Development of Microbial Characterization Techniques for Crystalline Rock

NWMO-TR-2018-05 **April 2018**

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ABSTRACT

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Abstract

The development of techniques for the characterization of potential indigenous microbial communities in crystalline rock environments was undertaken using core obtained from the Grimsel site in Switzerland. Based on previous reports, low microbial abundances (10^3-10^4 cells/mL) were expected to be associated with water samples, while pristine rock matrix was hypothesized to be sterile and thus provide an effective assessment of detection limits. The approaches used were adapted from methods developed previously for sedimentary rock cores (NWMO-TR-2013-17). A combination of cell counting, phospholipid fatty acid (PLFA) and molecular genetic approaches were tested on filtered water and rock core samples. Water samples were collected prior to drilling, during drill and post-drilling activities and included drilling fluids and groundwater samples. Drill core was collected and both exterior surfaces and pristine interior samples were characterized. Pristine interior core samples were collected by paring away 0.5 to 1 cm of core outer surfaces, followed by pulverization of core interiors under sterile conditions. Given the expected low biomass for these samples, extensive blank testing and control comparisons were undertaken.

Results for PLFA and molecular genetic analysis of all core samples yielded negligible evidence of microbial biomass, consistent with the hypothesis of sterility. Cell counting was not applied to solid samples. Detection limits for PLFA analysis were extended beyond previous work (NWMO-TR-2013-17) via the utilization of gas chromatography flame ionization detection (GC-FID). The laboratory analytical blank was characterized to be 435 +/- 235 pmoles PLFA in 100 microliters which, using conventional conversion factors, is equivalent to a detection limit of 1.8 to 4.3×10^4 cells/gram for a 400 gram extraction. Procedural blank testing indicated that, despite best efforts, the handling and processing of the large masses (circa 400 grams pulverized rock) resulted in slightly increased blanks equivalent to 700 to 1400 pmoles, equivalent to 2 to 4×10^5 cells/gram. Analysis of triplicate pulverized rock samples yielded PLFA concentrations that were consistent with or below the procedural blanks and thus could not be confidently interpreted to indicate the presence of viable bacterial biomass in these samples. Concurrent molecular genetic analysis of sub-samples of the same pulverized rock yielded very low biomass, below the detection limit of the Qubit dsDNA High Sensitivity Assay kit. The 16S rRNA gene results showed that no common operational taxonomic unit occurs in all replicate extraction samples. The amount of DNA recovered was too low to generate robust and reproducible sample-specific 16S rRNA gene sequencing data. The samples are considered below detection limits, preventing confident conclusions about microbial communities in these core samples.

Water filters yielded no detectable PLFA when compared to concurrent field blank samples. Calculations of detection limits based on volumes of water indicated that this non-detection was equivalent to less than 0.7 to 1.7×10^4 cells/mL. DNA extraction and 16S rRNA gene analysis verified low biomass in groundwater and drilling fluid samples. Only one sample (groundwater from borehole 13.001) yielded sufficient amounts of DNA to be quantified using the Qubit

dsDNA High Sensitivity Assay kit. When DNA was extracted from only 60 or 150 mL drilling fluid, the results from 16S rRNA gene sequencing was too unreliable for further analysis and the samples are considered below detection limit. The filtration of 1200 mL drilling or borehole liquid appears to be essential to receive sufficient biomass to generate robust and reproducible sample-specific 16S rRNA gene data. The results of this study show that very low biomass is present in these samples, less than 2 to 4 × 10^5 cells/gram. Based on current approaches, these values can be considered to be detection limits for these matrices. Detection limits were comparable by the three approaches used (PLFA, Molecular genetic analysis, cell counting). The comparability in detection limits indicates that in systems where low water volumes are present and/or where significant impact on *in situ* water has likely occurred via fluids introduced during drilling, rock core analysis may be an important additional technique to assess the potential for biomass living within microfractures or pore spaces.

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1. INTRODUCTION

The Nuclear Waste Management Organization (NWMO) is responsible for implementing Adaptive Phased Management (APM), Canada's plan for the long-term care of used nuclear fuel produced by Canada's nuclear reactors. The end point of APM is long-term containment and isolation of used nuclear fuel in a Deep Geological Repository (DGR) constructed at approximately 500 m depth in a low permeability host rock in a willing and informed community. The DGR includes an engineered barrier system consisting of used fuel containers surrounded by highly compacted bentonite (HCB) clay. The used fuel container has 3 mm of copper applied directly by electrodeposition and cold spray onto a steel container which holds 48 CANDU fuel bundles. The steel provides the used fuel container with strength, whereas copper is for corrosion protection.

Beyond this engineered barrier, development of a DGR includes selection of a site where the geologic context provides for long-term isolation and containment of stored materials. The increasing recognition that microbial life is pervasive in the Earth's subsurface (Sherwood Lollar 2011 and references therein, Onstott 2017 and references therein) has identified that microorganisms have the potential to affect the geochemical conditions of used nuclear fuel repositories (Hallbeck et al. 2012, Sherwood Lollar 2011, Stroes-Gascoyne et al. 2007), including potentially affecting the engineered barrier system. Thus, characterization of the presence, and metabolic capabilities of, microbial communities is an important component of site assessment and selection.

Studies of subsurface microbial life in terrestrial environments have ranged from depths of hundreds of meters to kilometers in a range of rock types (e.g. Pedersen 1993, 2001, Chapelle et al. 2002, Moser et al. 2005, Lin et al. 2006, Hallmann et al. 2008, Chivian et al. 2008, Itavaara et al. 2011, Fukada et al. 2010, Davidson et al. 2011, Hallbeck et al. 2012). These studies have demonstrated that chemolithotrophic microbial communities can survive and grow utilizing energy stored in chemical disequilibria that occur in many subsurface environments (Pedersen 1992a, 1992b, 2000). These and other studies have further demonstrated that microbial communities in these terrestrial subsurface systems are often tolerant of elevated salinity and temperature, and often utilize metabolisms that involve sulphate reduction, reduction of metals, fermentative processes and/or the production and consumption of methane and acetate although an increasing diversity of metabolic strategies is being documented (Lau et al. 2016).

The majority of studies of indigenous microbial communities in terrestrial subsurface environments have focused on groundwater produced from boreholes. However, sites proposed for development of deep geologic repositories of spent nuclear fuel are often sites with low permeability rocks where collection and analysis of groundwater un-impacted by drilling or development activities is difficult or impossible. Further, it is recognized that microbial cells will often be present at higher abundances on surfaces as opposed to free floating in water (Pedersen 1992a, Jagevall 2011). The lack of obtainable groundwater at proposed geologic repository sites implies that analysis of solid samples may be the only viable approach to characterize the *in situ* microbial community. While solid crystalline rock is expected to be sterile, there is the potential for microbial communities to be associated with micro-fractures and zones of connected porosity. It is therefore important to develop methods that allow the detection and characterization of potential indigenous microbial communities associated with rock core materials in addition to groundwater based approaches.

Microbial cell abundances in deep terrestrial subsurface systems are generally low. Studies of planktonic cells in groundwater samples obtained from deep terrestrial subsurface systems yield estimates of 10^2 to 10^5 cells/mL (e.g. Itavaara et al. 2011, Fukada et al. 2010, Davidson et al. 2011; Konno et al. 2013, Lau et al. 2016, Onstott 2017). Although cell abundances associated with solid rock samples have not been extensively assessed, they are likewise expected to be low. In low biomass marine subsurface systems, sediment-associated cellular abundances have been estimated to be in the range of 10^2 to 10^5 cells/g (Kallemeyer et al. 2012) based on direct counting techniques. In the deep terrestrial subsurface systems abundances are hypothesized to be at the low end of this range. In crystalline rock environments the solid rock matrix which does not have interconnected pore-spaces cannot support life. However, it is possible for life to establish in water saturated zones of microfractures or interconnected pores that may be present within the solid rock matrix. Previous work has reported on methods for assessing planktonic versus attached microorganisms (Erickson et al 2016) and organisms associated with biofilms (Jägeval et al 2011).

Broadly speaking, approaches to detect and assess subsurface organisms include direct counting, characterization of biomarkers such as PLFA, molecular genetic (DNA based) characterization and culturing. While culturing provides some of the strongest and most insightful evidence regarding indigenous microbial communities, it is also the most difficult to achieve. On average 5-10% of organisms can be cultured from crystalline rock (Hallbeck et al. 2012). While this can be even higher in some cases, it still does not capture the majority of the microbial community present. Similarly, direct cell counting can provide direct evidence of the presence of microbial communities and is often successfully applied to fluid samples. However, it is a difficult approach to successfully apply to solid matrices, particularly those that require pulverization prior to sampling as this can lead to cell damage. Further, cell counting can provide only limited insight into the identity and metabolic activity of observed cells. Therefore, for most environmental applications, the identification and characterization of indigenous organisms focuses on biomarker and molecular genetic techniques.

The NWMO has an active technical program to develop techniques required to characterize *in situ* subsurface microbial communities that may be present at sites proposed for the development of a DGR. This program includes the development and application of approaches to characterize low-abundance microbial communities present in low porosity systems. Previous work reported the development and application of such approaches to low-permeability sedimentary systems (Slater et al. 2013, NWMO-TR-2013-17). Here we report the testing and application of approaches to crystalline rock environments. The three primary components of this study were:

1) Constraining potential sources of microbial contamination associated with drilling, sampling and downstream analysis
2) Assessment of potential indigenous microbial communities by PLFA analysis
3) Assessment of potential indigenous microbial communities by nucleic acid analysis

This study was carried out as part of activities related to NWMO's participation in the **Ma**terials **Co**rrosion **Te**st (MaCoTe) at the Grimsel Test Site (GTS) in Switzerland in collaboration with the Swiss (NAGRA), British (RWM), and Czech (SURAO) nuclear waste agencies. The GTS is a center for underground research and development situated in the Swiss Alps. Established in

1984, the GTS hosts international partners from Europe, Asia and North America who collaborate on a wide range of research projects focused on the geological disposal of radioactive waste. The underground research facilities exist in granitic rock and consist of approximately 1000 m of tunnels constructed 450 m below the earth's surface. The GTS location and tunnel layout are shown in Figure 1A and B.

The MaCoTe project (Figure 1C) involves the drilling of a number of boreholes into the granite host rock into which modules are emplaced to test the potential corrosion of canister materials and the inhibition of microbial activity by bentonite within the engineered barrier system. Ongoing MaCoTe activities include drilling several boreholes from which granitic core and associated groundwater samples are to be obtained and used in this project.

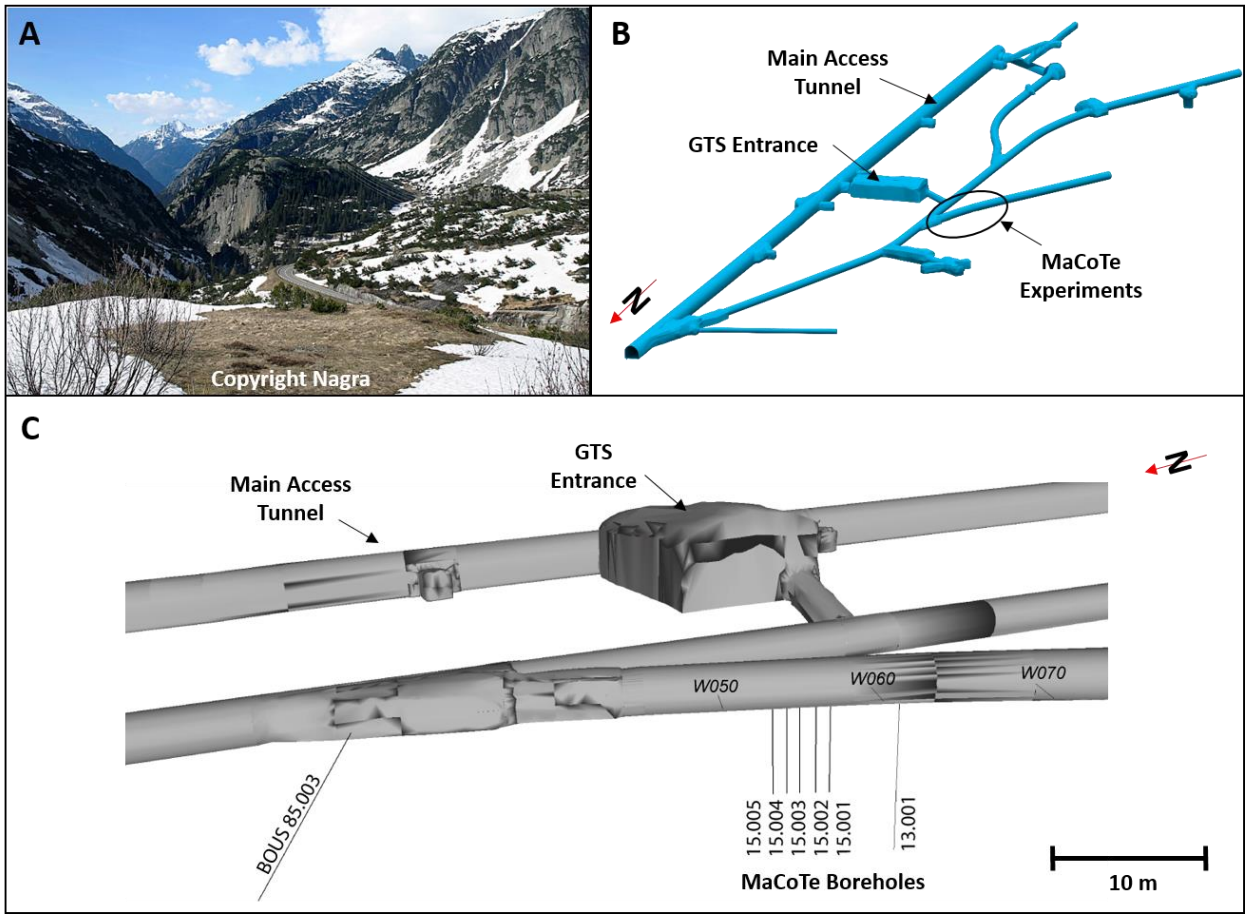

Figure 1: A. View from the access tunnel used to enter the Grimsel Test Site showing the surrounding Swiss Alps mountains. B. Layout of the Grimsel Test Site underground research laboratory with the location of the MaCoTe project indicated (map taken from www.grimsel.com). C. The specific locations of the MaCoTe boreholes (13.001 and 15.001 – 15.005) within the underground research laboratory. Drilling water was sourced from BOUS 85.003.

This project built on previous work characterizing indigenous microbial communities in low permeability sedimentary rocks (NWMO TR-2013-17) by testing the application of these approaches in a crystalline rock system. As recognized previously, the potential for contamination is high when searching for life in such low biomass environments, particularly when investigating solid rock samples (Onstott et al. 1998). Here we used the characterization of drilling fluids, groundwaters and outer core surfaces to constrain potential contamination inputs to the rock samples that were the focus of this project. We also removed circa 1 cm of the outer surfaces of all core segments via paring. Based on previous reports, this should be sufficient to remove matrix material that was impacted during sample collection (Slater et al. 2013, Sherman et al. 2007).

A key component of this project was assessing the sensitivity of the approaches used. Given the expectation that the crystalline rock being studied would be sterile, the core analysis component of these studies represent an effective test of the sensitivity of the approaches on this matrix. In order to increase the sensitivity of PLFA biomarker-based detection relative to the previous study in sedimentary rock (Slater et al. 2013) the analysis by gas chromatography flame ionization detection (GC-FID) was added to the previous gas chromatography mass spectrometry (GC-MS) analysis. Although less capable in the identification of organic compounds, GC-FID detection is linear to much lower concentrations and was expected to enable increases in sensitivity of an order of magnitude or more. GC-MS is the ideal approach when concentrations of organic molecules are sufficiently high for detection as it also provides confirmation of the compounds identity (beyond retention time). However, the GC-MS response is non-linear at low concentrations. The detection limits of molecular genetic approaches were tested via analysis of replicate samples of core, groundwater and drilling fluid. The detection limits are expected to be determined by the point at which observed responses in replicate samples are driven by the noise associated with sample processing, rather than DNA derived from the sample itself.

2. MATERIALS AND METHODS

2.1 SITE DESCRIPTION AND SAMPLE COLLECTION

2.1.1 Overview and Site Description

The principal objective of the GTS is to perform *in situ* experiments to improve understanding of fracture flow and DGR site characterization techniques in crystalline rock formations (Martel and Peterson 1991). The site is 130 km south of Zurich, Switzerland, and 450 m below Grimsel Pass, a mountain pass in the Swiss Alps. The test site is built within the Aare Massif, a large body of 370-270 Ma old granitoids that formed by magmatic intrusion into older basement rocks (Greber et al. 2011 and references therein). The granite and granodiorite was foliated by deformation between 25 and 15 Ma (Konno et al. 2013 and references therein).

The MaCoTe project involves emplacement of modules containing copper coupons surrounded by bentonite into underground boreholes to test engineered barrier system performance. Microbial characterization of the groundwater and core presented in this report provides a baseline to which the bentonite retrieved from the modules over time will be compared.

2.1.2 Water Sample Collection

Borehole 13.001 - Groundwater. This borehole was drilled in 2013 and packed with up to 9 borehole modules in September 2014. Water samples were collected in September 2014 from the borehole prior to insertion of modules in order to characterize the groundwater in the borehole that will saturate the bentonite. An additional sample was taken from this borehole in October 2015 to dewater the borehole prior to retrieval of the first modules for testing. The results of bentonite analysis from the retrieved module are described in a companion report (Engel et al. 2018 NWMO-TR-2018-04).

Boreholes 15.003 and 15.004 – Drilling fluids. In June 2015, drilling fluid was collected during the drilling of boreholes 15.003 and 15.004. The drilling water used at GTS is sourced from borehole (BOUS) 85.003 located ~35 m down the tunnel from the MaCoTe site. The water is stored overnight in a tank under argon (Ar) headspace to maintain anoxic conditions before it is used during drilling. To characterize contamination, water samples were taken from BOUS 85.003 directly, from the storage tank, and from water upwelling adjacent to the drill string to characterize the drilling fluid and potential contamination.

Water sampling for DNA and PLFA analysis. Water samples for PLFA analysis were collected via disposable sterilized syringe onto 0.22 µm polyvinylidene fluoride (PVDF) membrane syringe filters. Up to 1200 mL of water sample was filtered in replicate for PLFA analysis. In addition, a blank filter that had no water put through it was collected. Water samples for DNA were collected via disposable sterilized syringe onto 0.22 µm sterivex syringe filters.
Up to 1200 mL of liquid (Table 5) was collected as replicates along with an air blank filter.

Water sampling for Cell Counts. Water samples for cell counting were collected into sterile 60 mL centrifuge tubes preloaded with 3 mL of 50% w/w glutaraldehyde fixative (5% of 60 mL sample volume). Centrifuge tubes were filled with water, leaving no headspace, then immediately capped, minimizing entrainment of oxygen in the water samples. Air blank samples were also taken to assess the abundance of airborne microbes via cell counting. These were collected by opening a glutaraldehyde loaded centrifuge tube, waiting 10 seconds and then reclosing the tube. The caps of the sample tubes were reinforced with electrical tape to protect the seal during transport from the field site to the laboratory.

2.1.3 Core Sample Collection

Rock core sampling occurred in June 2015 as part of the MaCoTe project. At this time, five 6 m deep boreholes were drilled at the GTS. Two of these boreholes, MaCoTe 15.003 (primary core analyzed in this study) and MaCoTe 15.004 (backup core), were sampled for this study. Samples for microbiological method development were selected from the bottom 3 m of the boreholes. Each of the boreholes are drilled into a single unit of massive crystalline granite thought to be chemically and physically similar and therefore were considered replicate samples. Samples for microbiological characterization were broken into 30 cm lengths using a chisel, bagged and frozen for return to McMaster University for sample preparation and subsequent analysis.

Table 1: Sample Collection Sequence

Borehole	Substrate	Sampling Date
13.001	Groundwater in borehole prior to module emplacement	September 23 2014
13.001	Groundwater in borehole that was dewatered during collection of module	October 21 2015
15.003	Core Drilling fluid	June 23 & 24 2015
15.004	Core Drilling fluid	June 23 & 24 2015

2.2 ANALYTICAL METHODS

2.2.1 Cell Counting of Water Samples

Direct microscopic counts were performed using DAPI (4,6-Diamidino-2-Phenylindole, Thermo Scientific Pierce) staining, following the methods of Sherr et al. 2001 and King et al. 1988, as described in Watt 2016. All reagents used were filtered immediately before use with 0.10 µm filters to remove any microbial contamination (Kallmeyer 2011, Kallmeyer et al. 2012). All glassware was autoclaved to sterilize surfaces through high temperature steam treatment. Other equipment requiring repetitive use, such as filter forceps, were flame sterilized as needed. To concentrate cells for enumeration, the water samples were filtered using 0.2 µm pore-size black polycarbonate membrane filters (Whatman filters). Immediately before filtration, the samples were agitated by hand for 30 seconds and then vortexed lightly (setting 3) for 10 s to break apart cell aggregates and ensure an even spatial distribution of cells within the fluid. The sample was drawn through the filter using a vacuum pump. An even distribution of the vacuum was achieved by placing a 0.45 µm pore-size nitrocellulose filter (Whatman filters) below the delicate polycarbonate filter. DAPI was added to a final concentration of 10 µg/mL, and left to stain for 10 minutes. PBS (polyphosphate buffered saline, Amresco Inc.) was then used to rinse away any residual DAPI. Filters were mounted with type A immersion oil (Cargille immersion oil) onto glass microscope slides and covered with a glass coverslip. The slides were observed using a Nikon microphot FXA microscope with a 100x oil immersion fluorescence objective lens and a UV-2A filter block. 10-20 fields of view within the active filter area were randomly selected. Photographs were taken using Fuji X-TRA 400 ISO film and a microscope camera attachment. Numbers of microbes were visually counted for each field of view, averaged, and then scaled up to the active filter area to attain the total number of cells captured on the filter. The number of cells per unit volume of sample filtered was calculated. The blank for this analysis was determined by assessing the maximum counted cellular abundance whose variance overlapped with the cellular abundance of the air blank samples. This resulted in an assessed blank of 1.2×10^3 cells/mL.

2.2.2 Core Subsampling and Pulverization

Core samples were prepared as per the methods developed in Slater et al. 2013. Outer surfaces of each core sample were rinsed with solvent for PLFA analysis of potential surface contamination. Subsequently, the outer 0.5 to 1 cm of core was pared away from all surfaces via sterilized hydraulic splitter and/or hammer and chisel. Interior core pieces were broken into

smaller sizes using a solvent rinsed stainless steel mortar and pestle. Core pieces were ground to a fine powder (sieved to 1 mm) using a solvent rinsed stainless steel puck mill. Precombusted silica sand was ground in the puck mill between preparations of each core section to assess potential blank and or carryover contributions between samples.

200 to 400 g aliquots of powdered rock core and 100 to 200 g aliquots of pulverized sand blanks were extracted for PLFA analysis as per Slater et al. 2013. Sample and sand blank masses are listed in Table 4.

100 g aliquots of rock powder were collected from the same pulverized core samples were transferred to precombusted (450 °C) glass vials with Teflon faced septa sealed with polyurethane tape and transported to University of Waterloo for molecular genetic analysis at 4 °C.

2.2.3 PLFA Analytical Approaches

PLFA were extracted from all samples using a modified Bligh and Dyer PLFA extraction method consistent with the methods developed in Slater et al. 2013. Extraction of total lipid extracts (TLE) was achieved overnight (~18 hrs) at room temperature using a mixture of dichloromethane (DCM), methanol (MeOH), and phosphate buffer in a ratio of 1:2:0.08. TLE were separated into (F1) non-polar, (F2) neutral and (F3) polar fractions using silica gel chromatography (Guckert et al. 1985). Phospholipids recovered from the polar fractions were converted to fatty acid methyl esters (FAMEs) via mild-alkaline methanolysis and subsequently purified by a secondary silica gel chromatography (Guckert et al. 1985). Microbial FAMEs were initially identified and quantified using GC-MS on an Agilent instrument (Agilent Technologies Inc., Santa Clara, CA, USA) with a DB-5MS capillary column (30 m × 0.25 µm film thickness). The temperature program for the GC was maintained at 50 °C for one minute, then increased the temperature at 20 °C/min to a temperature of 130 °C, then increased at 4 °C/min to 160 °C, and finally increased at a rate of 8 °C/min to a temperature of 300 °C which was held for 5 minutes. The detection limit for the GC-MS based on the lowest concentration standard that responded linearly was 0.5 to 1.5 µg/mL injected.

For samples with low concentrations of PLFA, or where PLFA were not detected by GC-MS, samples were re-run on an Agilent GC-FID system (Agilent Technologies Inc., Santa Clara, CA, USA). This system enabled much greater sensitivity for detection of PLFA. The system was equipped and operated under the same conditions as the GC-MS system. GC-FID analysis used a DB-5MS capillary column (30 m x 0.25 µm film thickness). The temperature program was identical to the GC-MS experiment described above. The detection limit for the FID for this system was determined to be 70 ng/mL based on the lowest standard that responded linearly with sufficient peak area, approximately an order of magnitude more sensitive than the GC-MS system.

All reported concentrations considered only quantifiable peaks. Identities of the fatty acids are listed with the following nomenclature: total number of carbon atoms followed by the total number of double bonds (e.g., 16:1 represents a 16 carbon monounsaturated fatty acid). Terminal-branching fatty acids are indicated by the prefixes *i (iso)* and *a (anteiso)*. Mid-branching positions are represented by the number of carbon atoms from the carboxyl group to the methyl group (e.g., 10Me16:0). Cyclopropyl fatty acids are represented by the prefix *cy*.

2.2.4 DNA Analytical Approaches

2.2.4.1 DNA Extraction from Drilling Fluid

DNA from fluids was extracted as described previously by Slater et al. 2013. Briefly, 60 to 1200 ml fluid (Table 5) was filtered using a Sterivex-GP 0.22 µm filter (Millipore, MA, USA, cat. no. SVGP01050). Sterivex housings were opened with flame sterilized (70% ethanol) and flame-treated (to burn DNA) pliers. Filter membranes were removed using a sterile, DNA-free, single-use razor blade. One quarter of each filter was used for extraction with the PowerSoil DNA Isolation Kit (MO BIO Laboratories, CA, USA, cat. no. 12888). After addition of lysis solution, the PowerBead tube was incubated at 65 °C for 30 min, followed by bead beating using FastPrep instrument (MP Biomedicals, OH, USA) at 5.5 m/s for 45 s. The remainder of the extraction was carried out following the manufacturer's instructions. DNA was eluted into 60 µL and stored in 2 × 30 µL aliquots at −20 °C until PCR analysis.

2.2.4.2 DNA Extraction from Rock Powder

Total genomic DNA from rock powder was extracted using MoBio Power Max Soil DNA Kit (MO BIO Laboratories, CA, USA, cat # 12988-10). Ten grams of rock powder were added to PowerBead tubes. After addition of PowerBead solution and lysis solution, the tube was incubated at 65 °C for 30 min before bead beading for 10 min at 30 Hz (Mixer Mill MM 400, Retsch, Germany). The remainder of the extraction was carried out following the manufacturer's instructions. DNA was eluted using 2 mL elution buffer. Nucleic acids were precipitated using 4 µL per mL Co-Precipitant Linear Polyacrylamide (Bioline, Germany, cat. no. BIO-37075), 0.1 volumes of 5 M NaCl (prepared in molecular biology grade water and 0.2 µm filter sterilized), 1 volume of isopropanol (HPLC grade), then stored at −20 °C overnight. DNA was pelleted by centrifugation at 13,000 × g for 30 min. Pellets were washed with 80% ethanol (HPLC grade), air dried, and eluted in 120 µL of elution buffer. Aliquots were frozen at −20 °C until PCR analysis.

2.2.4.3 DNA quantification

Genomic DNA was quantified using the Qubit dsDNA High Sensitivity Assay kit (Invitrogen, CA, USA, cat. no. Q32854) on a Qubit 2.0 fluorometer.

2.2.4.4 DNA amplification and Sequencing

The V3-V4 region of 16S ribosomal RNA genes (16S rRNA genes) were amplified using universal prokaryotic primers Pro341F and Pro805R (Takahashi et al. 2014). Each primer contained a unique six base index sequence for sample multiplexing, as well as Illumina flow cell binding and sequencing sites (Bartram et al. 2011). The 25 µL PCR mix contained 1× ThermoPol Buffer, 0.2 µM forward primer, 0.2 µM reverse primer, 200 µM dNTPs, 15 µg bovine serum albumin (BSA), 0.625 U *Taq* DNA polymerase (New England Biolabs, MA, USA, cat. no. M0267L), and 2 µL of template (up to 10 ng). Each PCR was prepared in triplicate. DNA extraction "kit controls" (i.e., simulated DNA extraction from kit buffer instead of sample) and no-template controls (NTCs) were included. PCR was performed in two rounds by adding 1 µL product from PCRa into PCRb. The PCRs were performed as follows: 95 °C for 3 min, 35 (PCRa) or 15 (PCRb) cycles of 95 °C for 30 sec, 55 °C for 30 sec, 68 °C for 1 min, and a final

extension of 68 °C for 7 min. The first PCR of all samples and controls showed some amplification of the kit controls, thus a second DNA extraction and PCR was conducted on all samples. For the purpose of comparison, all samples and controls from the first and second extraction and PCR were included in all downstream steps. Triplicate PCR products were pooled and equal quantities of each amplicon were pooled into a single tube. For controls that did not show an amplicon on the agarose gel, 30 µL each were added to the pool. The pooled 16S rRNA amplicons were excised from an agarose gel and purified using Wizard SV Gel and PCR Clean-Up System (Promega, WI, USA, cat. no. A9282). The Illumina library was denatured and diluted following Illumina guidelines (Document no. 15039740 v01). A 5 pM library containing 5% PhiX Control v3 ((Illumina Canada Inc, NB, Canada, cat. no. FC-110-3001) was sequenced on a MiSeq instrument (Illumina Inc., CA, USA) using a 2 × 250 cycle MiSeq Reagent Kit v2 (Illumina Canada Inc, cat. no. MS-102-2003).

2.2.4.5 Illumina sequence analysis

Reads were assembled using the paired-end assembler for Illumina sequences (PANDAseq version 2.8, Masella et al. 2012) using a 0.9 quality threshold, 8 nucleotide min overlap and 300 nucleotide minimum assembled read length. Assembled reads were analyzed using Quantitative Insights Into Microbial Ecology (QIIME version 1.9.0, Caporaso et al. 2010b), managed by automated exploration of microbial diversity (AXIOME version 1.5, Lynch et al. 2013). Sequences were clustered into operational taxonomic units (OTUs) using the UPARSE algorithm USEARCH version 7.0.1090 (Edgar 2013) at 97% identity and aligned with the Python Nearest Alignment Space Termination tool (PyNAST version 1.2.2, Caporaso et al. 2010a). All representative sequences were classified by the Ribosomal Database Project (RDP version 2.2, Wang et al. 2007) with a stringent confidence threshold (0.8); the Greengenes database (McDonald et al. 2012) was used to assign taxonomy. AXIOME generated principal coordinate analysis (PCoA) plots using Bray-Curtis distances with samples rarefied to the smallest sample size (smallest read count) included in the analysis. Due to the low read counts of controls, some PCoA plots were rarefied to only 252 reads. Bubble charts showing taxonomy profiles were created using the "ggplot2" package (Wickham 2009) in R v.3.4.0 using OTU tables generated by AXIOME. Those OTU tables were not rarefied and will be referred to as “unrarefied”. To visualize the differences in read counts between controls and samples, we reported read counts at the end of the sample name. Only OTUs at or above 2% relative abundance were shown. Proportions increased to up to 5% for more diverse sample groups. All sequences were deposited in the European Nucleotide Archive (https://www.ebi.ac.uk/ena) with study accession number PRJEB24856.

3. RESULTS

3.1 CELL COUNTING RESULTS

Cell counting results are listed in Table 2. The laboratory method blank for cell counting was determined to be 1.2×10^3 cells/mL. The only sample that had detectable cells was the groundwater sample from borehole 13.001 collected in October 2015. The standard deviation of this calculated cell abundance did overlap with the assessed blank. However, the abundance of cells observed in microscope fields where positive detection was made was well above this level (see Figure 2). This highlights the variability in cellular abundance that was present.

Table 2: Cell Counting Results

Sample (date)	Cells/mL	St Dev	Replicates
Samples associated with Boreholes 15.003 and 15.004 core sampling			
BOUS 85.003 (June, 2015)	BD	NA	3
Storage tank (June, 2015)	BD	NA	3
Drilling fluid (June, 2015)	BD	NA	3
Samples associated with Borehole 13.001 groundwater characterization			
Groundwater (Oct 2015)	2.2E+06	2.2E+05	3

BD = below assessed detection limit of 1.2×10^3 cells/mL. NA = not applicable

The cell counting observations for samples above background contamination are illustrated in Figures 2 and 3 (Watt 2016).

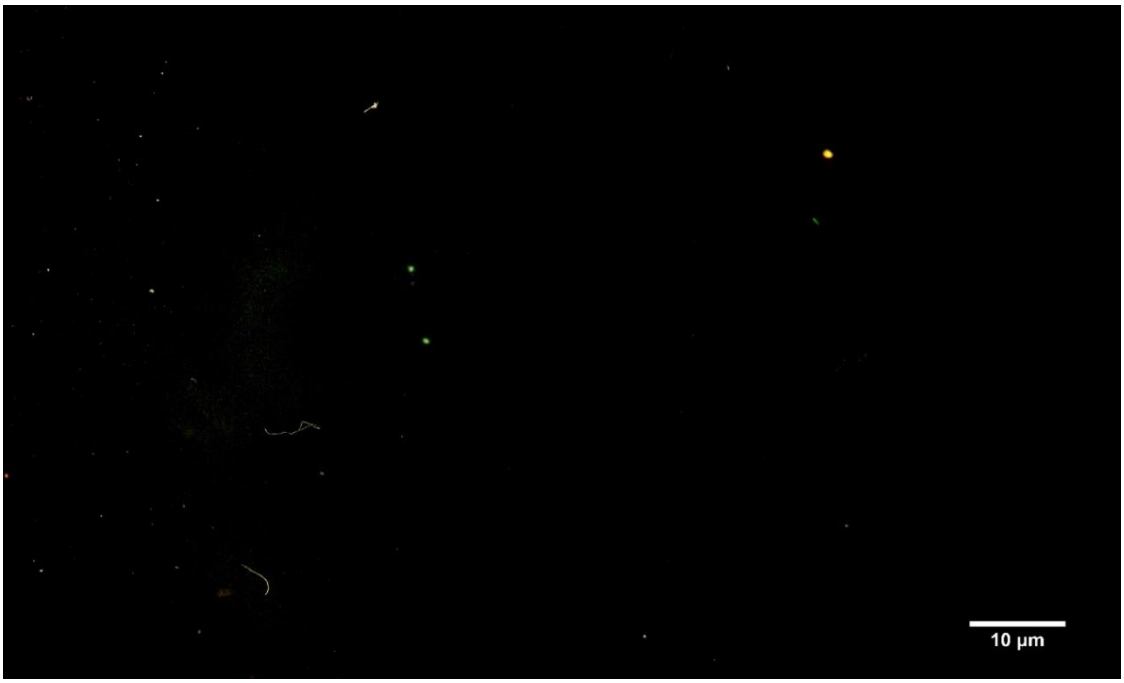

Figure 2: **Photo of BOUS 85.003 displaying cell morphologies of diameter 1 µm or less.** Taken at 1000x magnification. This sample was below the assessed detection limit of the approach used. Contrast adjusted to for low light. (Watt 2016)

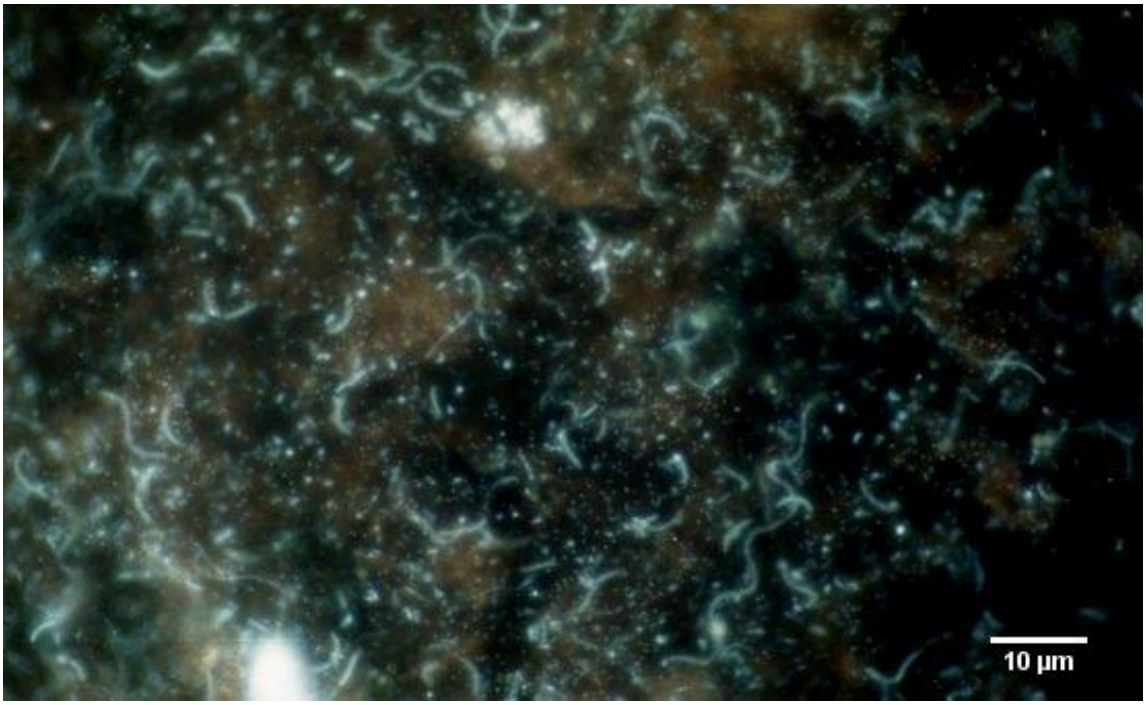

Figure 3: Photo of Borehole 13.001, October 2015, displaying coccoid and elongate morphologies. Taken at 1000x magnification, the scale bar represents 10 µm. This image illustrates the positive detection of biomass in this sample as compared to non-detection (Figure 2). Aggregates of cells appear as larger white clusters. (Watt 2016)

3.2 PLFA RESULTS

Results of PLFA analysis for all samples are shown in Tables 3 and 4. Total masses and concentrations of PLFA are presented along with estimated cell abundances based on conversion using a factor of 4×10^4 cells/pmol (Green and Scow 2000). Results for each subset of analyses are described below. Tables also include field blanks, process blanks (method blanks run at the time of each set of analyses) and sand blanks (pre-combusted quartz sand processed through puck mill pulverization). Also included is the compiled blank for the PLFA method (method blank) which is based on the results of n=18 tests using the GC-FID analysis. No process blank is detectable via GC-MS analysis. Results are presented first based on PLFA mass detected as this is the measured quantity. Conversions to cell numbers and/or cell abundances per volume or mass are given for context and will be revisited in the discussion.

Table 3: Water Sample and Blank PLFA Results

Sample ID	Type	Date	Volume/Mass (mL/g)	Total pmol PLFA	Total cells	Cells/mL
Samples associated with Borehole 13.001 groundwater characterization						
MCT-14-01 blank	Field filter blank	September 2014	na	3650	1.5E+08	na
MCT 14-01 PLFA	Filter	September 2014	1200	1587	6.3E+07	6.3E+04
MCT 14-02 PLFA	Filter	September 2014	1200	2935	1.2E+08	1.2E+05
Filter blank MaCoTe 21.10.15	Field Filter blank	October 2015	na	248	9.9E+06	na
PLFA1 MaCoTe Q1 21.10.15 filter broken > 120 mL	Filter	October 2015	120	609	2.4E+07	2.0E+05*
PLFA 2 MaCoTe Q1 21.10.15 960 mL	Filter	October 2015	960	715	2.9E+07	3.0E+04
Process blank	Filter	October 2015	na	361	1.4E+07	na
Lab filter blank	Lab filter blank	October 2015	na	326	1.9E+00	na
Samples associated with Drilling fluid sampling for Boreholes 15.003 and 15.004						
#1 Filter blank	Field filter blank	June 2015	na	100	1.4E+07	na
#3 BOUS	Filter	June 2015	1200	146	2.0E+07	1.7E+04
#4 from tank	Filter	June 2015	1200	109	1.5E+07	1.2E+04
Process blank	Filter	June 2015	na	137	1.9E+07	1.6E+04
Lab filter blank	Lab Filter blank	June 2015	na	148	2.1E+07	na
Laboratory Blank Characterization						
Compiled method blank	Laboratory		na	437 +/- 235 in 100 µL (4.37 pmol on column)	1.7E + 07 +/- 1.0E +07	na

*Total cells reports total number of cells in the sample based on conversion of total picomoles of PLFA to cells via conversion factor of 4 × 10^4 cells/pmol (Green and Scow 2000). Cells/mL reports the equivalent cellular abundance for the volume of water filtered. Blank filters had no water passed through them and thus volume and cells/mL are not applicable. na = not applicable; * = value potentially artificially inflated due to low water volume passed through filter*.

Table 4: Rock Core PLFA Results for Borehole 15.003

Sample ID	Type	Mass (g)	Total pmol PLFA	Total Cells	Cells/g
23.06.2015-GTS 15.003 PLFA 1	Core interior	449.34	1715	5.4E+07	1.2E+05
23.06.2015-GTS 15.003 PLFA 2	Core interior	263.86	1374	3.6E+07	1.4E+05
23.06.2015-GTS 15.003 PLFA 3	Core interior	405.74	1436	5.7E+07	1.4E+05
Process blank	Core rinse	na	1300	5.2E+07	na
Rinse outside of plfa 1	Core rinse	na	3018	1.2E+08	na
Rinse outside of plfa 2	Core rinse	na	5123	2.0E+08	na
Rinse outside of plfa 3	Core rinse	na	3544	1.4E+08	na
Process blk for PLFA 1 & 2	Method blank	na	1044	2.1E+07	na
Proocess blk for PLFA 3	Method blank	na	630	2.5E+07	na
Sand in puck mill after plfa 1	Sand Blank	124.08	717	2.9E+07	2.3E+05
Sand in puck mill after plfa 2	Sand Blank	110.96	1405	3.8E+07	3.5E+05
Sand in puck mill after plfa 2	Sand Blank	113.38	552	2.2E+07	1.9E+05
Sand in puck mill after plfa 3	Sand Blank	229.36	1262	5.0E+07	2.2E+05
Compiled method blank	Lab method blank	na	437 +/- 235	1.7E+07 +/- 1.0E+07	na

Total cells reports total number of cells in the sample based on conversion of total picomoles of PLFA to cells via conversion factor of 4 × 10^4 cells/pmol (Green and Scow). Cells/g reports the number of cells per gram of rock extracted. na = not applicable

3.2.1 Results from PLFA laboratory and Process Blanks

Extensive testing of the method blank associated with this PLFA analysis was undertaken using the GC-FID approach. While no PLFA had been detected in previous GC-MS based blank analyses, GC-FID analysis did detect PLFA in the blanks. This was due to an increase of over an order of magnitude of the limit of quantification (i.e., the lowest concentration standard detectable) by the GC-FID as compared to the GC-MS analysis. Based on 18 method blank analysis a compiled method blank for the PLFA analysis was found to be 437 +/- 235 pmol of total PLFA in 100 µL (or 4.4 pmol in 1 µL injected on the column) as FAMES. Testing indicated that this blank was at least partially related to impurities in the solvents despite utilization of the

highest purity commercially available. Other research groups have undertaken distillation of solvents to reduce such blank issues. In general, process blanks run at the same time as the filter analyses were comparable (100 to 361 pmol total PLFA in 100 µL, n=4) to the compiled method blank. Process blanks run at the same time as the core samples and rinses tended to be slightly higher (630 to 1300 pmol total PLFA in 100 µL, n=3).

3.2.2 Water filter PLFA results

PLFA results for the groundwater filter samples in September 2014 and drilling water samples in June 2015 detected no PLFA above the level of the relevant field and/or process blanks. Notably, PLFA results for the September 2014 groundwater filters had significantly higher masses of PLFA (1500 to 2900 pmol total PLFA in 100 µL) than other filter samples. However, the corresponding field filter blank had commensurately high PLFA masses. The cause of these high values is not known. Potentially some contamination occurred during sample handling/transport that resulted in the samples and blank being affected. However, the high levels observed for the blank negate any significance to the masses observed on the filters. For the June 2015 drilling fluid samples, all PLFA masses were comparable with the process blanks and with the compiled method blank of PLFA analysis. The only sample which had indication of PLFA masses above the level of the blank was the October 2015 groundwater sample (borehole 13.001) PLFA 2 which has a PLFA mass slightly above the level of the compiled method blank and above the range of the concurrent process blank. The replicate samples PLFA 1 also had higher PLFA concentrations that were at the upper range of the compiled blank and were in excess of the concurrent field blank.

3.2.3 Core sample PLFA results

PLFA amounts for core interior samples from borehole 15.003 ranged from 1400 to 1700 pmol total PLFA in 100 µL with a mean of 1500 +/- 180 pmol of total PLFA in 100 µL as FAMEs. While these masses are in excess of the compiled method blank, and the concurrent process blanks, they were comparable to masses observed for sand blanks processed between samples (range 550 to 1400 pmol total PLFA in 100 µL, mean 980 +/- 410 pmol). When these masses were normalized to mass of rock/sand processed, the core interiors in fact have lower masses than the sand blanks (see cell abundances listed in Table 3). Based on the similarity to the blanks, no PLFA could be detected with confidence in the core interior samples. Core exterior rinses did have higher PLFA masses (3000 to 5000 pmol total PLFA in 100 µL) indicating some impact on the surface during sampling.

3.3 DNA RESULTS

Samples from June 2015 (core and drilling fluid from Borehole 15.003) and October 2015 (groundwater from Borehole 13.001) were analyzed by the University of Waterloo (Table 5 and 6).

Core. All DNA extracts from rock powder from core of borehole 15.003 and associated drilling fluid samples were below the detection limit and 16S rRNA gene PCR yielded mostly weak amplifications in nested PCR (Figure 4).

Groundwater. From borehole 13.001 (#43) we were able to recover 2.5 ng/µL DNA (Table 6). The first PCR amplification of samples showed amplification even from kit controls (Figure 4, top) and, as a result, the PCR was repeated successfully without any amplification in the controls (Figure 4, bottom). When 60 mL (#48) or 150 mL (#45) of drilling fluids were filtered, no PCR amplicons were detected. A weak amplicon could be detected in the agarose gel only when 1200 mL liquid was filtered (#46, #47, #49; Figure 4, bottom gel).

Table 5: List of samples analyzed by University of Waterloo researchers.

Sample	Date	Sampling description	Description
Samples associated with core from Borehole 15.003			
39		Rock powder	Core sample 15.003 PLFA 3
40		Rock powder	Core sample 15.003 PLFA 1
41		Rock powder	Core sample 15.003 PLFA 2
44	2015-Jun-23	-	Air blank (15.003)
45	2015-Jun-23	Drilling fluid	150ml drilling water filtered (15.003; clay on filter)
46	2015-Jun-24	Drilling fluid	1200ml filtered, drilling water direct from tank
47	2015-Jun-24	Drilling fluid	1200ml filtered; BOUS 85.003 tank source borehole
48	2015-Jun-24	Drilling fluid	60ml water filtered during drilling (15.004; clay on filter)
49	2015-Jun-24	Drilling fluid	1200ml filtered, drilling water from tank after drilling (15.004)
Samples associated with groundwater from Borehole 13.001			
42	2015-Oct-21		Air blank
43	2015-Oct-21	Groundwater	Borehole 13.001 unknown volume

Table 6: DNA extraction and PCR results from borehole module samples.

Sample	Material	Extraction Kit	gDNA (ng/µL)	Amplicon PCRa[#]	Amplicon PCRb[#]
39	Rock powder	PowerMax	too low	no	yes
40	Rock powder	PowerMax	too low	no	yes
41	Rock powder	PowerMax	too low	no	yes
42	Sterivex filter	PowerSoil	too low	no	no
43	Sterivex filter	PowerSoil	2.5	yes	yes
44	Sterivex filter	PowerSoil	too low	no	no
45	Sterivex filter	PowerSoil	too low	no	no
46	Sterivex filter	PowerSoil	too low	yes	yes
47	Sterivex filter	PowerSoil	too low	yes	yes
48	Sterivex filter	PowerSoil	too low	no	no
49	Sterivex filter	PowerSoil	too low	yes	yes

[#]Observations are based on the second PCR replicate (without amplification in the kit controls)

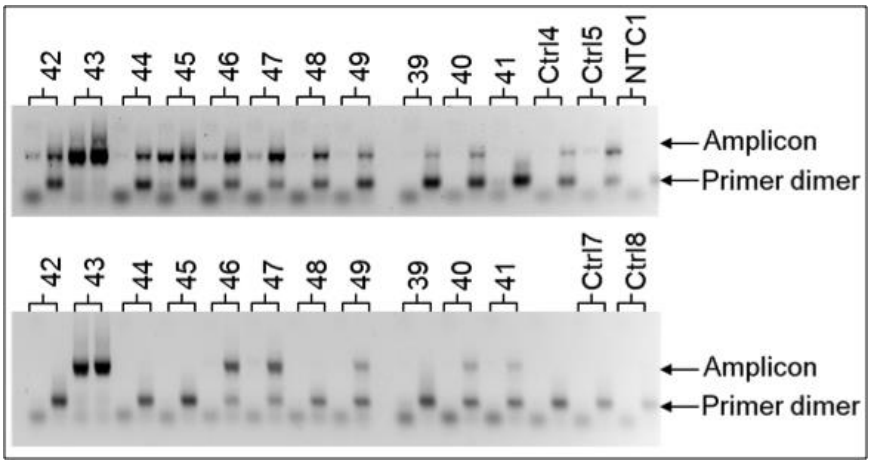

Figure 4: Agarose gels showing 16S rRNA gene amplicons (~0.5 kb) from drilling fluid and rock powder samples. The top gel represents the first PCR of all extracted samples, with amplification in the kit control extraction samples (Ctrl). The bottom gel represents the same samples but from a second extraction, without amplification of the kit controls (Ctrl). Amplicons were separated on a 1.5 % agarose gel stained with GelRed (Biotium, cat. no. 41002). For each sample and control, 5 µL of PCRa and PCRb were loaded next to each other. Samples are labeled according to sample ID in Table 5. NTC is no template control.

3.4 SEQUENCING RESULTS FOR BOREHOLE 15.003 (GROUNDWATER OCTOBER 2015)

For all samples, a total of 336 operational taxonomic units (OTUs) were identified in the resulting sequence data. Beta diversity measures using principal coordinate analysis (PCoA) plots showed that bacterial communities in the extraction kit controls did not differ detectably from all samples in the first two dimensions of the ordination (Figure 5). Samples that are closer together with the controls on the ordination have communities that are likely more similar to one another. Due to low DNA concentration in those samples, the PCR might have only amplified background contamination but no sample-specific DNA. However, samples from source borehole (#47) and 1200 mL of filtered drilling fluid (#46, #49) group closely together in the PCoA plot (group I, Figure 5) and were distinct from the extraction kit and Sterivex air blank controls. Notably, both replicate extraction samples from the source borehole and 1200 mL filtered drilling fluid group closely together (group I, Figure 5). PCoA ordination analysis was based on rarefied data resulting in only 252 and 286 reads per sample when all samples are included (panel A and C in Figure 5). To investigate the effect of low sequence counts on the grouping of samples, we also removed samples with few sequences from the analysis, increasing the number of reads per sample to 2,048 and 2,046 (panel B and D in Figure 5). However, grouping of samples did not change detectably.

Bubble charts showing taxonomy profiles of controls and samples (Figure 6 and 8) highlight the dominant OTUs detected in each library. Depending on the diversity of samples included, only OTUs at or above 2% or 5% relative abundance were shown. In the source borehole fluid, the top two OTUs were unclassified bacteria (Figure 6). Within the top 10 OTUs shared between both replicates, three are affiliated with *Ignavibacteriales*, and one each with *Nitrospirales* and *Rhizobiales*. Those OTUs were not detected in the corresponding kit extraction controls. The microbial communities in 1200 mL filtered drilling water before and after drilling (#46, #49) were similar (Figure 6). In both samples, the top OTU was affiliated with *Caulobacter henricii*, an

aerobic bacterium originally isolated from well water (Bowers et al. 1954). Further, OTUs in the top 5 abundance group were affiliated with *Pseudomonadaceae* and *Comamonadaceae.* All OTUs listed above were absent from the corresponding extraction kit controls. When only 60 or 150 mL drilling fluid were filtered (#45, #48), samples do not separate well from corresponding extraction kit control in the first extraction (group III, Figure 5) and do not separate well from extraction kit control and Sterivex air blank in the second extraction (group IV, Figure 5). The filtration of 1200 mL drilling or borehole liquid appears to be essential to receive sufficient biomass for robust and reproducible 16S rRNA gene analysis. In all three core samples (#39, 40, 41), no OTU can be identified that occurs in all replicate extraction samples. *Gardnerella, Clostridium*, and *Megasphaera* were the most abundant OTUs in one core sample (#41b) but mostly absent from the other two. Core samples grouped closely together in the PCoA plot (cluster II, Figure 5) but extraction kit controls group closely as well. The amount of DNA recovered from 10 g of rock powder was too low to generate a reproducible 16S rRNA gene sequencing result.

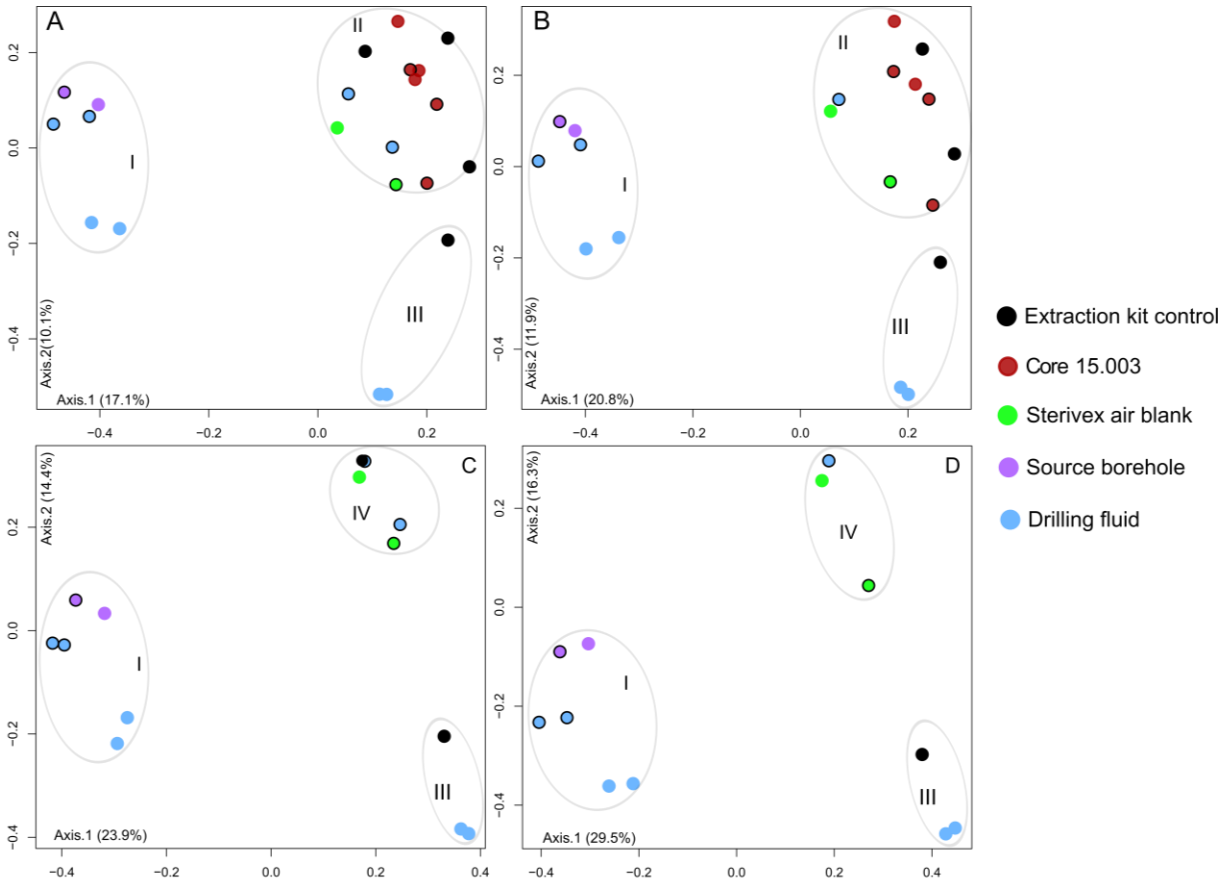

Figure 5: PCoA plot based on the Bray-Curtis distance metric, showing drilling fluid samples with (top) or without (bottom) inclusion of rock core samples. The left panels include samples with very low read count, resulting in data being rarefied to 252 (A) or 286 (C) reads per sample. Samples with lower read counts have been removed in the analysis shown in the right panels, resulting in the data being rarefied to 2048 (B) or 2046 (D) reads per sample. Each sample was extracted twice; the result of the second extraction is marked with a black circle around the data point. All extractions from 1200 mL drilling or borehole fluid are contained within group I and separate well from extraction kit and Sterivex blank controls. Proportion of variations explained are given in brackets for each axis.

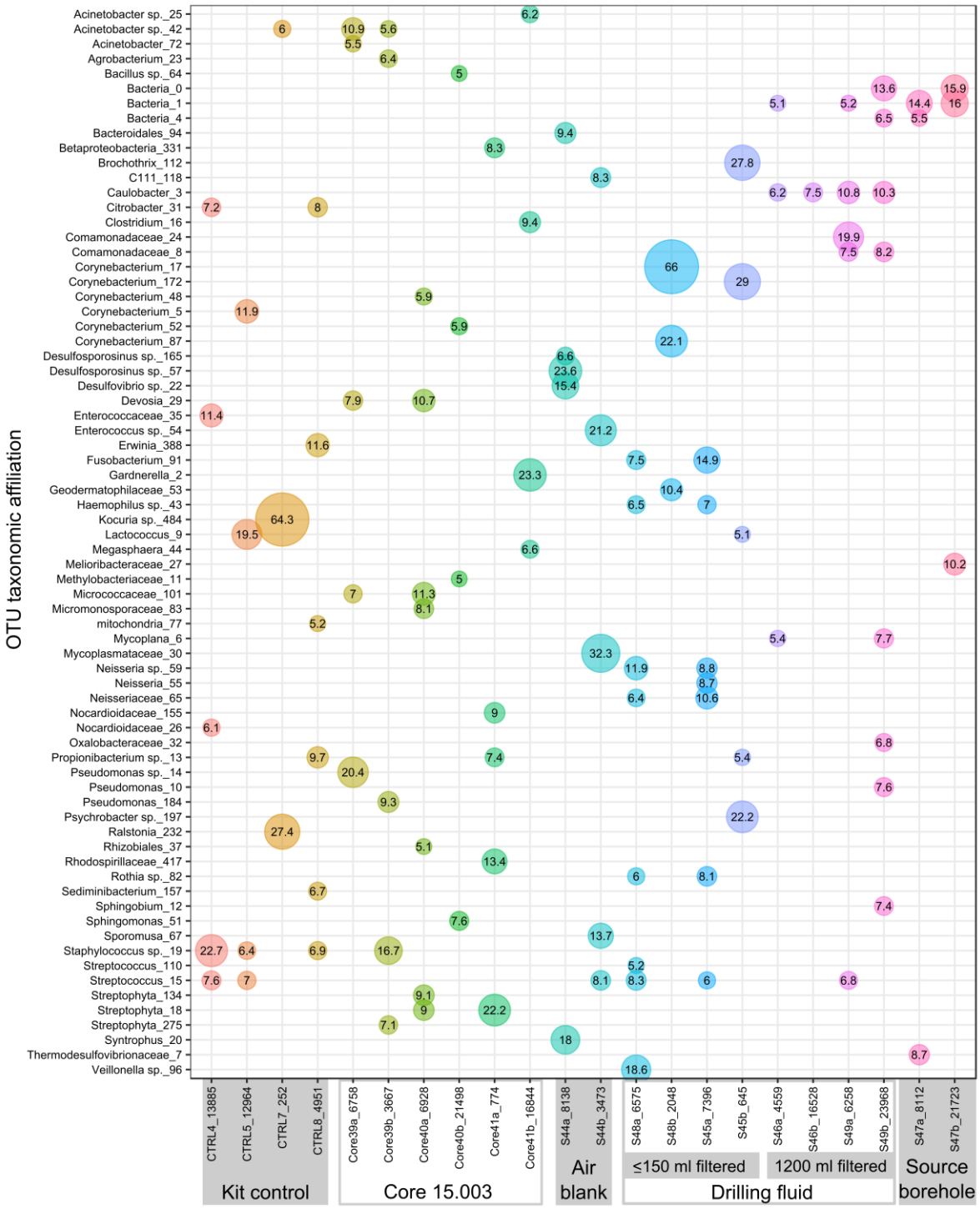

Figure 6: Bubble chart showing 16S rRNA profiles of drilling fluid and core extracts. Graph is based on unrarefied data and only OTUs at or above 5% abundance in every sample are shown. Data from replicate extractions (a and b) are shown. Read count for each sample is shown at the end of the name. OTU taxonomic affiliation is shown on the y-axis followed by a random OTU number.

3.5 SEQUENCING REULTS FOR FLUID FROM BOREHOLE 13.001

When module 1A and 2A were recovered from the Grimsel borehole 13.001 in October 2015, borehole fluid was filtered through a Sterivex filter. The borehole had been drilled approximately 2 years previous to this, and filled with up to 9 borehole modules in September 2014. We extracted DNA from the filter (#43) and were able to recover 2.5 ng/µL DNA (Table 6: DNA extraction and PCR results from borehole module samples.

Sample	Material	Extraction Kit	gDNA (ng/µL)	Amplicon PCRa[#]	Amplicon PCRb[#]
39	Rock powder	PowerMax	too low	no	yes
40	Rock powder	PowerMax	too low	no	yes
41	Rock powder	PowerMax	too low	no	yes
42	Sterivex filter	PowerSoil	too low	no	no
43	Sterivex filter	PowerSoil	2.5	yes	yes
44	Sterivex filter	PowerSoil	too low	no	no
45	Sterivex filter	PowerSoil	too low	no	no
46	Sterivex filter	PowerSoil	too low	yes	yes
47	Sterivex filter	PowerSoil	too low	yes	yes
48	Sterivex filter	PowerSoil	too low	no	no
49	Sterivex filter	PowerSoil	too low	yes	yes

[#]Observations are based on the second PCR replicate (without amplification in the kit controls)

6). The 16S rRNA gene PCR yielded a very strong amplicon (Figure 4). Both DNA extraction replicates from borehole 13.001 (#43) group very closely together in the PCoA plot (group III, Figure 7, both samples are on top of each other) and were distinct from the source borehole and 1200 mL filtered drilling fluid (group I, Figure 7) as well as extraction kit and Sterivex blank controls (group II, Figure 7). The first replicate of the Sterivex air blank sample groups closely together with borehole 13.001 samples, which was likely caused by cross-contamination in the 96-well plate; both samples were next to each other during the PCR. However, all other controls are distinct to borehole 13.001 samples. A total of 139 OTUs were identified in the borehole fluid and the most abundant OTUs are affiliated with *Desulfosporosinus meridiei, Syntrophaceae,* and *Desulfovibrio mexicanus* (Figure 8). All three organisms are putative sulfate reducers.

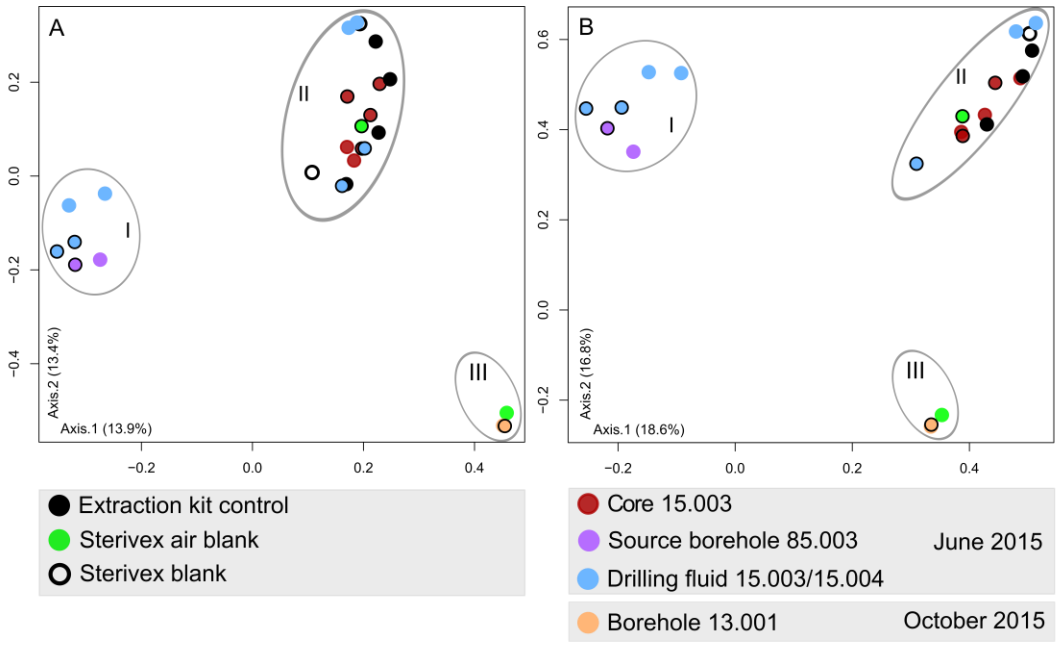

Figure 7: PCoA plot based on the Bray-Curtis distance metric, showing core, drilling and borehole fluid samples. The left panels include samples with very low read counts, resulting in data being rarefied to 189 reads per sample. Samples with lower read counts have been removed in the analysis shown in the right panel, resulting in the data being rarefied to 2048 reads per sample. Each sample was extracted twice; the result of the second extraction is marked with a black circle around the data point. All extractions from 1200 mL drilling or source borehole fluid are contained within group I and separate well from extraction kit and Sterivex blank controls. Both replicate extractions from October 2015 borehole fluid are contained within group I and separate well from extraction kit and Sterivex blank controls. Proportion of variation explained are given in brackets for each axis.

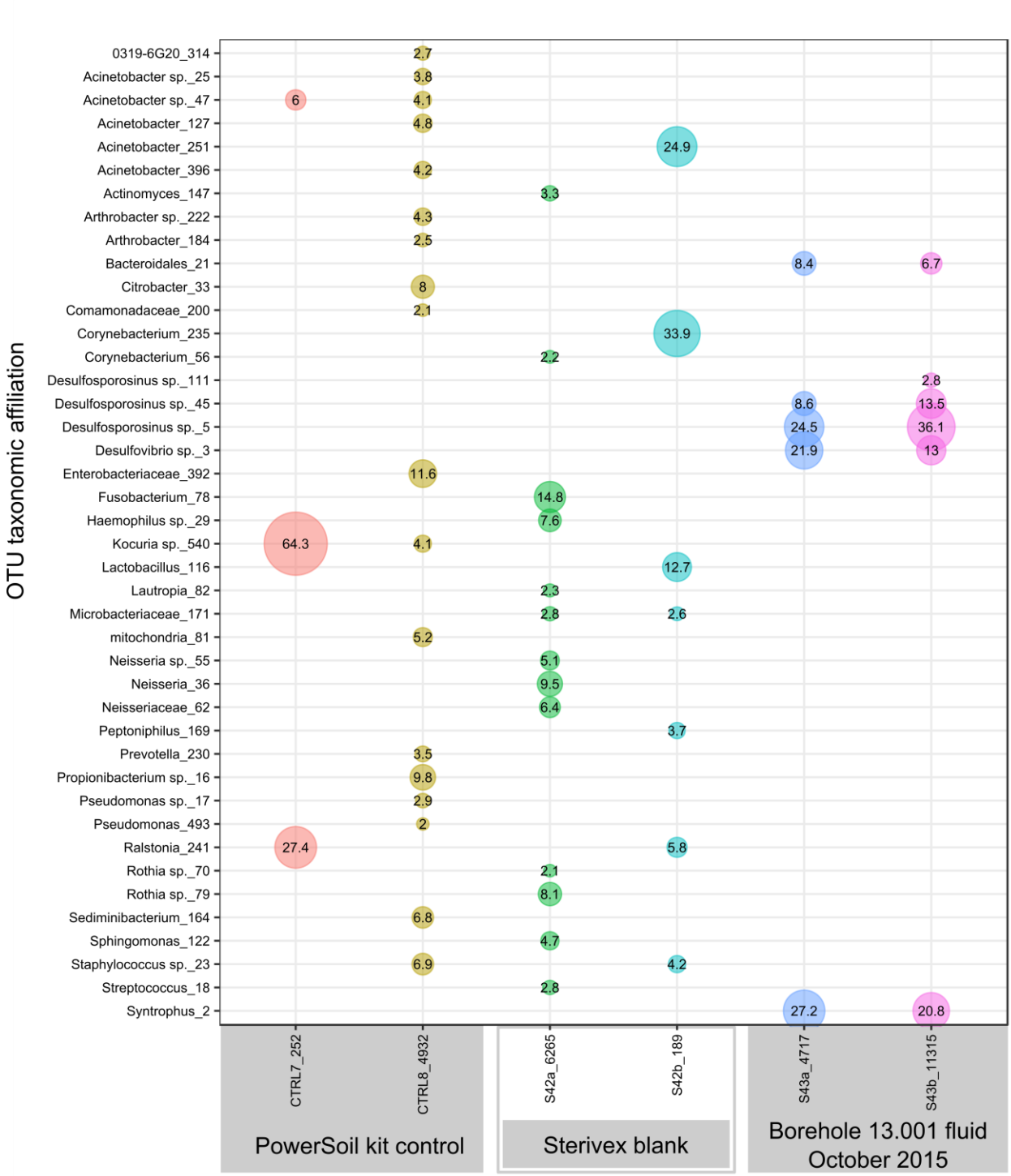

Figure 8: Bubble chart showing 16S rRNA profiles of borehole fluid extracts. Results of two replicate extractions using the PowerSoil DNA Isolation Kit are shown. The chart is based on unrarefied data and read counts for each sample are shown at the end of the name. Only OTUs at and above 2% abundance are shown. OTU taxonomic affiliation is shown on the y-axis followed by a random OTU number.

4. DISCUSSION

Overall the results of this study indicated very low or no biomass in the drilling fluid and core samples from the Grimsel site at the time of the drilling of boreholes 15.003 and 15.004. However, fluid from a two year old borehole (13.001) indicated the presence of microorganisms based on cell counts, PLFA and 16S rRNA gene sequencing.

4.1 CELL COUNTING RESULTS

Cell counting yielded very low biomass for all of the samples, with the exception of the October 2015 samples. Cell counts in the June 2015 samples were in the 10^2 to 10^3 cells/mL range. However, variance for these results overlapped with the variance on the blank, making confident detection of biomass impossible. Notably, these cell abundances are slightly below those observed by Konno et al. 2013 who reported cell abundances of 10^3 to 10^4 cells/mL in samples from the Grimsel site. However, the sites sampled were slightly different, which may account for the variability and in both cases biomass is extremely low. October 2015 samples originate from groundwater of a two year old borehole (13.001). Here larger biomass was found with cell counts on the order of 2×10^6 cells/mL. As indicated in Figure 3 these cell counts were associated with noticeably more plentiful cells present in clusters.

4.2 PLFA RESULTS

PLFA analysis of rock core interior samples yielded no evidence of indigenous microbial communities. PLFA masses were slightly above the method blank, but comparable to or below the pre-combusted sand blanks for the sample pulverization process. The PLFA masses observed for the sand blanks run between the core samples ranged from 700 to 1400 pmol total PLFA in 100 µL. These masses are above the level of the compiled method blank for the PLFA method which was 435 +/- 235 pmol total PLFA in 100 µL. This indicates that some addition of PLFA was occurring during sample handling and processing. The PLFA masses in the sand blanks were equivalent to cell abundances of 2 to 4×10^5 cells/g based on an average conversion factor of 4×10^4 cells/pmol (Green and Scow 2000). Thus, based on the results of this investigation, the rock core interiors contained less than 2 to 4×10^5 cells/g cell abundances.

As noted, the utilization of GC-FID based detection was shown to increase the sensitivity of the PLFA analysis beyond that achieved by GC-MS alone and beyond what was reported in Slater et al. 2013. Detection limits by GC-FID were found to increase by an order of magnitude over GC-MS based detection. The increased sensitivity resulted in the detection of a PLFA method blank of 435 +/- 235 pmol of total PLFA in 100 µL or 4.4 pmol of PLFA injected on the column. This blank was not observable by GC-MS approaches. If this blank is used to calculate a PLFA based detection limit, it yields a range of 1.8 to 4.3×10^4 cells/g for a 400 g extraction.

Water filter samples in this study yielded only trace detection of PLFA. No PLFA were detected above the level of the method blank in the June 2015 samples that were collected during drilling of boreholes 15.003 and 15.004. Based on the filtration of 1000 mL of water for these samples, the method blank derived detection limit is 0.7 to 1.7×10^4 cells/mL. Cell abundances calculated for water filter samples collected at the time of rock core sampling were all below 1.4×10^4 cells/mL. This result is consistent with previously reported cell abundances of 10^3 to 10^4

for water at the Grimsel site reported by Konno et al. 2013. Water filter samples collected prior to drilling activities yielded higher PLFA masses. However, the presence of higher PLFA abundances associated with the field blank filter make confident interpretation of these results impossible.

Water filter samples collected in October 2015 from a two year old borehole (13.001) were the only samples that showed some evidence of PLFA above the level of the method blank. Sample PLFA 1 which filtered 960 mL of water had PLFA masses that were just in excess of the upper range of the method blank. The observed PLFA masses were also almost three times those observed on the concurrent field blank. When converted to cell abundance estimates these abundances were equivalent to 3×10^4 cells/mL. Sample PLFA 2 collected only 120 mL of water due to the filter breaking. However, this sample also had elevated PLFA mass. In this case however, it was not outside the range of the method blank. When divided by the small volume of water filtered, this PLFA mass yielded a cell abundance estimate of 2×10^5 cells/mL. This value is artificially inflated due to the small volume of water that the PLFA blank is being allocated to. This illustrates the importance of sampling consistent volumes of water that are sufficiently large to overcome the influence of the small, consistent methodological blanks. Based on the similarity in PLFA masses between PLFA 1 and PLFA 2 from this sample collection, there is a question as to how robust the detection of biomass by PLFA 1 is. The finding of increased biomass is however supported by the increased cell counts found for this sample.

4.3 COMPARISON TO LOW POROSITY SEDIMENTARY ROCK RESULTS

The previous development of methods to study low biomass microbial communities in sedimentary rock environments (NWMO-TR-2013-17) did not detect the presence of indigenous microbial communities in either water filter or rock core samples from a high salinity, low porosity sedimentary subsurface environment (Slater et al. 2013). The study did identify the presence of PLFA at concentrations equivalent to 1 to 3×10^5 cells/g for rock core samples, despite no detection of DNA for these same samples. The reason for this contrast was not clear. It was hypothesized that matrix interferences were potentially blocking the detection of DNA. Indeed, tests of DNA detection from the rock matrix showed a detection limit of 5×10^5 cells/g, insufficient to detect the observed cell abundances indicated by the PLFA results.

The results of this current study are comparable to the previous study. However, the more extensive blank testing completed here yielded process blank PLFA concentrations of 2 to 4×10^5 cells/g that are comparable to the PLFA concentrations observed in the previous study. The similarity between the value of the process blank in this study and the PLFA concentrations observed in the previous study suggests that the previous study may also have been affected by process blank contributions that were not sufficiently well constrained. If this was the case, it would reduce the confidence in the observation of PLFA in rock core interiors in the previous study of low permeability sedimentary rocks. In this scenario, the DNA and PLFA results in Slater et al. 2013 can be reconciled, and a stronger conclusion made that the low permeability sedimentary rock system previously investigated in 2013 has no biomass present above a detection limit of 1 to 5×10^5 cells/g.

The implications of both of these studies are that characterization of low biomass subsurface environments using the core-based extraction and characterization methods of these two studies are limited to the ability to detect organisms present at abundances on the order of 10^5

cells/g. While increased detection limits may be achieved using distilled solvents, it appears that artefacts introduced during rock core pulverization represent a larger concern. Further improvements in sample handling may address these issues and decrease detection limits to those of the method (equivalent to 2 to 4 × 10^4 cells/g). However, if cellular abundances are higher on solid surfaces (i.e. rock core matrix) than in groundwater, as indicated by previous studies (Pedersen 1992a, Jägevall 2011) then these detection limits would be comparable to much lower groundwater cell densities, depending on the proportion of cells associated with the surfaces.

4.4 16S rRNA SEQUENCING RESULTS

Reagent and laboratory contaminant sequences can contribute a large proportion of detectable DNA in samples associated with extremely low biomass. As a result, careful reagent and workspace decontamination was performed to decrease the contamination background. By PCR, we amplified 16S rRNA genes in rock powder and drilling fluid but their low biomass required careful examination of the sequencing results to differentiate sample-specific signal from noise. Contamination from DNA extraction kit reagents were reported previously (Salter et al. 2014), therefore we verified the absence of dominant OTUs in samples from extraction kit controls. If samples did not separate well from controls in a PCoA plot, it can be due to cross-contamination and/or reagent contamination. However, sequencing results of those samples needed close examination and were mostly discarded as noise. Furthermore, if replicate extractions did not yield similar sequencing results, these samples were also discarded from the analysis. Randomization, duplicate DNA extractions, and sequencing of reagent controls are essential for identifying potential contamination, especially for low biomass (Salter et al. 2014). Note that OTUs affiliated with *Staphylococcus* and *Actinobacter* were present in extraction kit controls (Figure 6) and are likely contaminants from kit reagents. The microbial community in 1200 mL filtered drilling water before and after drilling (#46, #49) was similar and the top OTU is affiliated with *Caulobacter henricii.* When only 60 or 150 mL drilling fluid were filtered (#45, #48), samples did not separate well from corresponding extraction kit controls and were considered too unreliable for further analysis. The filtration of 1200 mL drilling or source borehole liquid appears to be essential to receive sufficient biomass to generate robust and reproducible sample-specific 16S rRNA gene data.

In all three 15.003 core samples (#39, 40, 41), no OTU can be identified that occurs in all replicate extraction samples. *Gardnerella, Clostridium*, and *Megasphaera* are the most abundant OTUs in one core sample (#41b) but mostly absent from the other two. The amount of DNA recovered from 10 g of rock powder was too low to generate reproducible sample-specific 16S rRNA gene sequencing data. The samples are considered below detection limits, preventing confident conclusions about microbial communities in these core samples.

Biomass was high in the liquid of Grimsel borehole 13.001 in October 2015 and we were able to recover 2.5 ng/µL of DNA. A total of 139 OTUs were identified in the borehole fluid and the most abundant OTUs are affiliated with *Desulfosporosinus meridiei, Syntrophaceae,* and *Desulfovibrio mexicanus*. Cell counting also detected high biomass in this sample (Section 4.1) and, for PFLA analysis, it was the only sample that showed levels above the method blank (Section 4.2). Borehole 13.001 was drilled in 2013, approximately two years before the current sampling. No sample was taken at that time for microbial analysis, which prevents direct comparison of biomass levels. However, it may be that initial biomass was as low as in the

source borehole 85.003 and biomass increased in 13.001 over the two year timeframe. Currently, borehole 13.001 is dominated by potential sulfate-reducing bacteria.

5. CONCLUSIONS

PLFA analysis of rock core interiors from the GTS yielded non-detect for triplicate analyses when compared to PLFA present in process blanks. These observations were consistent with the expectation that the crystalline rock would be sterile. As such, they enabled characterization of the detection limit of this approach. The detection limits for PLFA analysis of rock core samples and water filters were characterized to be 2 to 4 × 10^5 cells/g and 0.7 to 1.7 × 10^4 cells/mL respectively. Concurrent DNA extractions of aliquots of the same pulverized rock core samples were likewise below detection. PLFA detection limits for rock core samples were impacted during rock pulverization procedures. However, if microbial biomass is associated with rock matrix surfaces then it would be expected that analysis of solid matrix material would compare favorably to the detection limit based on analysis of groundwater samples.

Groundwater based PLFA analyses yielded no observed PLFA with the exception of water filters collected in October 2015 from borehole 13.001. This was concurrent with the observation of increased cell mass by cell counting techniques and nucleic acid analysis of these same samples. Thus, while PLFA and DNA-based detection were close to the limits of detection for samples analyzed in this report, the convergence of these multiple approaches indicates that increased biomass was present in groundwater post drilling.

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