higher redox level. At the time of sampling, SLW was slowly re-filling [60], implying that lake waters were being replenished, consistent with the addition of fresh oxidants via inflow. If this replenishment scenario is the case, then the active hydrological processes may be governing substrate availability for resident microorganisms.

Irrespective of its origin, the presence of the redox minimum close to the water–sediment interface has potentially important consequences for the biogeochemistry and microbial ecology of SLW. Interestingly, estimates of reduction potentials for various half-reactions indicate that conditions at the redox minimum may be sufficiently reducing for the reduction of sulfate to sulfide and sulfur oxyanions to elemental sulfur to take place (figure 4b). As a result, these

(a)



(b)

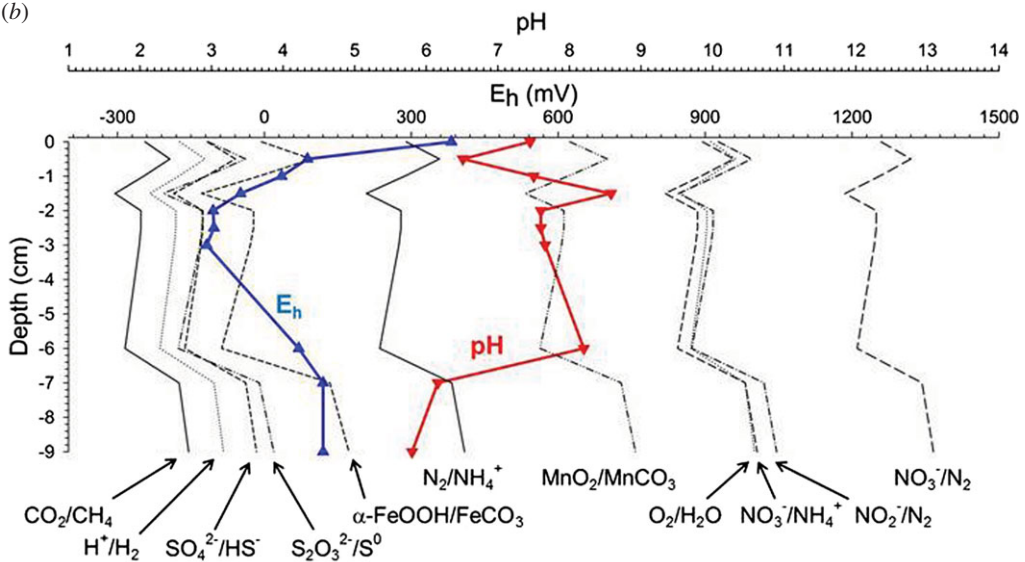


Figure 4. (a) Estimates of the *in situ* Gibbs free-energy yield (for two electron equivalents) for various redox couples in the water column of Subglacial Lake Whillans (SLW). The reactions (as the reduction half-couple), their stoichiometry and Gibbs free energy at pH 7 are given in the electronic supplementary material, table S1. The Gibbs free-energy values are adjusted for *in situ* water column temperature and pH. (b) Redox potential (E_h) and pH measured in the upper 8 cm of SLW sediments along with estimates of the *in situ* reduction potentials for various reduction half-reactions.

reduction reactions, or the presence of pyrite or greigite framboids, could be a source of reduced sulfur or iron that can be coupled to the reduction of oxygen or nitrate to provide energy for species such as *Sideroxydans* or *Thiobacillus* at the sediment–water interface (figure 4a). The extraction of energy through a cryptic sulfur cycle, similar to that described in Blood Falls [8] and marine sediments [85,86], appears to be a widespread phenomenon in aquatic environments given the appropriate redox conditions [103]. Above the redox minimum zone, any resulting reduced nitrogen compounds could then be used by species such as *Candidatus Nitrotoga* or *Candidatus Nitrosoarchaeum* along with any processes involving the reduction of oxygen or nitrate that are observed higher in the water column.

6. Conclusion

Antarctica has often been promoted as a model for understanding potential extraterrestrial habitats (i.e. [104]). The presence of liquid water, a key ingredient for life, has been postulated on planets and moons within our Solar System, including Enceladus, Europa and Mars [105–109]. The findings of the WISSARD project further support the idea that life can exist wherever liquid water and suitable substrates are present, and that energy from sunlight is not required to support that life. The WISSARD project has also provided technical information on how to look for microbial life in ecosystems below thick glacial ice and what is required to conclude that it is not the result of contamination. However, while the search for life beyond the Earth is an aspirational endeavour, there remains an urgent need to understand ecosystem processes here on the Earth and this is particularly true in the underexplored polar regions. Both terrestrial and marine ecosystems are under increasing threat due to environmental change with polar ecosystems facing the greatest impacts of warming.

Thus, it becomes critically important to comprehend how largely unexplored ecosystems, such as SLW, function before any climate impacts take hold. Results from SLW show that chemosynthetic food webs actively cycle minerals and organic matter beneath 800 m of ice. Moreover, in terms of carbon uptake, the SLW microbial community is as productive as the chemolithoautotrophic communities being revealed in the dark oceans of the world [75,76,110]. SLW sediments contained a diversity of carbon fixation genes as well as potentially novel gene sequences, suggesting niche partitioning may be in play. While there is still much to untangle on how subglacial communities function, information regarding how ecosystems such as SLW persist, and which organisms are key players in subglacial biogeochemical processes, has important implications for the downstream environments into which they drain, as well as our conceptualization of the potential for life beyond our planet. Collecting samples and detecting viable microbial life from SLW represents an achievement decades in the making, requiring innovation across disciplines and high levels of collaboration, and still only marks the beginning of the ecological exploration of Antarctic subglacial lakes. Findings from SLW provide unequivocal evidence that an Antarctic subglacial lake and its sediments host active microbial communities, making it likely that all subglacial environments with liquid are biomes.

Data accessibility. DNA sequences: GenBank accessions (KT862526–KT862533) (<http://www.ncbi.nlm.nih.gov/genbank/>).

Authors' contributions. J.A.M., A.M.P., D.G. and A.C.M. collected and analysed sediment and water samples for microbiology and redox chemistry; P.A.L. contributed the estimates for the half-reaction reduction potentials and Gibbs free energy. J.A.M. collected the CTD data; K.D.M., J.A.M., A.T.F. and S.T. interpreted the results; S.C., M.R.S. and H.A.F. interpreted ICESat data and developed a model of SLW hydrological inputs. T.H., J.C., R.S. and R.P. analysed sediment cores for mineralogy and palaeomarkers. A.A.A. provided 16S rRNA gene data. J.A.M. and P.A.L. wrote the manuscript with significant written contributions from all authors; all authors helped interpret data and provided significant editorial remarks.

Competing interests. We have no competing interests.

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