Development of protocols for sampling and assessment of bentonite and environmental samples associated with *in situ* proof tests of engineered barrier systems

NWMO-TR-2018-04 **April 2018**

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Document History

Title:	Development of protocols for sampling and assessment of bentonite and environmental samples associated with *in situ* proof tests of engineered barrier systems		
Report Number:	NWMO-TR-2018-04		
Revision:	R000	Date:	April 2018
Author Company(s)			
Authored by:	Katja Engel (University of Waterloo) Sara Coyotzi (University of Waterloo)		
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Nuclear Waste Management Organization			
Reviewed by:	Jennifer McKelvie, Jeff Binns, Peter Keech, Paul Gierszewski		
Accepted by:	Derek Wilson		

Revision Summary		
Revision Number	**Date**	**Description of Changes/Improvements**
R000	2018-04-04	Initial issue

ABSTRACT

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Report No.: **NWMO-TR-2018-04**
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Company: [1]University of Waterloo, [2]McMaster University
Date: April 2018

Abstract

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. In preparation for the Canadian work, NWMO is participating in the Grimsel Underground Research Laboratory (URL) Materials Corrosion Test (MaCoTe) in Switzerland. Test modules containing metal coupons surrounded by highly compacted MX-80 bentonite were retrieved from ca. 9 m deep boreholes at the URL MaCoTe site after 394 days of storage. We developed protocols for sampling of bentonite and environmental samples associated with the engineered barrier systems (EBS) and assessed microbial communities and their distributions using DNA-based methods. Swab and bentonite samples were taken from various surfaces and materials of borehole modules 1a and 2a. Genomic DNA was extracted from 79 borehole module samples using the PowerSoil or PowerMax DNA Isolation Kit (MO BIO Laboratories). Only 18 DNA extractions yielded sufficient genomic DNA to be quantified using the Qubit fluorometer. Only 55% of samples yielded an amplicon in a conventional 16S rRNA gene PCR with 35 cycles of amplification. A "nested" PCR protocol increased the proportion of successful amplification to 75%, but many amplicons were still weak. Borehole module samples, extraction kit, and no-template controls were sequenced on a MiSeq instrument (Illumina) and assembled sequences were clustered into 1,612 operational taxonomic units (OTUs).

The borehole fluid was dominated by OTUs affiliated with *Desulfosporosinus meridiei* and *Desulfovibrio mexicanus,* which are putatively involved in sulfate-reduction. Also abundant were OTUs affiliated with *Syntrophus*, which likely grow in a symbiotic association with sulfate-reducing bacteria. The relative abundance of these OTUs was lower for samples from the inside of the borehole module and were almost undetectable in samples of inner layer bentonite. *Pseudomonas stutzeri* was the dominant bacterium detected in case and filter samples and is considered a denitrifying bacterium. Its relative abundance reduces when moving towards the inside of the borehole module. *Streptomyces* spp*.* were dominant bacteria detected in the bentonite core samples, presumably as metabolically inactive spores or extracellular "relic" DNA. Subsequent work will be required to quantify the absolute abundance of these taxa and determine whether the detected bacteria were viable and capable of contributing metabolically to the proof test modules.

In addition to DNA analysis, six bentonite samples were assessed via phospholipid fatty acid (PLFA) analysis. PLFA concentrations were consistent for all samples at 24 +/- 7 ng/gm, which yielded estimates of cellular abundances of 1 to 3 × 10^6 cells/gm. Notably, PLFAs observed included iso and anteiso C15:0, which are often associated with sulfate-reducing bacteria, and C18:2ω9,12, often considered a biomarker of fungi. The distributions of PLFAs were generally similar for the bentonite samples from modules 1a and 2a and variations between outer and inner layer bentonite were minor.

TABLE OF CONTENTS

LIST OF TABLES

Page

LIST OF FIGURES

Page

LIST OF ABBREVIATIONS

APM	adaptive phased management
AXIOME	automated exploration of microbial diversity
BDL	below detection limit
BSA	bovine serum albumin
cm	centimeter
cat. no.	catalogue number
DGR	deep geological repository
EBS	engineered barrier systems
FAMEs	fatty acid methyl esters
g	gravitational force
GC MS	gas chromatography mass spectrometry
gm	gram
GTS	Grimsel test site
HCB	highly compacted bentonite
kb	kilobase
m	meter
MaCoTe	materials corrosion test
MIC	microbiologically influenced corrosion
min	minute
mm	millimeter
ng	nanogram
nt	nucleotides
NTC	no-template control
NWMO	The Nuclear Waste Management Organization
OTU	operational taxonomic unit
PCoA	principal coordinates analysis
PLFA	phospholipid fatty acid
QIIME	Quantitative Insights Into Microbial Ecology
rRNA	ribosomal RNA
s	second
µl	microliter
URL	Grimsel Underground Research Laboratory

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.

The NWMO has an active technical program to demonstrate that corrosion will not compromise the integrity of the used fuel container. Under anoxic conditions, sulfate-reducing bacteria in the repository have the potential to produce sulfide, which could diffuse through the HCB to the container and cause microbiologically influenced corrosion (Scully *et al.*, 2016). Based on laboratory pressure cell experiments that investigated the numbers of microorganisms present in saturated HCB, a design criteria of 1.6 g/cm^3 dry density has been established for the HCB, since it was demonstrated that growth of bacteria and germination of spores did not occur when bentonite dry densities exceeded this density (Stroes-Gascoyne *et al.*, 2010). This corresponds to a cessation of growth at water activities <0.96 and a swelling pressure of at least 2 MPa (Stroes-Gascoyne *et al.*, 2010; Motamedi *et al.*, 1996). In addition to protecting the container from microbiologically influenced corrosion, the HCB is designed to: i) limit the rate of liquid movement by diffusion, ii) provide mechanical support to the container, iii) retain radionuclides in the event of container failure, and iv) provide a thermally conductive medium to transmit heat to the surrounding host rock.

To further demonstrate that microbiologically influenced corrosion (MIC) will not occur, NWMO is participating 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 1. The main goals of the MaCoTe program are to: (i) confirm the long-term anaerobic corrosion rate of candidate canister materials in compacted bentonite under repository-relevant conditions, and (ii) to provide experimental evidence for the inhibiting effect of bentonite buffer on microbial activity and MIC. Experiments place test modules containing candidate canister materials embedded in bentonite into 9 m boreholes and saturates the modules with ground water retrieved from the BOUS 85.003 borehole. The location of these boreholes within the GTS with their respective identification numbers are indicated in Figure 1C.

The MaCoTe program will have a duration of 10 years, with the retrieval of modules occurring at various intervals throughout. Once retrieved, samples of candidate materials and bentonite will be analyzed with a full suite of corrosion and microbiological tests to evaluate the corrosion performance and the ability of bentonite to supress microbial activity.

The overall objective of the work presented herein was to characterize microbial community structures and relative abundances of bacteria in bentonite from the first test module retrieved from the experiment using high-throughput sequencing. This test module was emplaced in borehole 13.001 for 394 days. The results will serve as an important baseline against which the microbial diversity in future retrieved modules will be compared. Future samples from retrieved modules will be analyzed using the same methods to ensure results can be compared. The cell count and phospholipid fatty acid analysis (PLFA) of the groundwater from borehole 13.001 is described in a companion report (Slater *et al.*, 2018 NWMO-TR-2018-05).

Most prior assessments of microbial diversity in bentonite have relied upon PLFA (Mauclaire *et al.*, 2007; Stroes-Gascoyne *et al.*, 2007) because the strong binding affinity of nucleic acids to clay materials hinders extraction processes (Filip, 1973; Paget *et al.*, 1992; Walker *et al.*, 1989). As such, an important additional objective of this work was to develop protocols for DNA extraction and 16S rRNA gene sequencing to enable genomic analysis of microorganisms in bentonite samples. In addition, given the low biomass expected in these samples, development of methods to characterize background contamination and improve detection limits for microbiological analyses was necessary.

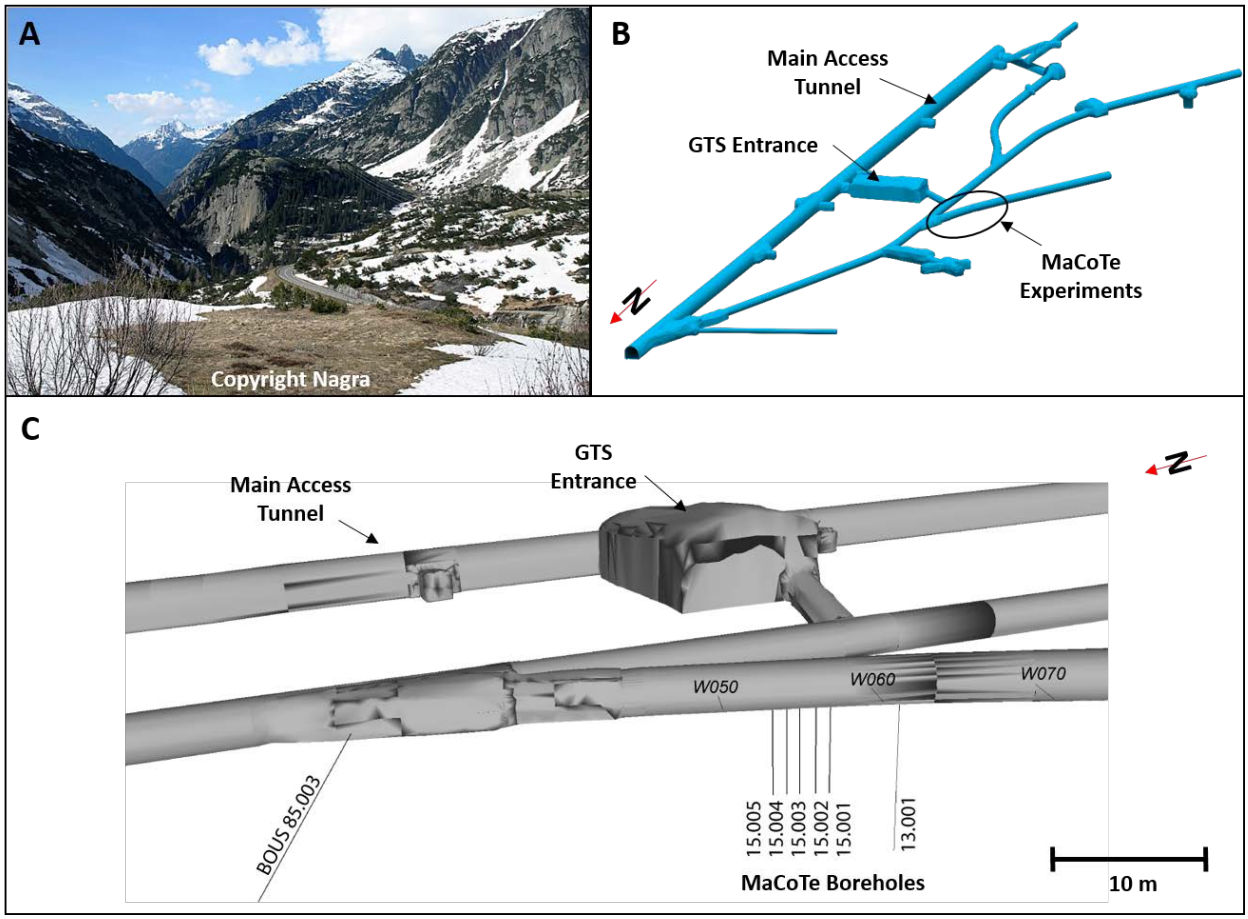

Figure 1. Location and outline of the Grimsel Test Site (GTS) in Switzerland. The underground research facility consists of approximately 1000 m of tunnels constructed 450 m below the Earth's surface. The GTS location and tunnel layout are shown in panel 1A and B. Borehole modules analysed in this research were retrieved from the borehole 13.001 (panel 1C).

2. METHODS

2.1 GRIMSEL BOREHOLE MODULES AND EXPERIMENTAL SET-UP

Stainless steel borehole modules, containing corrosion test pieces embedded in bentonite with two different densities, were prepared under anoxic conditions by AMEC Foster Wheeler (Harwell, UK). The borehole modules were 308 mm in height with a diameter of 126 mm (Figure 2). The filter was made of sintered stainless steel with a pore size of 18 µm. Disc-shaped corrosion coupons with a diameter of 20 mm were manufactured from carbon steel (10 mm height), cold spray copper (3 mm height), electrodeposited copper (3 mm height), wrought copper (10 mm height), and stainless steel (4 mm height). Coupons were embedded in the bentonite core in four layers, with three coupons each (Figure 2). Wyoming MX-80 bentonite at two different densities was used in this experiment. Targeted dry densities were 1.25 and 1.50 gm/cm^3 using a mixture of pellets and powdered bentonite. After assembly of the modules, the bentonite was fully saturated using deionised water. Borehole modules were placed in borehole 13.001 at the URL in Switzerland (Figure 1) in September 2014. The borehole contained natural groundwater. Diffusive holes in the modules permitted the exchange of water with the environment. After placement of the modules, the borehole was flushed with argon and sealed with a hydraulic packer to maintain anoxic conditions. A total of 8 modules were installed in the borehole and will be removed and analysed according to a schedule over a 10-year period.

2.2 SAMPLING FROM BOREHOLE MODULES 1a AND 2a

After 394 days of storage at the URL, two modules (Table 1) were removed from the borehole while purging with argon gas. Borehole fluid was sampled on the day of removal. Modules were transported to AMEC Foster Wheeler (Harwell, UK) for analysis in stainless steel shipping flasks, which were also purged with argon gas. Modules were disassembled in an anoxic glove box and samples taken from different locations in the module. Detailed description of the sampling (protocol, pictures, and drawings) can be found in appendices A to C.

Table 1. List of borehole modules retrieved after one year of storage in a borehole at the Grimsel Underground Research Laboratory.

Module number	MX-80 dry density (gm/cm^3)	Borehole number	Deposition date in borehole	Retrieval date from borehole	Module sampling date
1a	1.25	13.001	2014-Sept-22	2015-Oct-21	2015-Nov-10
2a	1.50	13.001	2014-Sept-22	2015-Oct-21	2015-Nov-12

AMEC Foster Wheeler retrieved and analyzed the metal coupons, which were embedded in each module for corrosion testing. We obtained swab samples from various locations on the module, clay imprints from metal coupons, liquid from the shipping flask, and various bentonite samples. Bentonite samples were also sent to McMaster University (Ontario, Canada) for phospholipid fatty acid (PLFA) analysis. A list of all samples analyzed in this report can be found in Table 2 and the subsample locations within the borehole modules is illustrated in Figure 2.

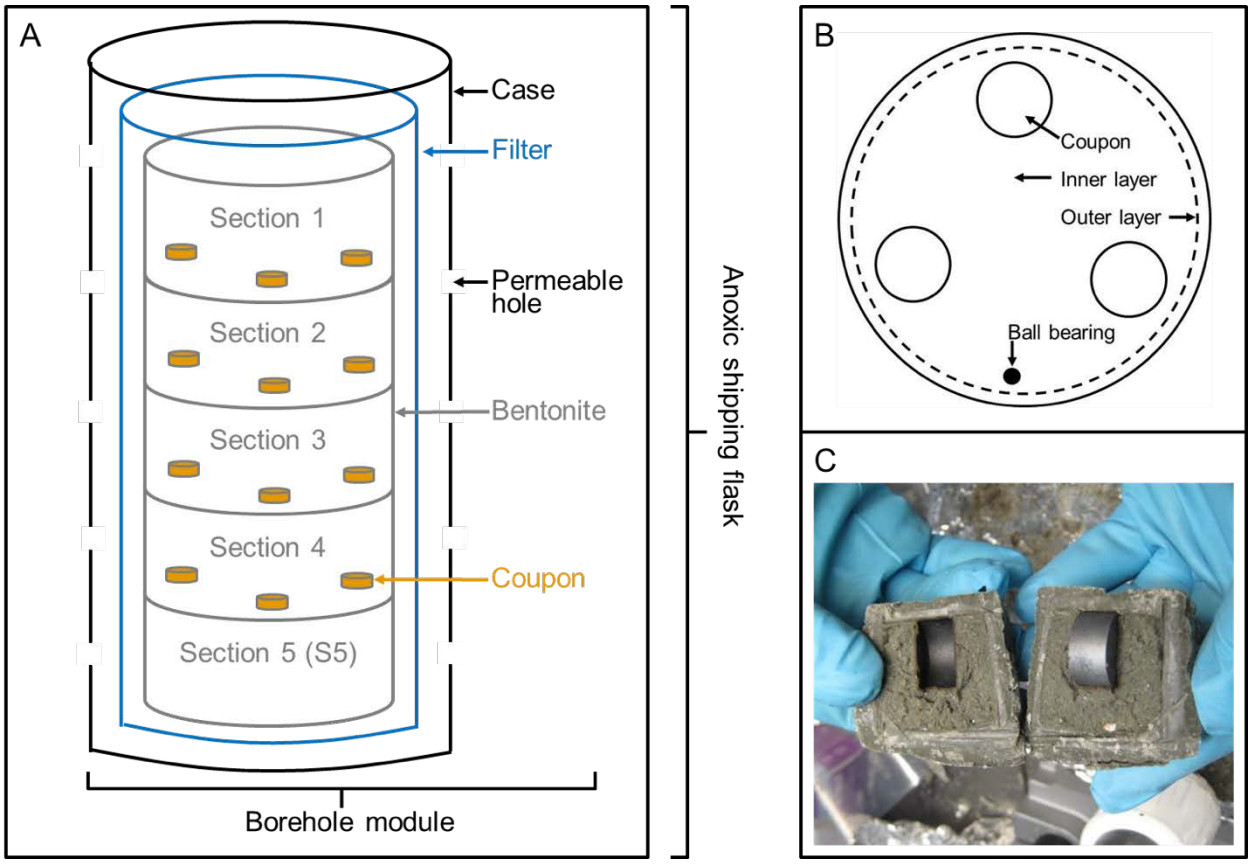

Figure 2. Schematic of the borehole module and bentonite core. A: Schematic showing the borehole module with metal coupons embedded in bentonite surrounded by a sintered stainless steel filter and metal case. Section 5 of the bentonite core does not contain coupons. The outer and inner layers of section 5 were sampled separately from the rest of the core. For shipment and storage the borehole module was placed in an anoxic shipping flask. B: Cross-section of the bentonite core indicating the location of inner and outer bentonite layers where the outer layer refers to the approximately 5 mm thick outer ring of bentonite around the core C: Image of a coupon embedded in bentonite and its imprint in bentonite (carbon steel coupon shown). The thin layer of clay in the immediate vicinity of the coupon is referred to as Coupon-Clay. The borehole module is 308 mm high and has a diameter of 126 mm.

Table 2. List of samples from borehole modules and borehole fluid analyzed in this report.

Sample	Module	Sampling date	Sampling location	Description	Sampling material
1a	-	2015-Nov-10	Control	Control 1, unused swab	Swab
1b	-	2015-Nov-10	Control	Control 1, unused swab	Swab
1c	-	2015-Nov-10	Control	Control 1, unused swab	Swab
2a	-	2015-Nov-10	Work surface day 1	Control 2 (Glovebox right wall)	Swab
2b	-	2015-Nov-10	Work surface day 1	Control 2 (Glovebox plexiglass)	Swab
2c	-	2015-Nov-10	Work surface day 1	Control 2 (Glovebox surface)	Swab
3	1a	2015-Nov-10	Shipping flask	Shipping flask outside	Swab
4	1a	2015-Nov-10	Shipping flask	Liquid in shipping flask	Liquid
5a	1a	2015-Nov-10	Case	Permeable hole	Swab
5b	1a	2015-Nov-10	Case	Permeable hole	Swab
6a	1a	2015-Nov-10	Case	Case outside	Swab
6b	1a	2015-Nov-10	Case	Case outside	Swab
6c	1a	2015-Nov-10	Case	Case outside	Swab
7	1a	2015-Nov-10	Case	Black deposit on inside of lid	Solid
8a	1a	2015-Nov-10	Case	Case inside, top, black deposit, and bentonite	Swab
8b	1a	2015-Nov-10	Case	Case inside, bottom, black deposit, and bentonite	Swab
9a	1a	2015-Nov-11	Filter	Filter outside	Swab
9b	1a	2015-Nov-11	Filter	Filter outside	Swab
10a	1a	2015-Nov-11	Filter	Filter inside	Swab
10b	1a	2015-Nov-11	Filter	Filter inside	Swab
21	1a	2015-Nov-11	Coupon-Clay imprint	Carbon steel	Bentonite
22	1a	2015-Nov-11	Coupon-Clay imprint	Wrought copper	Bentonite
23	1a	2015-Nov-11	Coupon-Clay imprint	Cold spray copper	Bentonite
24	1a	2015-Nov-11	Coupon-Clay imprint	Electrodeposited copper	Bentonite
25	1a	2015-Nov-11	Coupon-Clay imprint	Stainless steel	Bentonite
21S	1a	2015-Nov-11	Coupon-Clay imprint	Carbon steel	Swab
22S	1a	2015-Nov-11	Coupon-Clay imprint	Wrought copper	Swab
23S	1a	2015-Nov-11	Coupon-Clay imprint	Cold spray copper	Swab
24S	1a	2015-Nov-11	Coupon-Clay imprint	Electrodeposited copper	Swab
25S	1a	2015-Nov-11	Coupon-Clay imprint	Stainless steel	Swab
50	1a	2015-Nov-11	Coupon-Clay imprint	Wrought copper (WC1.2)	Bentonite
51	1a	2015-Nov-11	Coupon-Clay imprint	Carbon steel (CS1.2)	Bentonite
52	1a	2015-Nov-11	Coupon-Clay imprint	Cold spray copper (CSC1.1)	Bentonite
53	1a	2015-Nov-11	Coupon-Clay imprint	Wrought copper (WC1.5)	Bentonite
54	1a	2015-Nov-11	Coupon-Clay imprint	Electrodeposited copper (EDC1.2)	Bentonite
55	1a	2015-Nov-11	Coupon-Clay imprint	Electrodeposited copper (EDC1.4)	Bentonite
50S	1a	2015-Nov	Coupon-Clay imprint	Wrought copper (WC1.2)	Swab
51S	1a	2015-Nov	Coupon-Clay imprint	Carbon steel (CS1.2)	Swab
52S	1a	2015-Nov	Coupon-Clay imprint	Cold spray copper (CSC1.1)	Swab
53S	1a	2015-Nov	Coupon-Clay imprint	Wrought copper (WC1.5)	Swab
54S	1a	2015-Nov	Coupon-Clay imprint	Electrodeposited copper (EDC1.2)	Swab
55S	1a	2015-Nov	Coupon-Clay imprint	Electrodeposited copper (EDC1.4)	Swab
26	1a	2015-Nov-11	Bentonite core outer layer	Composite sample of bentonite core sections 1 to 4	Bentonite

Sample	Module	Sampling date	Sampling location	Description	Sampling material
27	1a	2015-Nov-11	Bentonite core inner layer	Composite sample of bentonite core sections 1 to 4	Bentonite
28	1a	2015-Nov-11	Bentonite core outer layer S5	Bentonite core section 5	Bentonite
29	1a	2015-Nov-11	Bentonite core inner layer S5	Bentonite core section 5	Bentonite
11a	-	2015-Nov-12	Work surface day 3	Control 3 (Glovebox surface)	Swab
11b	-	2015-Nov-12	Work surface day 3	Control 3 (Glove box plexiglass)	Swab
11c	-	2015-Nov-12	Work surface day 3	Control 3 (Glovebox gloves)	Swab
12	2a	2015-Nov-12	Shipping flask	Shipping flask outside	Swab
13	2a	2015-Nov-12	Shipping flask	Shipping flask inside	Swab
14	2a	2015-Nov-12	Shipping flask	Liquid in shipping flask	Liquid
15a	2a	2015-Nov-12	Case	Permeable hole	Swab
15b	2a	2015-Nov-12	Case	Permeable hole	Swab
16a	2a	2015-Nov-12	Case	Case outside	Swab
16b	2a	2015-Nov-12	Case	Case outside	Swab
17	2a	2015-Nov-12	Case	Black deposit on inside of lid	Solid
18a	2a	2015-Nov-12	Case	Case inside, top	Swab
18b	2a	2015-Nov-12	Case	Case inside, bottom	Swab
19a	2a	2015-Nov-12	Filter	Filter outside	Swab
19b	2a	2015-Nov-12	Filter	Filter outside	Swab
20a	2a	2015-Nov-12	Filter	Filter inside	Swab
20b	2a	2015-Nov-12	Filter	Filter inside	Swab
30	2a	2015-Nov-13	Coupon-Clay imprint	Carbon steel	Bentonite
31	2a	2015-Nov-13	Coupon-Clay imprint	Wrought copper	Bentonite
32	2a	2015-Nov-13	Coupon-Clay imprint	Cold spray copper	Bentonite
33	2a	2015-Nov-13	Coupon-Clay imprint	Electrodeposited copper	Bentonite
34	2a	2015-Nov-13	Coupon-Clay imprint	Stainless steel	Bentonite
30S	2a	2015-Nov-13	Coupon-Clay imprint	Carbon steel	Swab
31S	2a	2015-Nov-13	Coupon-Clay imprint	Wrought copper	Swab
32S	2a	2015-Nov-13	Coupon-Clay imprint	Cold spray copper	Swab
33S	2a	2015-Nov-13	Coupon-Clay imprint	Electrodeposited copper	Swab
34S	2a	2015-Nov-13	Coupon-Clay imprint	Stainless steel	Swab
35	2a	2015-Nov-13	Bentonite core outer layer	Composite sample of bentonite core sections 1 to 4	Bentonite
36	2a	2015-Nov-13	Bentonite core inner layer	Composite sample of bentonite core sections 1 to 4	Bentonite
37	2a	2015-Nov-13	Bentonite core outer layer S5	Bentonite core section 5	Bentonite
38	2a	2015-Nov-13	Bentonite core inner layer S5	Bentonite core section 5	Bentonite
42	-	2015-Oct-21	-	Air blank	Sterivex filter
43	-	2015-Oct-21	Borehole fluid	Dewatered (volume unknown); borehole 13.001	Sterivex filter

2.3 DNA EXTRACTION FROM BOREHOLE MODULE SAMPLES

DNA was extracted from sampled materials using DNA extraction kits from MO BIO Laboratories:

- PowerSoil DNA Isolation Kit (MO BIO Laboratories, CA, USA, cat. no. 12888).
- PowerMax DNA Isolation Kit (MO BIO Laboratories, CA, USA, cat. no. 12988).

Kit type varied based on the material and volume being extracted (Table 3, page 12). For each batch of extractions, a kit control was carried out containing no sample (reagent background).

2.3.1 DNA EXTRACTION FROM SWABS

Total genomic DNA from sterile DNA-free foam swabs (Puritan, ME, USA, cat. no. 25-1506 1PF BT) was extracted using the PowerSoil DNA Isolation Kit (MO BIO Laboratories). The swab tip was cut with flame-sterilized (70% ethanol) and flame-heated scissors (to burn DNA) into the PowerBead tube. After addition of lysis solution, the PowerBead tube was incubated at 70°C for 10 min, followed by bead beating using a 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 10 mM Tris and stored in aliquots at −20°C until PCR analysis.

2.3.2 DNA EXTRACTION FROM COUPON-CLAY IMPRINTS

DNA from coupon-clay imprints was extracted in two ways. Firstly, the surface of the coupon imprint was swabbed using a sterile, DNA-free foam swab (Puritan, ME, USA). Total genomic DNA was extracted from the swab as described in section 2.3.1. Secondly, the clay surrounding the imprint was removed using a sterile DNA-free single-use razor blade and was added to a PowerBead tube of the PowerSoil DNA Isolation Kit (MO BIO Laboratories). Cuttings were approximately 1 to 2 mm thick and weighed 0.19 to 0.30 gm. Four cuttings weighed above 0.30 gm (0.47 gm (#21), 0.34 gm (#33), 0.32 gm (#52) and 0.33 gm (#55)) and the clay absorbed all liquid in the PowerBead tube, preventing further extraction steps. For those samples, 0.40 gm coupon clay slurry was transferred to a second PowerBead tube for extraction. After addition of lysis solution, the PowerBead tube was incubated at 65°C for 30 min, followed by bead beating using a FastPrep instrument (MP Biomedicals) 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 10 mM Tris and stored in aliquots at −20°C until PCR analysis.

2.3.3 DNA EXTRACTION FROM INNER AND OUTER LAYERS OF BENTONITE

Total genomic DNA from bentonite was extracted using the PowerMax DNA Isolation Kit (MO BIO Laboratories) and PowerSoil DNA Isolation Kit (MO BIO Laboratories). Several millimeters of bentonite were removed from inner layer samples using a sterile, DNA-free single-use scalpel to remove potential contamination. All samples were cut into small pieces before placing into the bead beating tube to aid complete suspension in the extraction buffer. For the PowerSoil extraction, 0.19 to 0.22 gm of bentonite was added to the PowerBead tube. The remainder of the extraction was carried out as described in section 2.3.2. For the PowerMax extraction, 2.0 gm of bentonite were added to PowerBead tubes. After addition of PowerBead 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 in 2 ml 10 mM Tris. Nucleic acids were precipitated using 4 µl/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), and stored at −20°C overnight. DNA was precipitated by centrifugation at 13,000 g for 30 min. DNA pellet was 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.

2.3.4 DNA EXTRACTION FROM BOREHOLE FLUID

DNA from borehole fluid was extracted as described previously by Slater and colleagues (2013). Briefly, samples were 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) as described in section 2.3.1.

2.4 DNA QUANTIFICATION

Genomic DNA was quantified using the Qubit dsDNA High Sensitivity Assay kit (Invitrogen, CA, USA, cat. no. Q32854). Five µl of DNA extract was added to 195 µl of 200-fold diluted Qubit dsDNA HS reagent and mixed by vortexing. Standard solutions provided by the kit manufacturer were used to create a two-point calibration curve. After 3 min of incubation at room temperature standards and samples were measured on a Qubit 2.0 fluorometer (Life Technologies, CA, USA).

2.5 AMPLIFICATION OF 16S rRNA GENES 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 PCR was set up in a PCR workstation using ISO 5 HEPA-filtered air and UV light irradiated surfaces (AirClean Systems, ON, Canada, cat. no. AC632LFUVC). In addition, surfaces and equipment were cleaned beforehand with UltraClean Lab Cleaner (MO BIO Laboratories, cat. no. 12095-1000). Plasticware, PCR water (HyPure molecular biology grade water; Fisher Scientific, ON, CA, cat. no. SH3053801), and 10 mg/ml bovine serum albumin (BSA) solution were UV treated on a 302 nm transilluminator (ProteinSimple, CA, USA, cat. no. 76-12995) for 15 min. The 25 µl PCR mix contained 1× ThermoPol Buffer, 0.2 µM forward primer, 0.2 µM reverse primer, 200 µM dNTPs, 15 µg BSA, 0.625 units *Taq* DNA polymerase (New England Biolabs, MA, USA, cat. no. M0267L) and 2 µl of template (up to 10 ng). PCR was performed in two rounds. The first round PCR (PCR1) was performed as follows: 95°C for 3 min, 35 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. A second round of PCR (PCR2 or "nested PCR") was performed using 1 µl template from PCR 1 and amplified as described above for 15 cycles. Each PCR was prepared in triplicate. Replicate PCR products were pooled and visualized alongside 100 ng of 1 Kb Plus DNA Ladder (Invitrogen, CA, USA, cat. no.

10787018) on a 1.5% agarose gel containing GelRed (Biotium, CA, USA, cat. no. 41003). Uniquely indexed PCR amplicons from the nested PCR were quantified in a 1.5% agarose gel containing GelRed. Equal quantities of each amplicon were pooled into a single tube to a maximum of 30 µl. Controls were included in the Illumina sequencing pool (30 µl), even if amplicons were not detected. The pooled 16S rRNA gene 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).

2.6 DNA CONTAMINATION CONTROLS

We performed several controls to evaluate DNA contamination from laboratory reagents. DNA extraction “kit controls” (i.e., simulated DNA extraction from kit buffer instead of a sample) and “swab controls” (i.e., simulated DNA extraction from an unused swab) were included. Controls for DNA Isolation kit reagent (kit control) were performed with each batch of extraction. Negative controls for PCR master mix reagents were performed in single tubes. NTC1 is the no-template control of the first round PCR (PCR1, 35 cycles). NTC2 is the nested PCR amplification from NTC1 (50 cycles total). NTC3 is the no-template control for the nested PCR (PCR2, 15 cycles). NTCs were prepared for each PCR master mix in six different PCR runs (PCR I to PCR VI). All controls were included in the MiSeq sequencing, even if amplicons were not detected.

2.7 ILLUMINA SEQUENCE ANALYSIS

MiSeq reads (raw reads) were demultiplexed using Illumina MiSeq Reporter software version 2.5.0.5. Reads were assembled using the paired-end assembler for Illumina sequences (PANDAseq version 2.8, Masella *et al.*, 2012) with a quality threshold of 0.9, 8 nucleotide minimum 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 using 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.*, 2010). All representative sequences were classified using the Ribosomal Database Project (RDP version 2.2, Wang *et al.*, 2007) with a stringent confidence threshold (0.8) and the Greengenes database (McDonald *et al.*, 2012) was used to assign taxonomy. Chimeric sequences were filtered with UCHIME (Edgar *et al.*, 2011). For comparison of read counts between samples, only paired-end assembled and quality filtered reads were used. AXIOME generated principal coordinate analysis (PCoA) ordinations using Bray-Curtis distances with samples rarefied to the smallest sample size (smallest read count) included in the analysis. Samples with less than 100 reads were discarded before rarefication. Due to the low read counts of controls, some PCoA ordinations were rarefied to only 158 reads. Once controls were excluded, PCoA plots were rarefied to 4,451 or more reads. PCoA plots were used to assess the differences among sample groups with emphasis of differences between borehole module samples and controls. Bubble plots 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 each sample name. Only OTUs at or above 1% relative abundance were shown. The proportion was increased to as much as 5% for more diverse sample groups. We used the Functional Annotation of Prokaryotic Taxa tool (FAPROTAX version 1.0, Louca *et al*., 2016) to map OTUs to established metabolic or other ecologically relevant functions.

All sequences were deposited into European Nucleotide Archive (https://www.ebi.ac.uk/ena) with study accession number PRJEB24856.

2.8 PHOSPHOLIPID FATTY ACID (PLFA) ANALYSIS

Six bentonite samples from module 1a and 2a were analyzed by McMaster University for phospholipid fatty acid (PLFA) (Table 6, page 34). In addition to these samples, two replicate samples of powdered Volclay MX-80 (Caldic Canada; Lot 06525768; manufactured June 2015) were analyzed. This Volclay MX-80 powdered sample was used to validate the method before running the MaCoTe samples but it was not from the same batch used in the MaCoTe borehole modules. A sample of the MX-80 that was used to load the borehole modules was not available at the time of this analysis.

Combusted bentonite samples (combusted at 450°C for four hours) were analyzed as process blanks in addition to method blanks. Bentonite samples were freeze-dried for 24 hours, crushed with a mortar and pestle, and sieved to 1 mm grain size. Subsamples were weighed out according to Table 6. For module 1a and 2a outer layer bentonite, the entire crushed sample was used. All samples were extracted using a modified Bligh & Dyer protocol (White and Ringelberg, 1998). In brief, samples were extracted twice for 18 hours at room temperature using a 1:2:0.8 ratio of dichloromethane:methanol:phosphate buffer. All samples were fractioned on 6 gm of silica gel into three fractions: dichloromethane fraction 1 (F1), which will contain neutral lipids; acetone fraction 2 (F2), which will contain uncharged, polar lipids such as glycolipids; and methanol fraction 3 (F3), which will contain very polar, charged lipids including phospholipids. F1 and F2 were archived. F3 was evaporated until dry under nitrogen gas and methanolysis was performed under mildly alkaline conditions to convert all phospholipids to fatty acid methyl esters (FAMEs) of the phospholipid fatty acids (PLFA). PLFA present as FAMEs were purified via secondary silica gel prior to analysis by gas chromatography mass spectrometry (GC MS). PLFA extracts were evaporated under nitrogen gas to 100 µl and analyzed via GC MS. An Agilent 6890N gas chromatograph equipped with a DB5-MS column (30 m, 0.25 mm, 0.25 µm film thickness) was used with an Agilent 5973 inert mass selective detector (quadrupole). One µl of sample was injected per run. The splitless injection port temperature was 300°C with a column flow of 1.4 ml/min. The temperature program was as follows: oven hold at 50°C for 1 minute; ramp 20°C/min to 120°C; ramp 4°C/min to 160°C; ramp 8°C/min to 300°C; hold 5 minutes at 300°C. The acquisition mode was scanned with detection for masses between 50 and 450 (mass to charge ratio). Peaks were identified using retention times and molecular weights, an internal library data base, and comparison to a matreya standard.

3. RESULTS

3.1 DNA EXTRACTION AND 16S rRNA GENE PCR

Total genomic DNA was extracted from 79 samples (Table 2). Depending on sample type and material, two different commercially available DNA extraction kits were applied (Table 3). In addition to these samples, powdered MX-80 was analyzed, which was used to load the MaCoTe borehole modules before storage in the borehole. Only 18 DNA extractions yielded sufficient amounts of genomic DNA to be quantified using the Qubit fluorometer and only 42 samples yielded an amplicon in a 16S rRNA gene PCR with 35 cycles of amplification (Table 3). However, when using nested PCR, a total of 58 samples yielded an amplicon but many of them were very weak (Figure 3, Figure 4, and Figure 5). With the exception of kit controls 4 and 5, all kit controls and no-template controls showed no visible amplicon in agarose gels stained with GelRed (Figure 3, Figure 4, and Figure 5).

Table 3. Results of genomic DNA extraction and PCR amplification from borehole module samples. DNA was extracted using the PowerSoil (PS) or PowerMax (PM) DNA Isolation Kit. Genomic DNA concentration (gDNA) was determined using the Qubit dsDNA High Sensitivity Assay kit. Samples with concentrations below 0.5 ng/ml were indicated with BDL (below detection limit). The success of the first round PCR (PCR1, 35 cycles) and nested PCR (PCR2, total of 50 cycles) was summarized stating the presence (yes) or absence (no) of an amplicon on an agarose gel. A detailed description of the samples can be found in Table 2.

Sample	Module	Extraction kit	gDNA (ng/µl)	Amplicon PCR1	Amplicon PCR2
1a	-	PS	BDL	No	No
1b	-	PS	BDL	No	Yes
1c	-	PS	BDL	No	Yes
2a	-	PS	BDL	No	No
2b	-	PS	BDL	No	Yes
2c	-	PS	BDL	Yes	Yes
3	1a	PS	BDL	No	No
4	1a	PS	1.40	Yes	Yes
5a	1a	PS	BDL	Yes	Yes
5b	1a	PS	BDL	Yes	Yes
6a	1a	PS	BDL	Yes	Yes
6b	1a	PS	BDL	Yes	Yes
6c	1a	PS	0.49	Yes	Yes
7	1a	PS	0.09	Yes	Yes
8a	1a	PS	0.05	Yes	Yes
8b	1a	PS	BDL	Yes	Yes
9a	1a	PS	BDL	Yes	Yes
9b	1a	PS	BDL	Yes	Yes
10a	1a	PS	BDL	Yes	Yes
10b	1a	PS	BDL	Yes	Yes
21	1a	PS	BDL	Yes	Yes
22	1a	PS	BDL	No	No
23	1a	PS	BDL	No	Yes
24	1a	PS	BDL	No	No
25	1a	PS	BDL	Yes	Yes
21S	1a	PS	BDL	No	No
22S	1a	PS	BDL	No	Yes
23S	1a	PS	BDL	No	Yes
24S	1a	PS	BDL	No	Yes
25S	1a	PS	BDL	No	Yes
50	1a	PS	BDL	No	No
51	1a	PS	BDL	No	No
52	1a	PS	BDL	No	Yes
53	1a	PS	BDL	No	No
54	1a	PS	BDL	No	No
55	1a	PS	BDL	No	No
50S	1a	PS	BDL	No	Yes
51S	1a	PS	BDL	Yes	Yes
52S	1a	PS	BDL	No	Yes
53S	1a	PS	BDL	No	Yes
54S	1a	PS	BDL	No	No

Sample	Module	Extraction kit	gDNA (ng/µl)	Amplicon PCR1	Amplicon PCR2
55S	1a	PS	BDL	Yes	Yes
26	1a	PM	0.15	Yes	Yes
		PS	BDL	No	No
27	1a	PM	0.05	Yes	Yes
		PS	BDL	No	Yes
28	1a	PM	0.08	Yes	Yes
		PS	BDL	No	Yes
29	1a	PM	0.07	Yes	Yes
		PS	BDL	No	No
11a	-	PS	BDL	No	No
11b	-	PS	BDL	No	No
11c	-	PS	BDL	Yes	Yes
12	2a	PS	BDL	No	No
13	2a	PS	BDL	Yes	Yes
14	2a	PS	1.31	Yes	Yes
15a	2a	PS	BDL	Yes	Yes
15b	2a	PS	BDL	Yes	Yes
16a	2a	PS	0.15	Yes	Yes
16b	2a	PS	0.04	Yes	Yes
17	2a	PS	0.04	Yes	Yes
18a	2a	PS	BDL	Yes	Yes
18b	2a	PS	BDL	Yes	Yes
19a	2a	PS	BDL	Yes	Yes
19b	2a	PS	BDL	Yes	Yes
20a	2a	PS	BDL	No	Yes
20b	2a	PS	BDL	Yes	Yes
30	2a	PS	BDL	Yes	Yes
31	2a	PS	BDL	No	No
32	2a	PS	BDL	No	Yes
33	2a	PS	BDL	No	No
34	2a	PS	BDL	No	No
30S	2a	PS	BDL	No	Yes
31S	2a	PS	BDL	No	Yes
32S	2a	PS	BDL	No	Yes
33S	2a	PS	BDL	No	Yes
34S	2a	PS	BDL	No	Yes
35	2a	PM	0.06	Yes	Yes
		PS	BDL	No	No
36	2a	PM	0.07	Yes	Yes
		PS	BDL	No	No
37	2a	PM	0.05	Yes	Yes
		PS	BDL	No	No
38	2a	PM	0.05	Yes	Yes
		PS	BDL	No	Yes
42	-	PS	BDL	No	No
43	-	PS	2.49	Yes	Yes

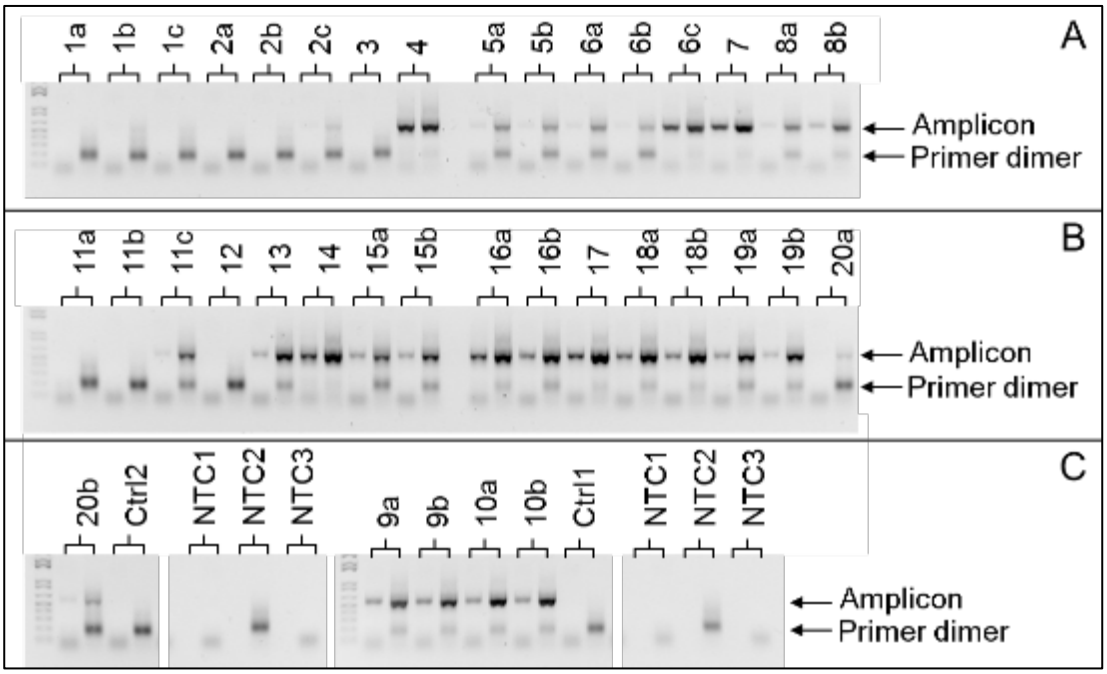

Figure 3. Agarose gels showing 16S rRNA gene amplicons (~0.5 kb) from case, filter, and shipping flask samples from modules 1a and 2a. Amplicons were separated on a 1.5% agarose gel stained with GelRed (Biotium, cat. no. 41002). For each sample, no-template control (NTC), and extraction kit control (Ctrl), 5 µl of PCR1 and nested PCR (PCR2) were loaded next to each other. The 1 Kb Plus DNA Ladder (50 ng, Invitrogen, cat. no. 10787018) was loaded in the first well in each row.

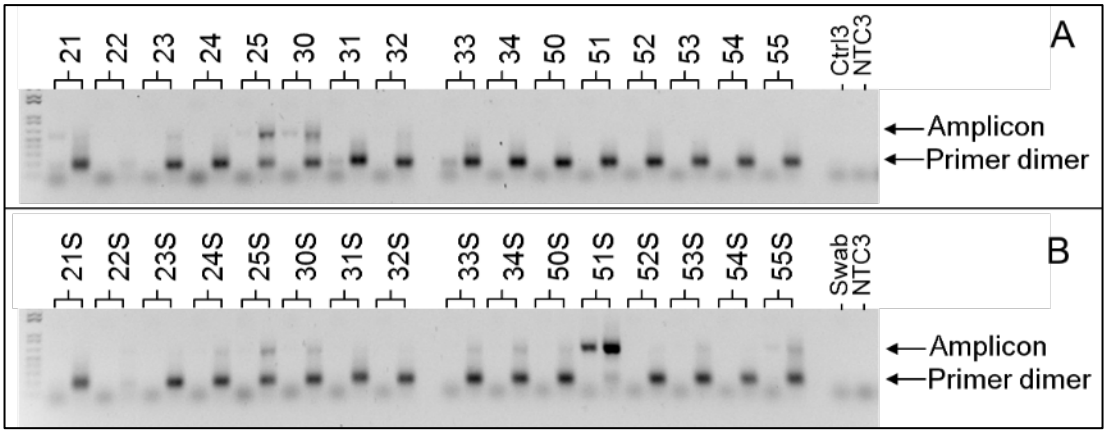

Figure 4. Agarose gels showing 16S rRNA gene amplicons (~0.5 kb) from coupon-clay samples from module 1a and module 2a. DNA was extracted from swabs of the coupon-clay surface (bottom) or directly from coupon-clay (top). Amplicons were separated on a 1.5% agarose gel stained with GelRed (Biotium, cat. no. 41002). For each sample, no-template control (NTC), and extraction kit control (Ctrl), 5 µl of PCR1 and nested PCR (PCR2) were loaded next to each other. The 1 Kb Plus DNA Ladder (50 ng, Invitrogen, cat. no. 10787018) was loaded in the first well in each row.

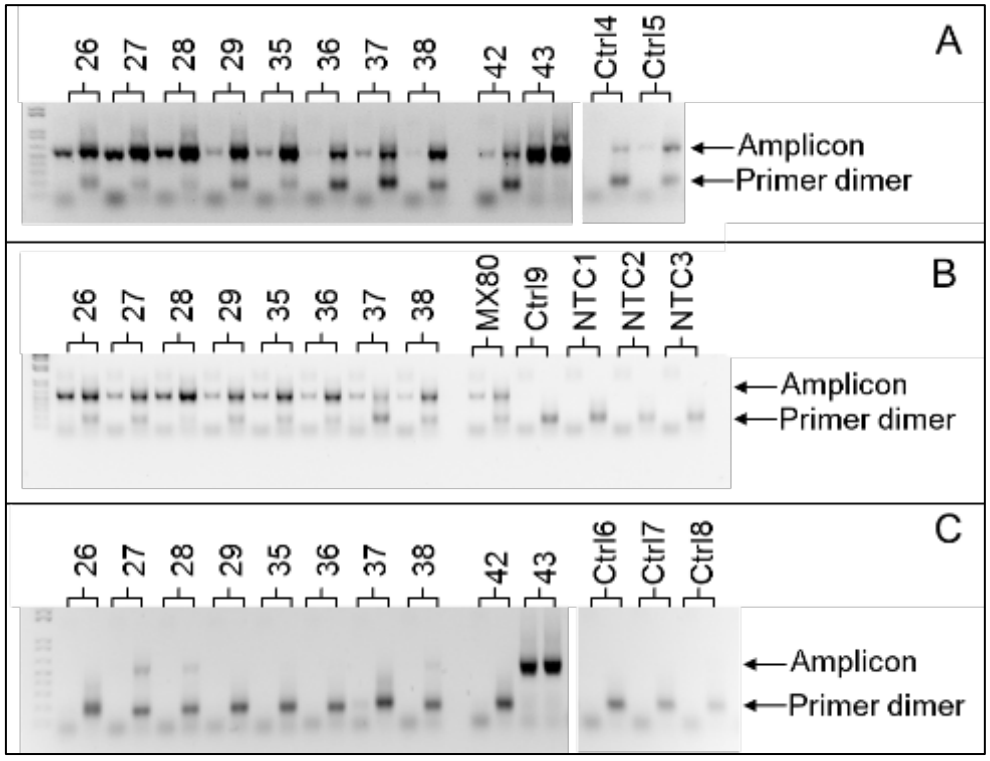

Figure 5. Agarose gels showing 16S rRNA gene amplicons (~0.5 kb) from bentonite and borehole fluid samples. Bentonite samples from module 1a (26 to 29) and module 2a (35 to 38) were extracted using the PowerMax (A, B) or PowerSoil (C) DNA Isolation kit. Gel A and B are replicate PowerMax extractions of bentonite samples. Borehole fluid (#43) and Sterivex blank (#42) were extracted twice (A, C) using the PowerSoil DNA Isolation kit. Amplicons were separated on a 1.5% agarose gel stained with GelRed (Biotium, cat. no. 41002). For each sample, no-template control (NTC), and extraction kit control (Ctrl), 5 µl of PCR1 and nested PCR (PCR2) were loaded next to each other. The 1 Kb Plus DNA Ladder (50 ng, Invitrogen, cat. no. 10787018) was loaded in the first well in each row.

3.2 16S rRNA GENE SEQUENCING

The sequencing of borehole module samples and controls on the MiSeq instrument generated 1,179,202 paired-end reads with an average length of 414 nt. Assembled reads were grouped into 1,612 OTUs. The MiSeq run also included core and groundwater samples from Grimsel that are reported in a companion report (NWMO-TR-2018-05). A total of 23.2% Illumina raw reads were removed in the demultiplexing step on the MiSeq instrument (Illumina) accounting for non-sample reads such as Illumina PhiX control library.

3.2.1 DNA CONTAMINATION CONTROLS

To identify contaminating DNA from reagents and materials, we used controls for unused swabs (swab control), DNA Isolation kit (kit control), and PCR reagents (NTCs). None of the controls (except kit control 4 and 5) showed a visible 16S rRNA gene amplicon in agarose gels stained

with GelRed (Figure 3, Figure 4, and Figure 5). The read counts for PCR1 reagent controls (NTC1, NTC3) were very low, with 0 to 167 each (Table 4). Average read counts for nested controls (NTC2) increased to 513 reads (Table 4) but OTUs were similar to NTC1 (Figure 6; except PCR III and PCR V). When comparing all six master mix controls, no dominant OTU could be identified that was present in all of them (Figure 6). The read counts in kit and swab controls were on average 12 times higher than in nested NTCs, but deviation was very high (Table 4). OTUs for kit and swab controls are shown in Figure 7. Beta diversity measures using principal coordinate analysis (PCoA) ordinations showed that bacterial communities in the controls did not differ detectably in the first two dimensions of the ordination from all samples (panel A in Figure 8). 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, controls (included in group IV) separate well from bentonite and filter samples as well as the majority of case samples. PCoA ordination analysis was based on rarefied data, resulting in only 158 reads per sample when controls were included. To investigate the effect of low sequence counts on the grouping of samples, we also removed controls from the analysis, increasing the number of reads per sample to 4,475. However, grouping of samples did not change detectably for this latter analysis (panel B in Figure 8).

Table 4. Comparison of sequencing read and OTU counts for kit controls, and no-template controls (NTCs). Read counts were determined after paired-end read assembly. For each control group, a varying number of replicate samples (n) were available. Standard deviation (SD) was determined for all groups.

Controls	n	Average read count	SD	Average OTU count	SD
Kit control (PowerMax)	2	4,368	531	42	19
Kit control (PowerSoil)	5	2,005	2,009	18	7
Unused swab control	4	6,738	5,366	50	16
NTC1 (35 cycles)	6	167	152	5	2
NTC2 (nested, 50 cycles)	6	513	559	15	3
NTC3 (15 cycles)	6	2	1	1	1

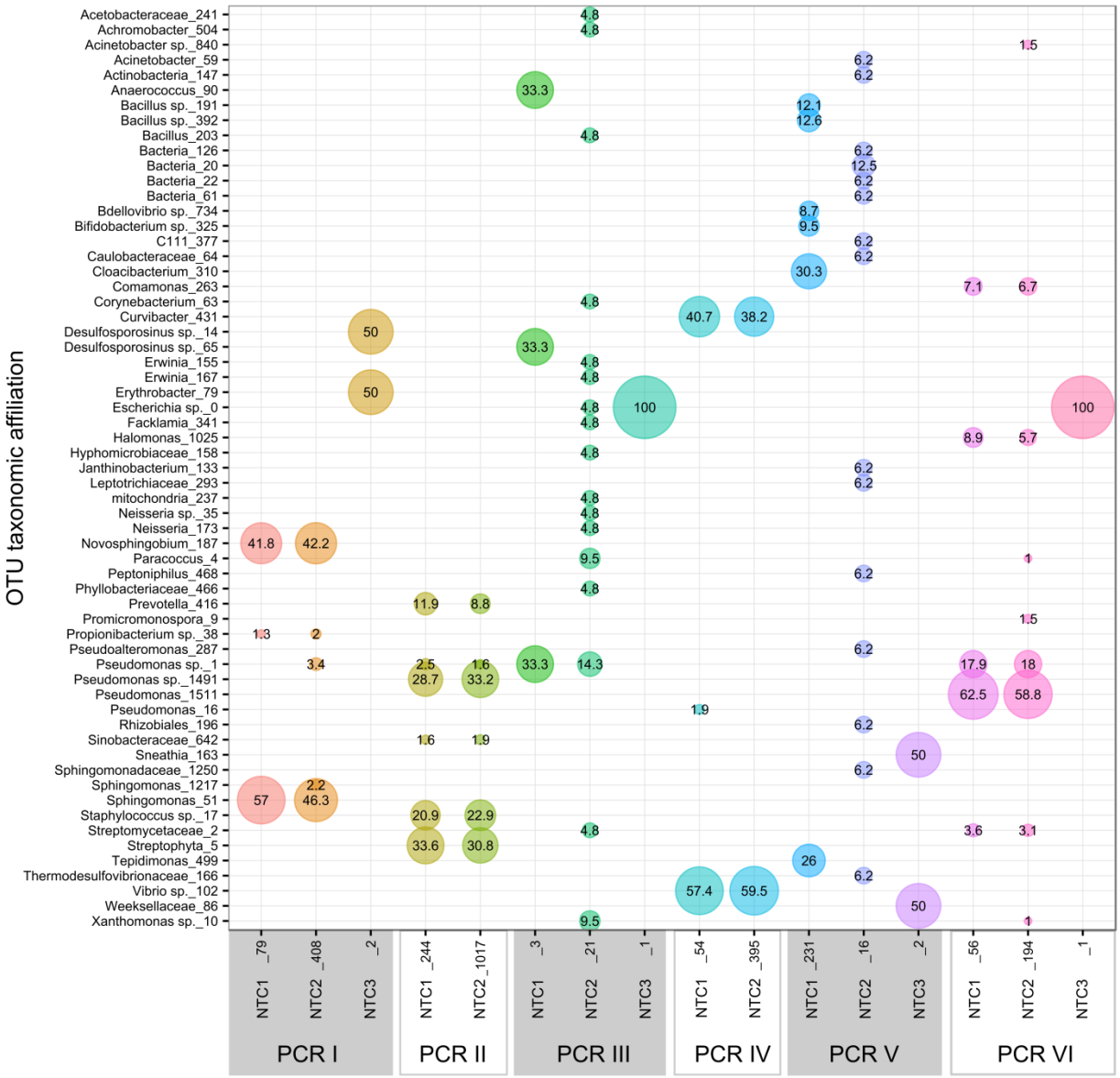

Figure 6. Bubble plot showing 16S rRNA gene profiles of no-template controls, which serve as PCR master mix reagent controls. No-template controls (NTCs) were amplified for 35 (NTC1), 50 (NTC2), or 15 cycles (NTC3) in six different PCR amplifications (PCR I to PCR VI), involving six independent master mixes. This plot is based on unrarefied data and read counts are shown at the end of the sample name. OTU taxonomic affiliation is shown on the y-axis followed by an OTU number. Only OTUs at or above 1% relative abundance are shown.

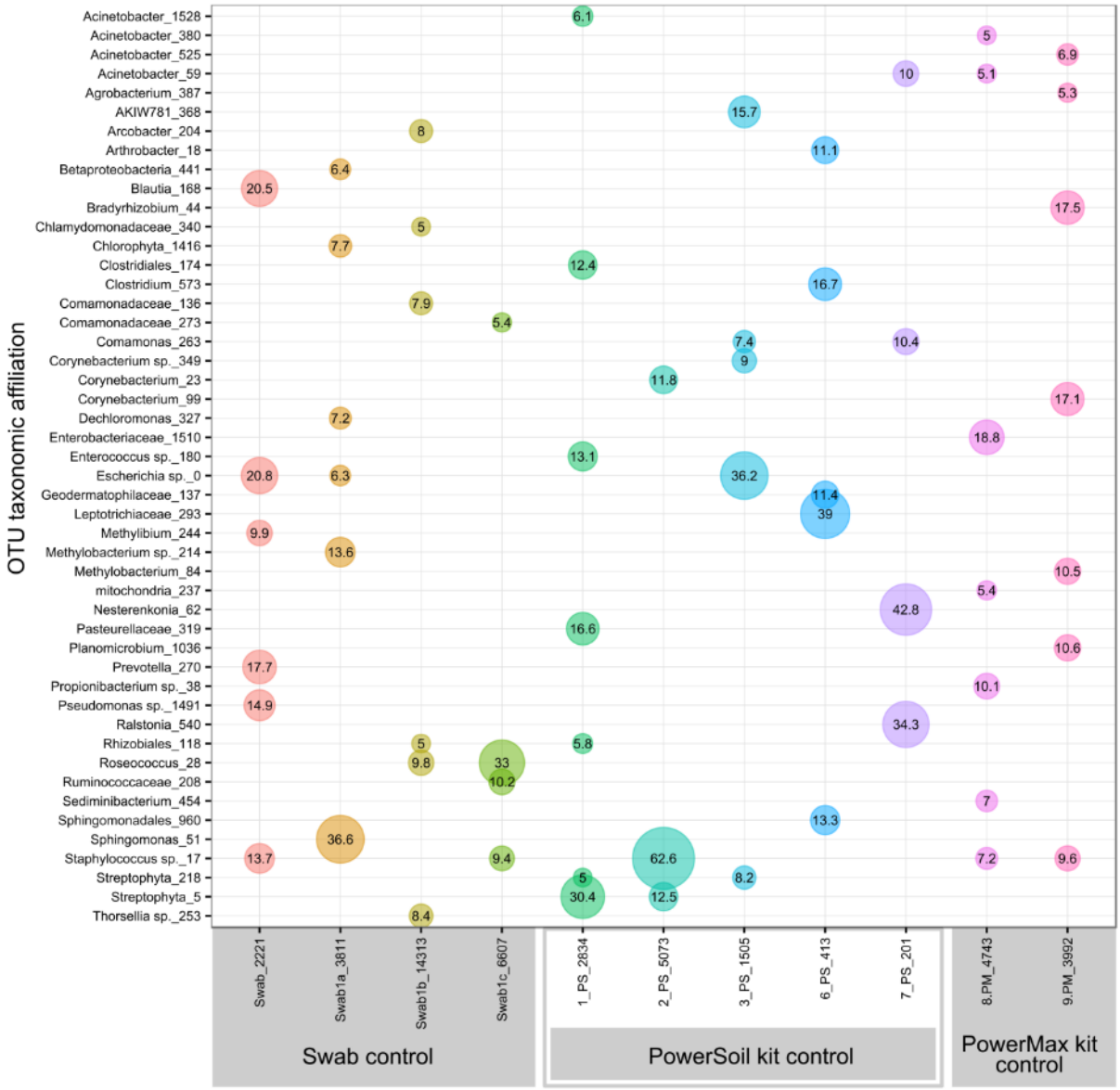

Figure 7. Bubble plot showing 16S rRNA gene profiles of swab and kit controls. This plot is based on unrarefied data and read counts are shown at the end of each sample name. OTU taxonomic affiliation is shown on the y-axis followed by an OTU number. Only OTUs at or above 5% relative abundance are shown.

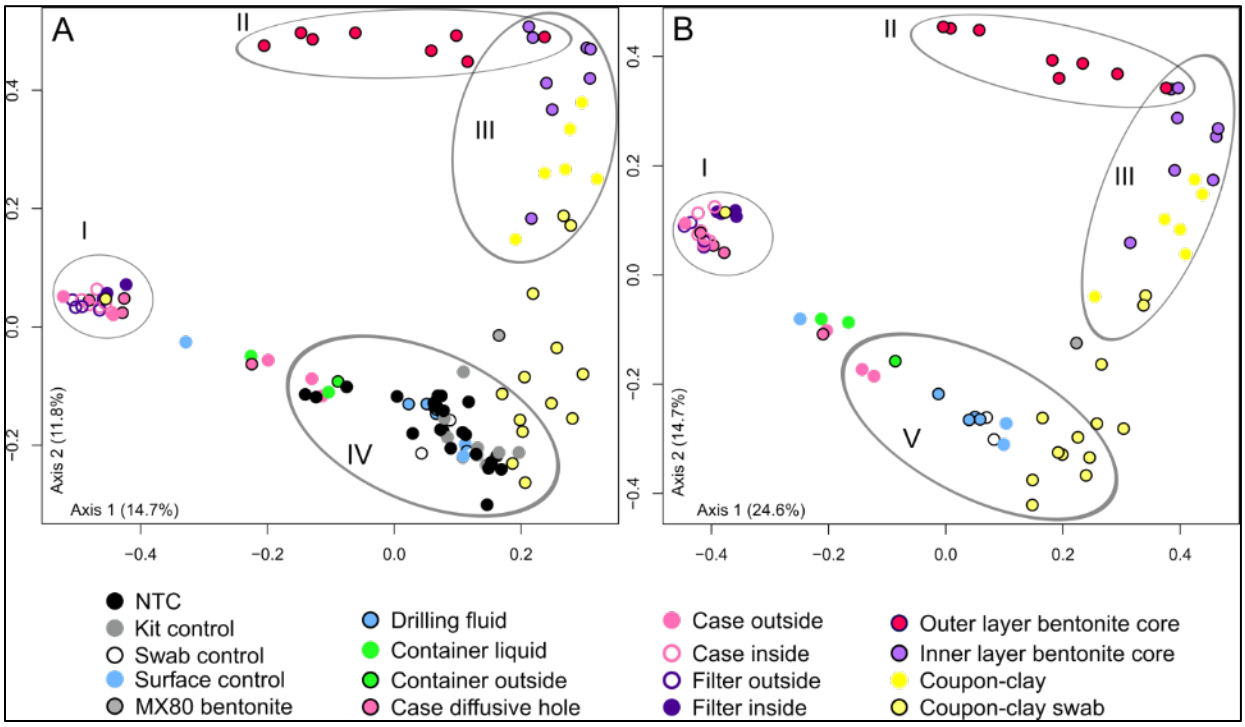

Figure 8. Grouping of samples and controls from the borehole modules in a PCoA plot based on Bray-Curtis metrics. PCoA plots were generated including (A) or excluding (B) controls (NTC and kit controls). NTCs with less than 100 reads were not included in analysis A. Samples are rarefied to the lowest sample size included in the analysis, resulting in 158 (A) or 4,475 (B) reads per sample. All kit controls and no-template controls (NTCs) are included in group IV. Groups I, II, and III separate well from control group IV. Group I includes non-clay samples (shipping flask, case, filter). Group II contains all samples from the outer layer of the bentonite core. Group III contains all samples from the inner layer of the bentonite core (coupon-clay and inner layer bentonite) but not all coupon-clay swab samples. Group V marks samples that were previously located closely to NTCs and kit controls.

3.2.2 SHIPPING FLASK AND CASE

A total of 28 samples were taken from the shipping flask, module body, and stainless steel filter from borehole modules 1a and 2a (Table 2). The DNA concentrations were below detection limit for the majority of samples (Table 3) but most yielded an amplicon in nested 16S rRNA gene PCR (Figure 3, Figure 4, and Figure 5). In samples from the shipping flask, case, and filter, a total of 550 OTUs were identified using 16S rRNA gene sequencing. Across all samples, the dominant OTU was affiliated with *Pseudomonas stutzeri* (99% nucleotide identity based on NCBI BLAST using the 16S rRNA database), a Gram-negative, motile, denitrifying bacterium. This OTU was absent from both kit controls (OTU #0 in Figure 9). The DNA extract from the shipping flask liquid of module 2a (#14) was dominated by an OTU affiliated with *Phenylobacterium*, a Gram-negative, strictly aerobic organism from the family of *Caulobacteraceae*. For module 2a, 26.7% of all reads belonged to this OTU whereas in module 1a (#4), this percentage was only 5.6% (Figure 9).

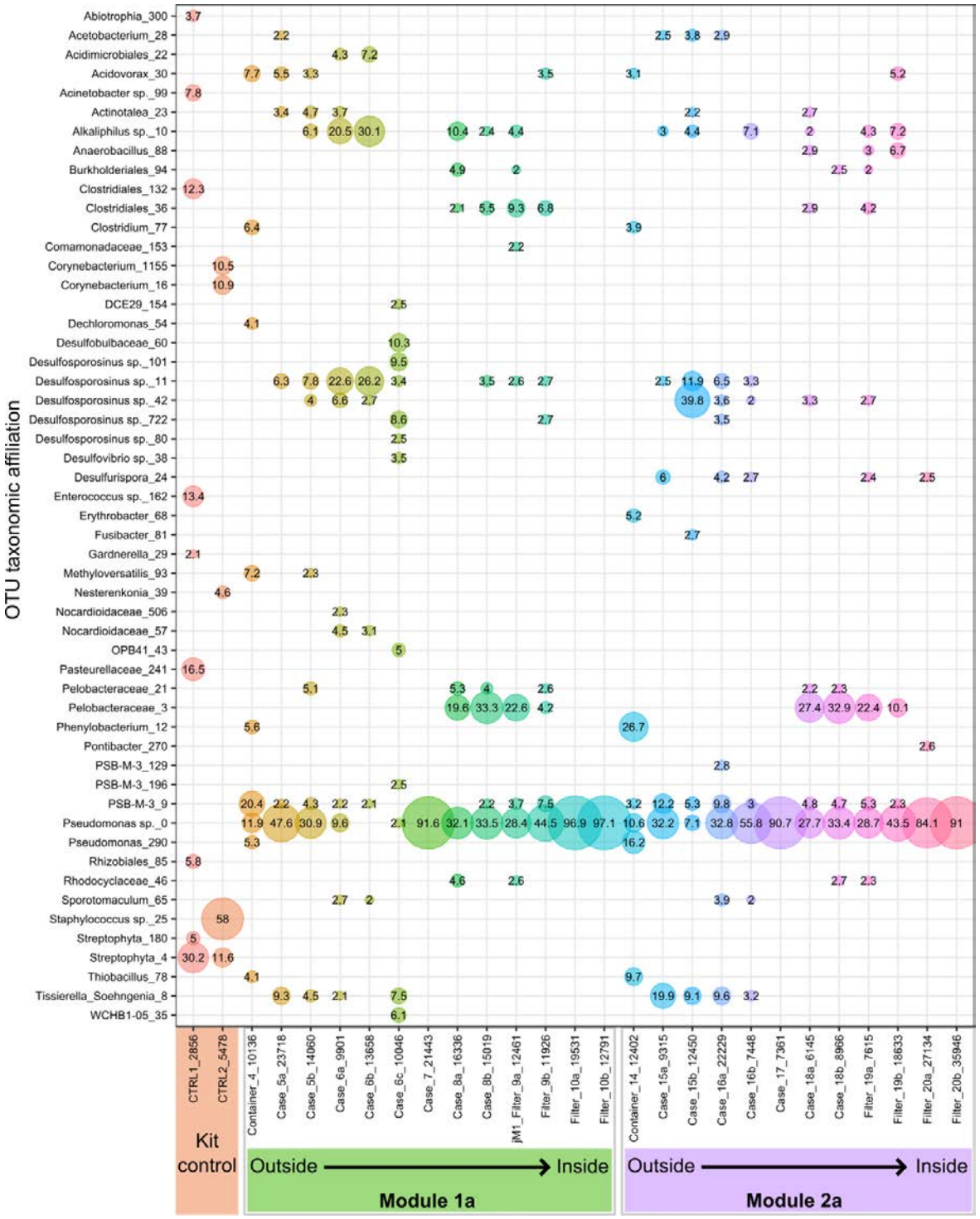

Figure 9. Bubble plot showing 16S rRNA gene profiles of module 1a and module 2a shipping flask, case, and filter samples. PowerSoil DNA Isolation kit control for module 1a (CTRL1) and module 2a (CTRL2) samples are shown. This plot is based on unrarefied data and read counts are shown at the end of each sample name. OTU taxonomic affiliation is shown on the y-axis followed by an OTU number. Only OTUs at or above 2% relative abundance are shown.

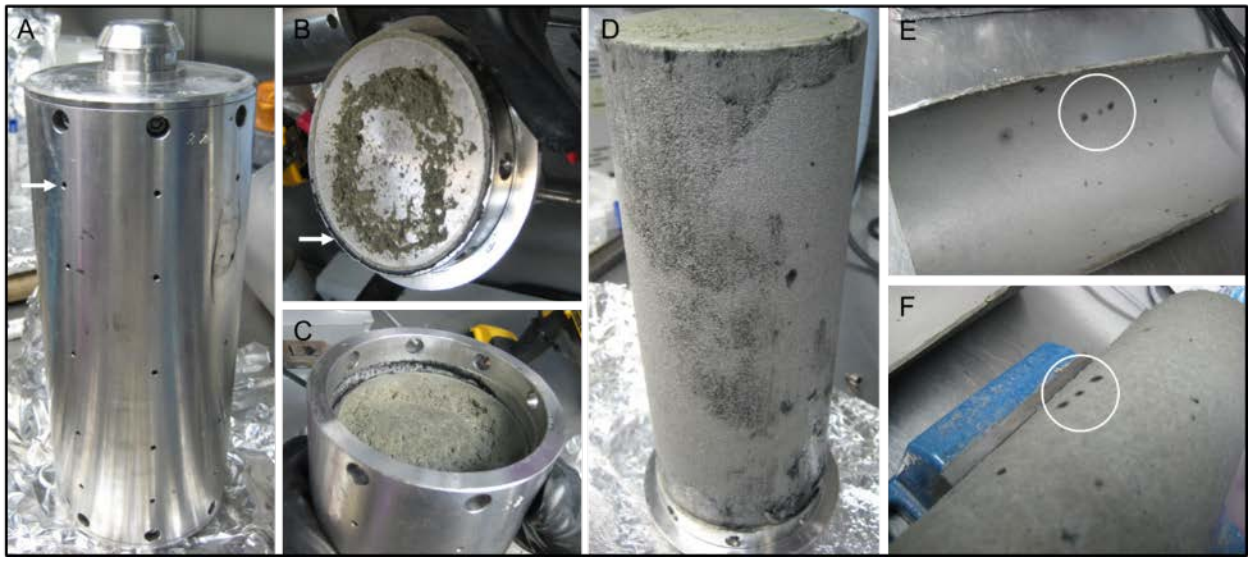

Figure 10. Borehole module and the location of black deposits. A: Metal case. White arrow points to one permeable hole. B: View of the inside surface of the case lid. White arrow points to a ring of black deposit. C: View of the open case with the filter and bentonite core inside. Black deposit can be seen at the top. D: View of the outside surface of the stainless steel filter with the bentonite core inside. Black stained areas can be seen on the filter surface. E: View of the inside surface of the stainless steel filter after the bentonite core was removed. Black spots can be seen on several locations on the surface. F: View of the outside surface of the bentonite core. Black spots can be seen on several locations. The white circle in images E and F point to the colocalization of spots on the filter and bentonite surface. The location of the black spots on the bentonite core do not colocalize with the diffusive holes in the metal case.

During the sampling of module 1a and 2a, small black spots (<0.5 cm diameter) were visible on several locations within the borehole module (i.e., on the interior and exterior of the stainless steel filter as well as on the bentonite; Figure 10). A ring of a black deposit was found on the inside of the case lid for both modules (Figure 10, image B) and DNA extraction from the black deposit yielded low but quantifiable amounts of DNA (sample #7 and #17 in Table 3). The 16S rRNA gene sequencing identified that 91 to 92% of the reads in both samples were affiliated with *Pseudomonas stutzeri* (OTU #0 in Figure 9).

Samples taken from the inside, top, and bottom region of the metal case (#8a, 8b for module 1a and #18a, 18b for module 2a) also contained black deposits that were mixed with hydrated (i.e. not compacted) bentonite (Figure 11). DNA concentrations in 3 out of 4 samples were below the detection limit but yielded a 16S rRNA gene amplicon (Figure 3). The 16S rRNA gene sequencing identified that 22 to 33% of the reads affiliated with *Pelobacteraceae* and 28 to 33% affiliated with *Pseudomonas stutzeri* (OTU #3 and #0 in Figure 9).

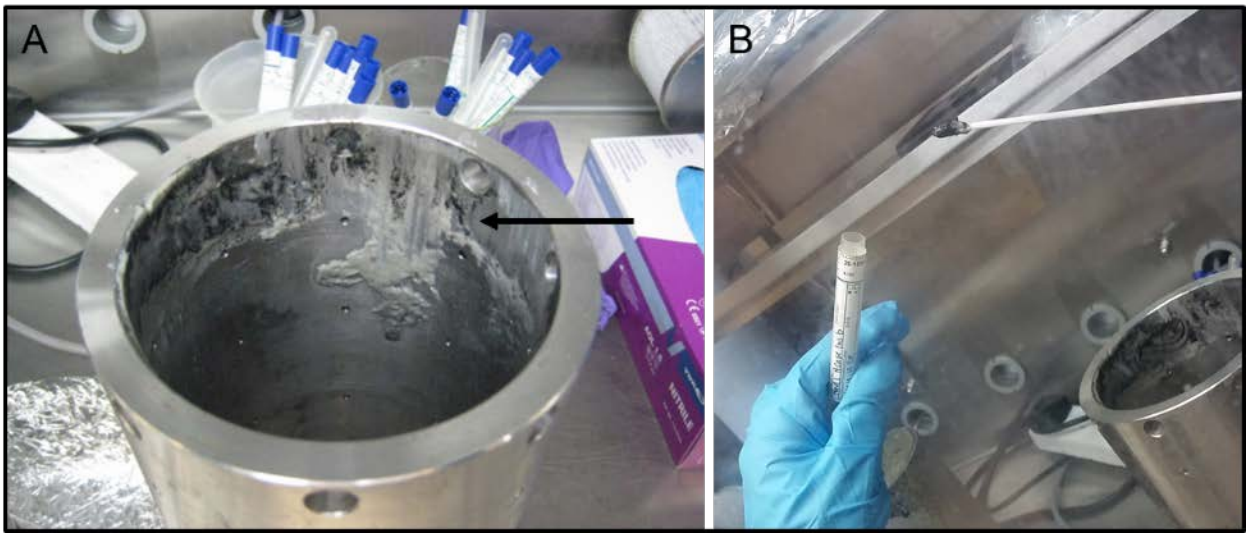

Figure 11. Sampling of the inside of the metal case. A: The top and bottom region of the inside of the metal case were decorated with hydrated bentonite (grey deposit) and a black deposit. The black arrow points to the area that was sampled using a swab (B).

Samples taken from the outside of the metal case (#5a, 5b, 6a, 6b and 6c for module 1a and 15a, 15b, 16a and 16b for module 2a) had OTUs affiliated with *Pseudomonas stutzeri* with varying relative abundance. Notably, these OTUs were less abundant than those found on the inside of the case and filter (Figure 9). Interestingly, *Desulfosporosinus meridiei* (OTUs #11 and #42 in Figure 9) was detected on the outside surface of the metal case with up to 30% and 57.1% relative abundance for module 1a (#61, 6b) and module 2a (#15b), respectively, while being essentially absent from the inside of the case and filter samples (Figure 9). Less than 0.1% of reads in bentonite core samples were affiliated with *Desulfosporosinus meridiei* (data not shown).

3.2.3 BOREHOLE FLUID

When module 1a and 2a were recovered from borehole number 13.001 in October 2015, borehole fluid was filtered through a Sterivex filter. DNA was extracted from the filter (#43) as well as one blank filter (#42) for comparison. We were able to recover 150 ng DNA from the borehole fluid filter, which is the highest amount of DNA recovered from any sample in this study (Table 3). The 16S rRNA gene PCR yielded a very strong amplicon (Figure 5). A total of 139 OTUs were identified in the borehole fluid and the most abundant OTUs were affiliated with *Desulfosporosinus meridiei*, *Syntrophus*, and *Desulfovibrio mexicanus* (OTUs #5, #2 and #3 in Figure 12).

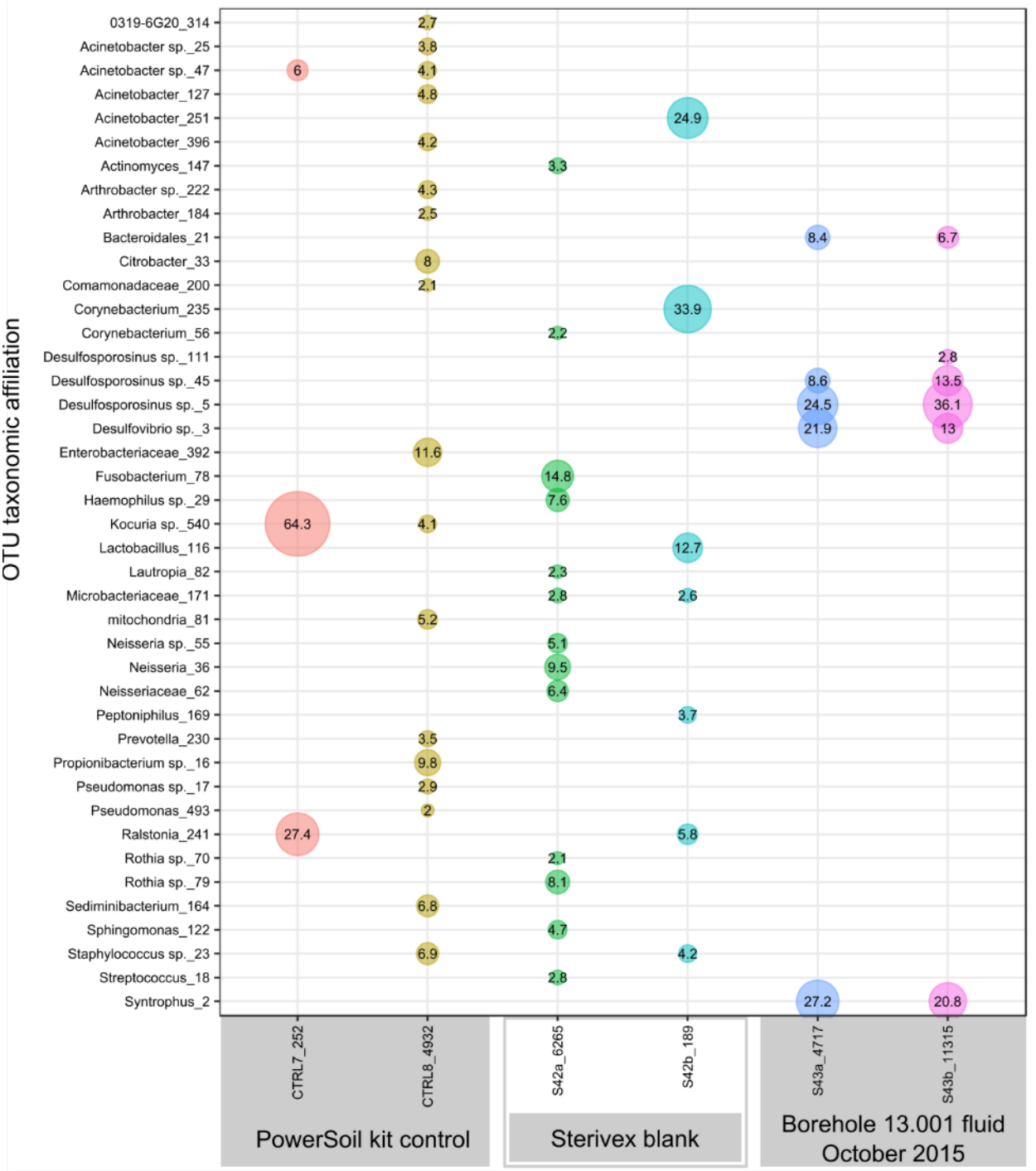

Figure 12. Bubble plot showing 16S rRNA gene profiles of borehole fluid. Results of two replicate extractions and kit controls using the PowerSoil DNA Isolation Kit are shown. The plot is based on unrarefied data and read counts for each sample are shown at the end of the name. OTU taxonomic affiliation is shown on the y-axis followed by an OTU number. Only OTUs at and above 2% relative abundance are shown.

3.2.4 INNER AND OUTER LAYER BENTONITE

Genomic DNA from bentonite was extracted using both PowerSoil and PowerMax DNA Isolation kits (Table 3). Only the PowerMax kit yielded DNA extracts that could be quantified using Qubit dsDNA High Sensitivity assay but concentrations were very low (Table 3). PCR analysis confirmed low DNA yield from 0.2 gm of bentonite using the PowerSoil DNA Isolation kit. Only 3 out of 8 samples yielded PCR bands which were faint and required a nested PCR protocol (27, 28, and 37 in Figure 5). The 16S rRNA gene sequencing showed very different taxonomic profiles for samples extracted with PowerSoil and PowerMax kits (Figure 13).
From 409 unique OTUs identified in bentonite core samples, only 30 OTUs were at or above 1% relative abundance. Those 30 OTUs represent 80.7 to 98.9% of total reads in each sample. The majority of OTUs were shared across samples (Figure 14) and the number of OTUs present in the inner layers of module 1a and 2a did not differ substantially (Table 5). The number of abundant OTUs (at or above 1%) was very similar for both modules and both compartments. The dominant OTUs across all bentonite core samples were affiliated with *Streptomyces*, *Pseudomonas stutzeri*, and *Promicromonospora* (OTUs #1, #0 and #5 in Figure 14).

A mixture of pellets and powdered Wyoming MX-80 bentonite was used to pack the MaCoTe modules before placing into the borehole. We analyzed samples of this MX-80 that were stored in plastic containers at room temperature until DNA extraction using the PowerMax DNA Isolation Kit. The dominant OTUs in both the powdered and pelleted bentonite, were affiliated with *Streptomycetaceae* and *Xanthomonadaceae* (OTUs #0 and #2 in Figure 15). The majority of the dominant OTUs identified in the inner layer of the bentonite core were also found in the original MX-80 sample. Only 0.8 to 3.0% of reads in the original MX-80 samples were associated with *Pseudomonas stutzeri* (data not shown).

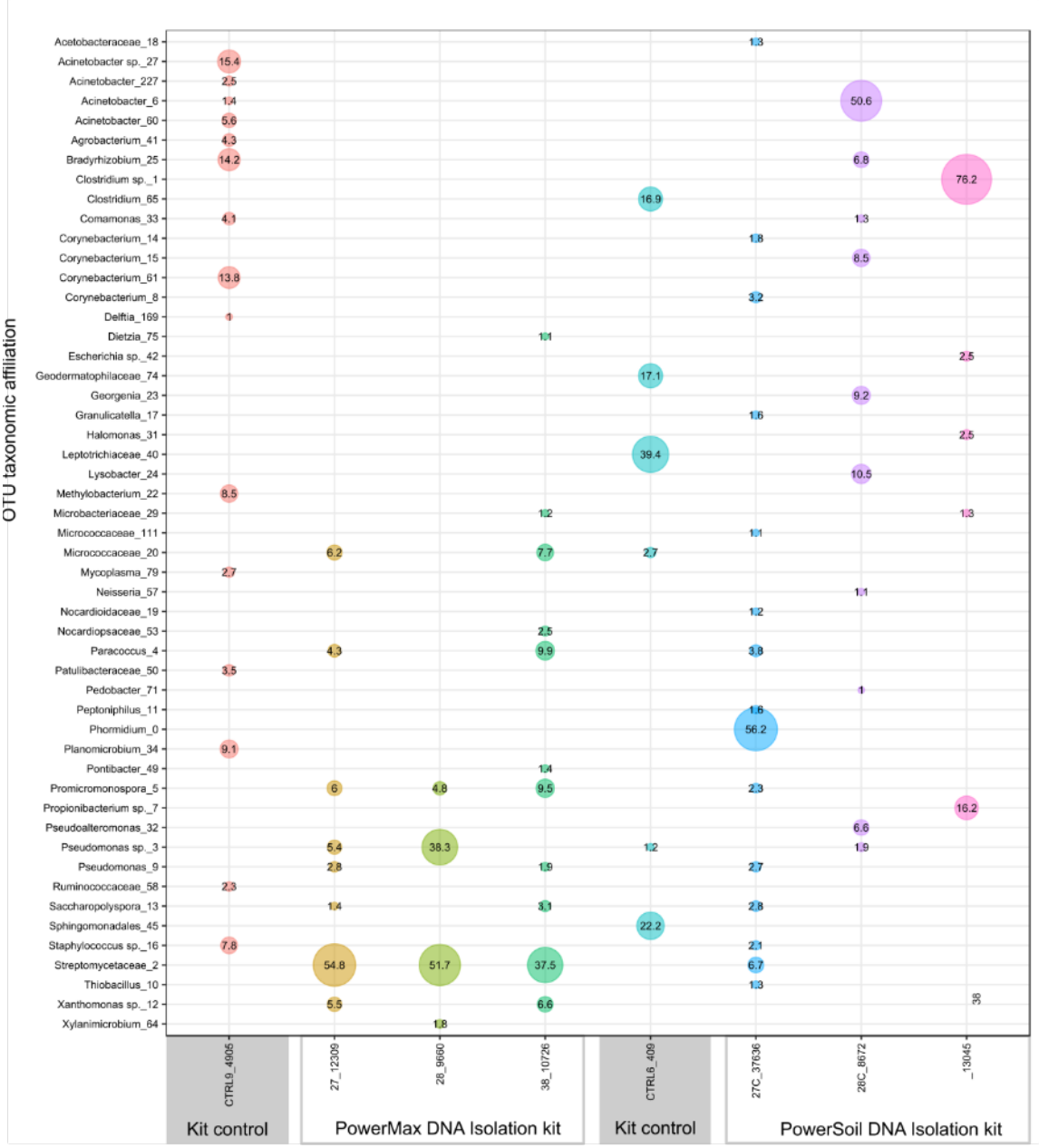

Figure 13. Bubble plot showing 16S rRNA gene profiles of bentonite DNA extracted using PowerMax or PowerSoil DNA Isolation Kit. Kit controls (CTRL) are shown alongside bentonite from module 1a (M1) and module 2a (M2). The plot is based on unrarefied data and read counts for each sample are shown at the end of the name. OTU taxonomic affiliation is shown on the y-axis followed by an OTU number. Only OTUs at and above 1% relative abundance are shown.

Table 5. OTU counts of bentonite core samples. Data are rarefied to 4,465 reads per sample. OTU counts are shown for full dataset and for OTUs at or above 1% relative abundance.

Bentonite	n	Average OTU count	SD	Average OTU count (≥1%)	SD
Module 1a outer layer	4	72	17	21	1
Module 1a inner layer	4	118	38	23	2
Module 2a outer layer	4	131	15	24	2
Module 2a inner layer	4	111	32	24	3

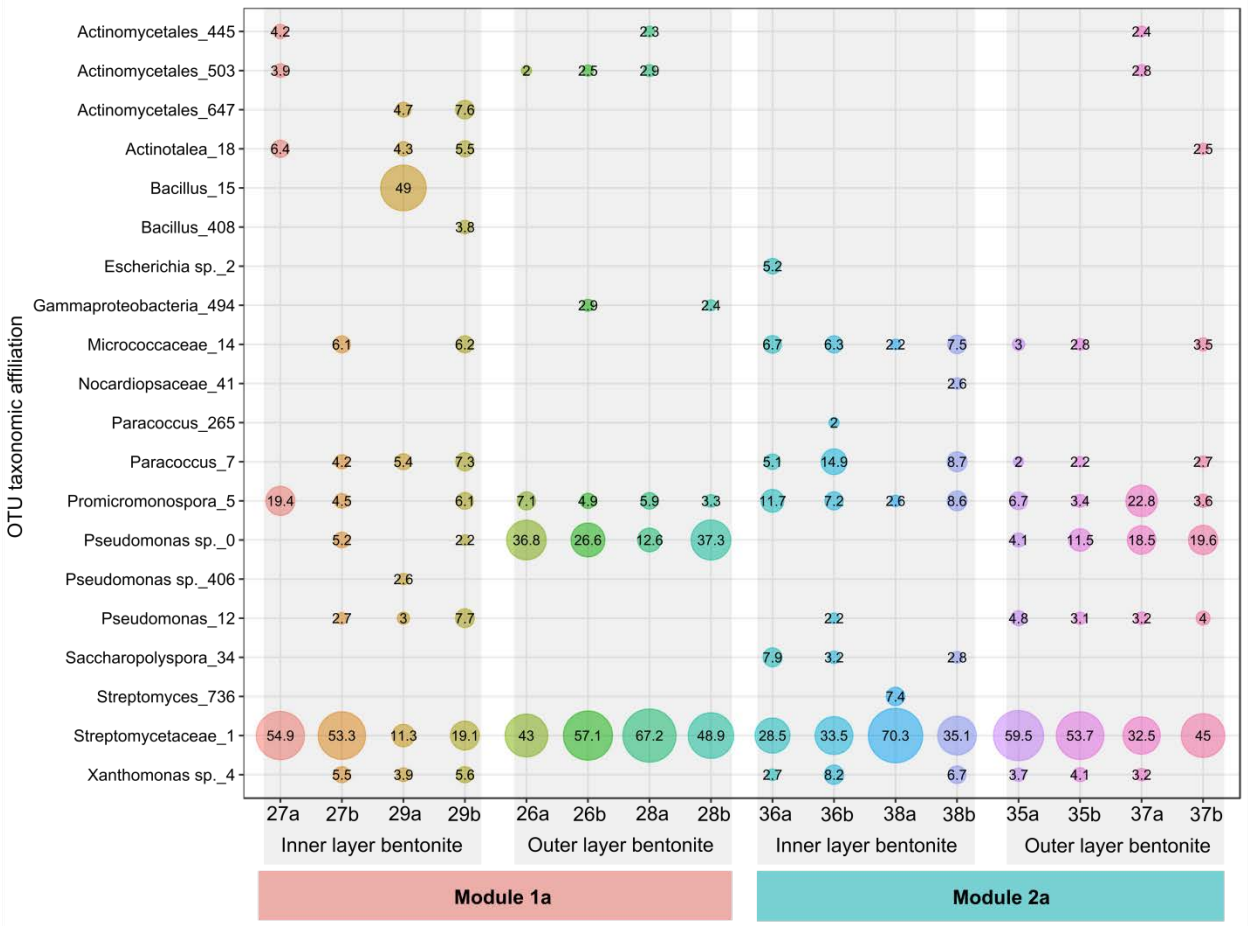

Figure 14. Bubble plot showing 16S rRNA gene profiles of inner and outer layer bentonite from module 1a and module 2a. Results of two replicate extractions using the PowerMax DNA Isolation Kit are shown for each sample. The plot is based on rarefied data (4,465 sequences per sample). OTU taxonomic affiliation is shown on the y-axis followed by an OTU number. Only OTUs at and above 2% relative abundance are shown.

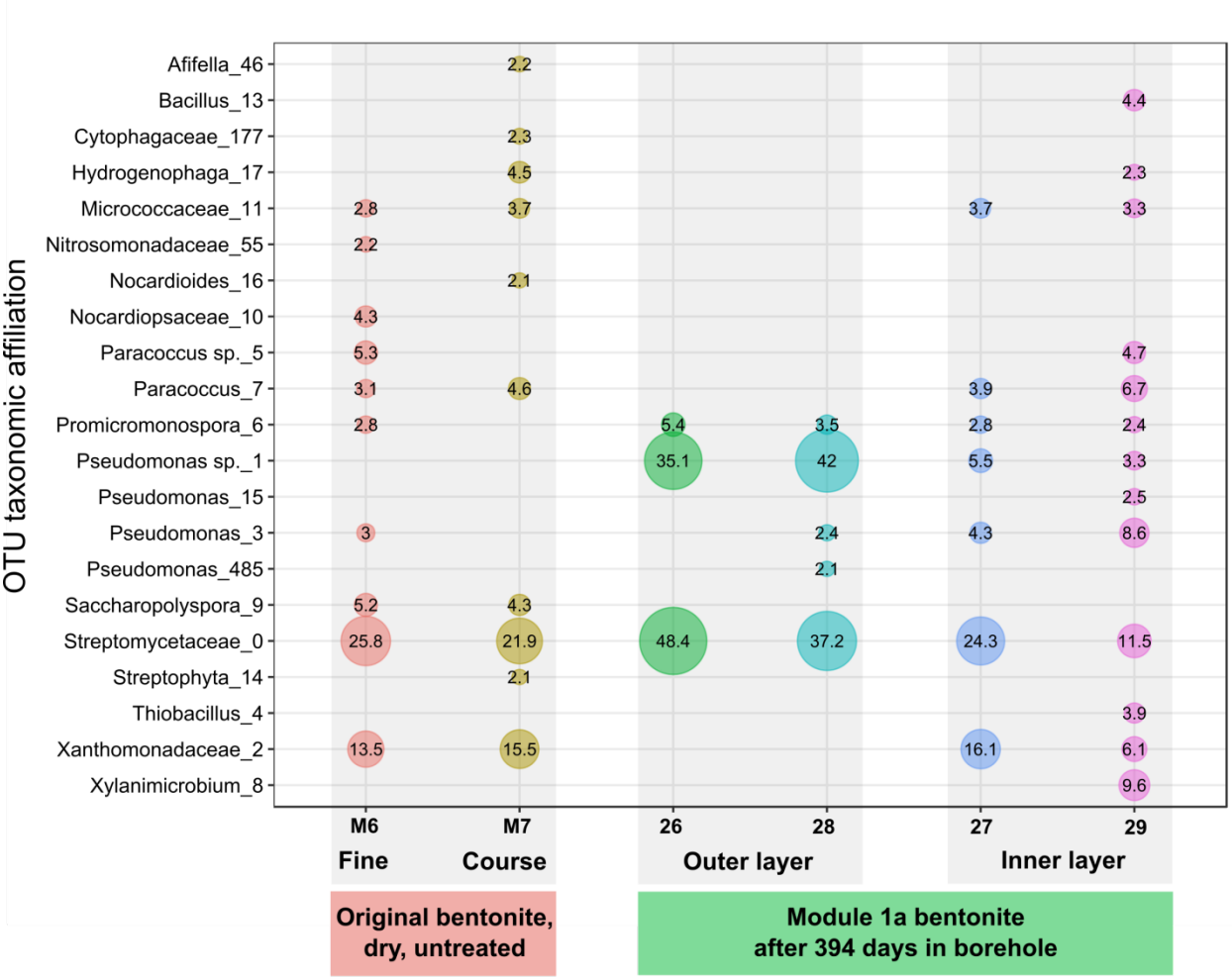

Figure 15. Bubble plot showing 16S rRNA gene profiles of bentonite before and after 394 days of storage in the borehole. A mixture of pellets (course) and powdered (fine) bentonite was used to pack borehole modules 1a and 2a. After assembly of the modules, the bentonite was fully saturated using deionised water and placed in borehole 13.001 at the URL in Switzerland for 394 days. Results of one replicate extraction using the PowerMax DNA Isolation Kit are shown. The plot is based on rarefied data (9,846 sequences per sample). OTU taxonomic affiliation is shown on the y-axis followed by an OTU number. Only OTUs at and above 2% relative abundance are shown.

3.2.5 BENTONITE FROM COUPON-CLAY

Genomic DNA from coupon-clay imprints was extracted in two ways. Firstly, the surface of the coupon imprint was swabbed using a sterile DNA-free foam. Secondly, approximately 1 to 2 mm thick clay surrounding the imprint was removed using a sterile DNA-free single-use razor blade. None of the extracts yielded DNA that could be quantified using Qubit dsDNA High Sensitivity assay (Table 3) and 16S rRNA gene PCR did not yield an amplicon for the majority of samples (Figure 4). The 16S rRNA gene sequencing showed very different taxonomic profiles for coupon-clay swabs (Figure 16) and no common OTU was identified except OTUs affiliated with *Escherichia coli*, which is likely a PCR contaminant (OTU #2 in Figure 7, PowerSoil kit control #3). OTUs affiliated with *Streptomyces* were identified in all coupon-clay samples but not in all coupon-clay swabs (OTU #1 in Figure 16).

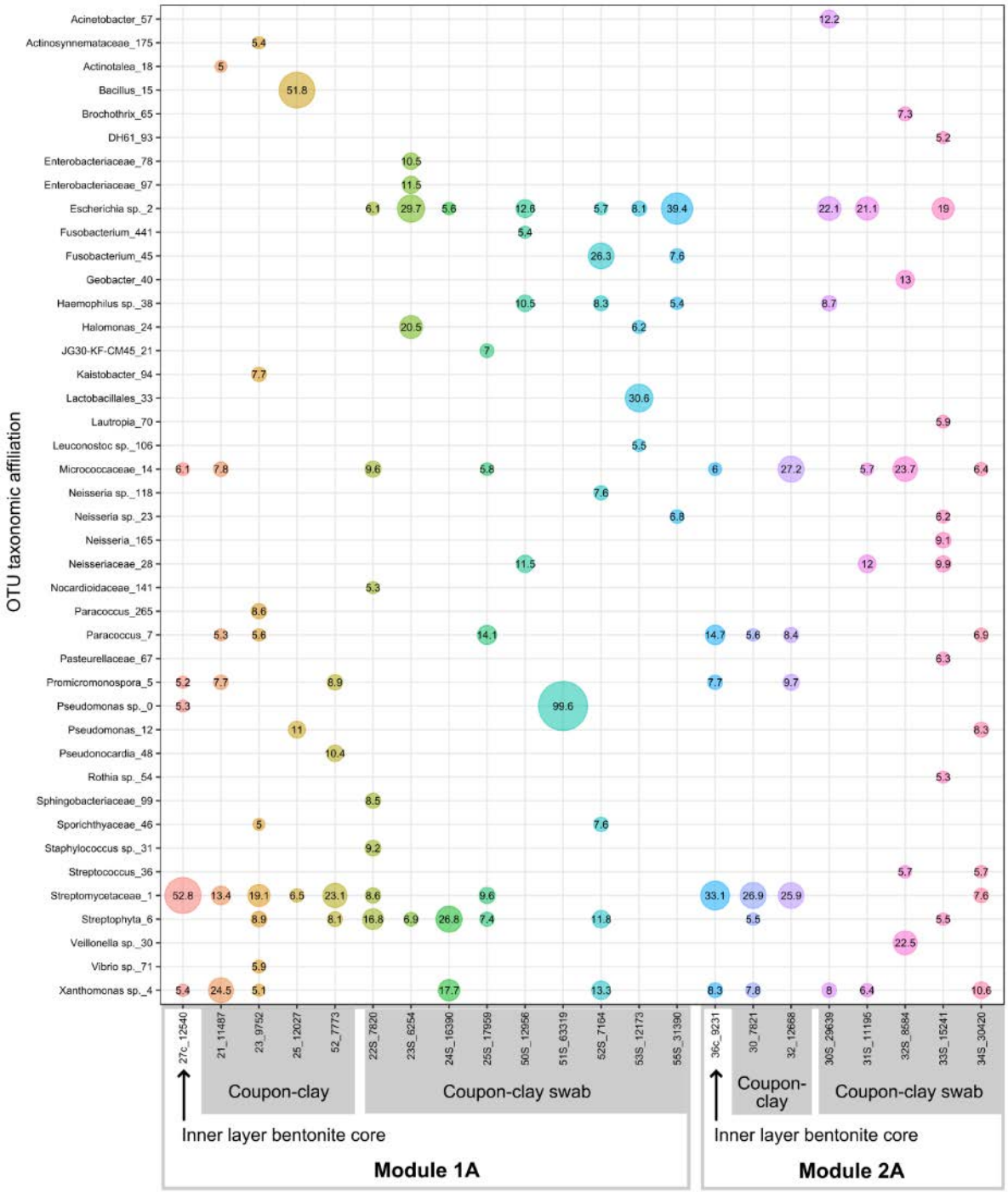

Figure 16. Bubble plot showing 16S rRNA gene profiles of coupon-clay samples from module 1a and 2a. DNA was extracted from swabs of the coupon-clay surface or directly from coupon-clay using the PowerSoil DNA Isolation kit. For comparison, the 16S rRNA gene profiles of inner layer bentonite from module 1a and 2a are shown. The plot is based on unrarefied data and read counts for each sample are shown at the end of the name. OTU taxonomic affiliation is shown on the y-axis followed by an OTU number. Only OTUs at and above 5% relative abundance are shown.

3.2.6 THE BOREHOLE MODULES FROM THE OUTSIDE TO THE INSIDE

Various samples were taken from borehole liquid, shipping flask, and borehole modules after 394 days of storage at the Grimsel Test Facility in Switzerland. The 16S rRNA gene sequencing showed distinct grouping (Figure 17) and different taxonomic profiles (Figure 18) when moving from the most outer to the most inner samples of the borehole modules. Each PCoA ordination was rarefied to the smallest sample size (smallest read count) included in the analysis, resulting in only 190 and 244 reads per sample when kit controls were included (panel A and C in Figure 17). Both plots show that bentonite core samples separate well from the kit controls. As mentioned previously, to investigate the effect of low sequence counts on the grouping of samples, we also removed controls from the analysis, increasing the number of reads per sample to 4,463 (panel B) and 4,451 (panel D). The grouping of samples changed but the overall pattern was similar (Figure 17). The PCoA plots showed that bacterial communities in the bentonite core differ from all case and filter samples as well as borehole fluid (panel D in Figure 17). Samples with more than 84% of reads affiliated with *Pseudomonas stutzeri* are contained within group IIb (#7, #10a, #10b, #17, #20a and #20b). Samples with more than 30% of reads affiliated with *Desulfosporosinus meridiei* (#6a, 6b and 15b) group close to the borehole fluid. From an original set of 832 OTUs, only 34 were at or above 5% relative abundance in at least one of the borehole module samples (Figure 18). Nevertheless, those 34 OTUs represented 70.7 to 98.5% of total reads in each sample. We generated functional profiles for those abundant OTUs using FAPROTAX, an automated annotation tool and database. FAPROTAX assigns each taxon to a particular metabolic function based on evidence from cultured microorganisms. FAPROTAX assigned 18 out of 34 of these abundant OTUs (52.9%) to at least one functional group (Figure 19). The borehole fluid was dominated by an OTU affiliated with *Desulfosporosinus meridiei* (OTU #37 in Figure 18) and FAPROTAX assigned this OTU to functional categories 8, 14 and 27 to 31 (Figure 18) based on peer-reviewed literature (Whitman, 2009). OTUs without a functional assignment were highlighted in Figure 18 and were grouped into a category called “other” in Figure 19. This category shows how well a sample is represented in the FAPROTAX analysis i.e. 97.1% of all OTUs in the borehole fluid (#43) could be assigned to a function but only 70-89% of inner bentonite (#29). The borehole fluid was also dominated by OTUs affiliated with *Desulfovibrio mexicanus* (assigned to categories 9, 27, and 28 in Figure 19), which are known as sulfate-reducing bacteria. Also abundant were OTUs affiliated with *Syntrophus* (assigned to categories 3 and 8 in Figure 19), which often grow in a syntrophic association with hydrogen/formate-utilizing partners. The relative abundance and potential activity of all three abundant OTUs reduced greatly when moving towards the inside of the borehole module and were almost absent from the inner layer of bentonite (Figure 18 and Figure 19).

Pseudomonas stutzeri (assigned to categories 1 to 3, 7, 11, 18 to 20, and 22 to 26 in Figure 19) was the dominant organism in the case and filter samples and is a putative denitrifying bacterium. Its relative abundance and potential activity reduces when moving towards the inside of the borehole module (Figure 18, Figure 19).

Streptomyces (assigned to categories 1 and 3 in Figure 19) was the dominant organism found in the bentonite core samples, presumably in the form of metabolically inactive spores or extracellular relic DNA. As an aerobic organism it would likely not be able to actively grow in the anoxic repository.

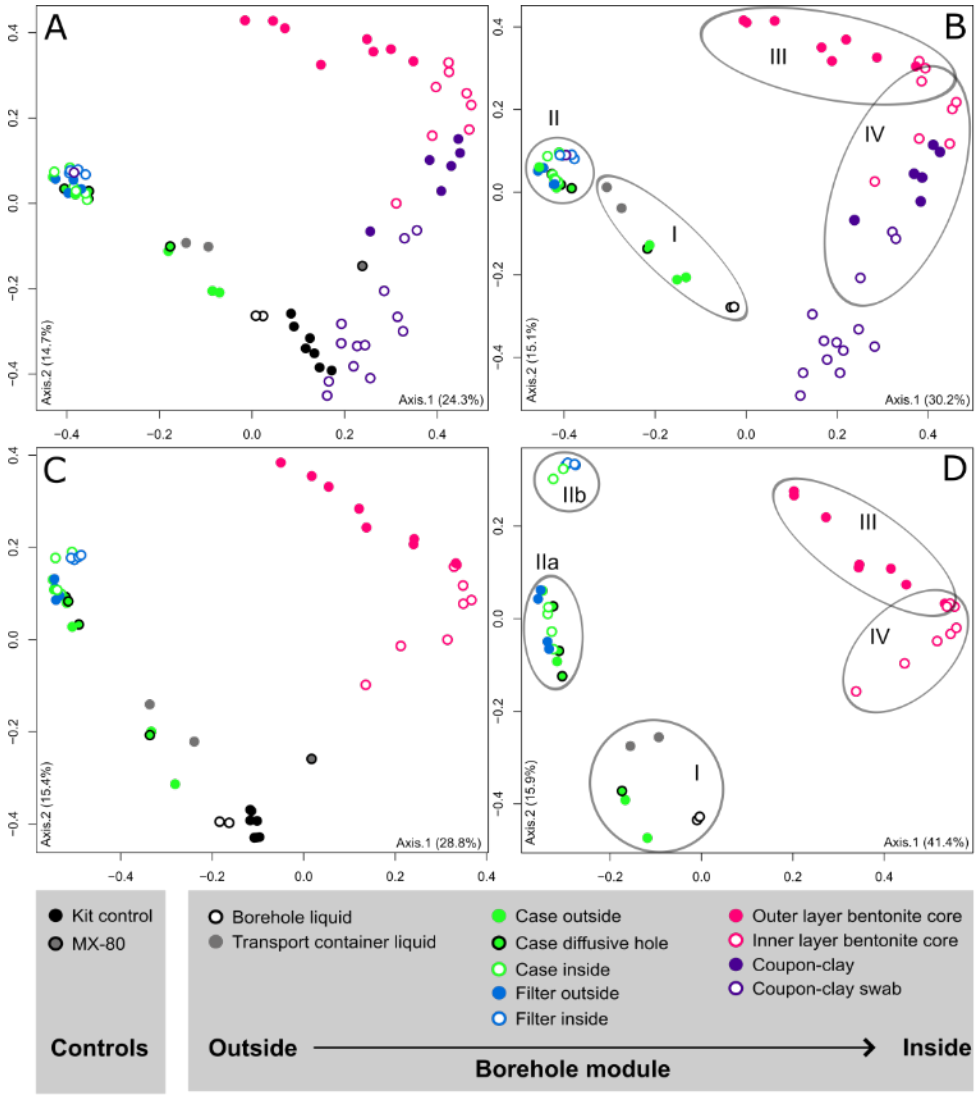

Figure 17. Grouping of borehole module samples in a PCoA ordination based on Bray-Curtis metrics. PCoA plots were generated including (top) or excluding (bottom) coupon-clay samples as well as including (left) or excluding (right) kit controls. Samples were rarefied to the smallest sample size included in the PCoA analysis, resulting in 190 (A), 4,463 (B), 244 (C), or 4,451 (D) reads per sample. Borehole and shipping flask liquid samples are contained in group I. Groups II, IIa, and IIb contain the majority of the case and filter samples and are well separated from all outer (group III) and inner (group IV) bentonite core samples.

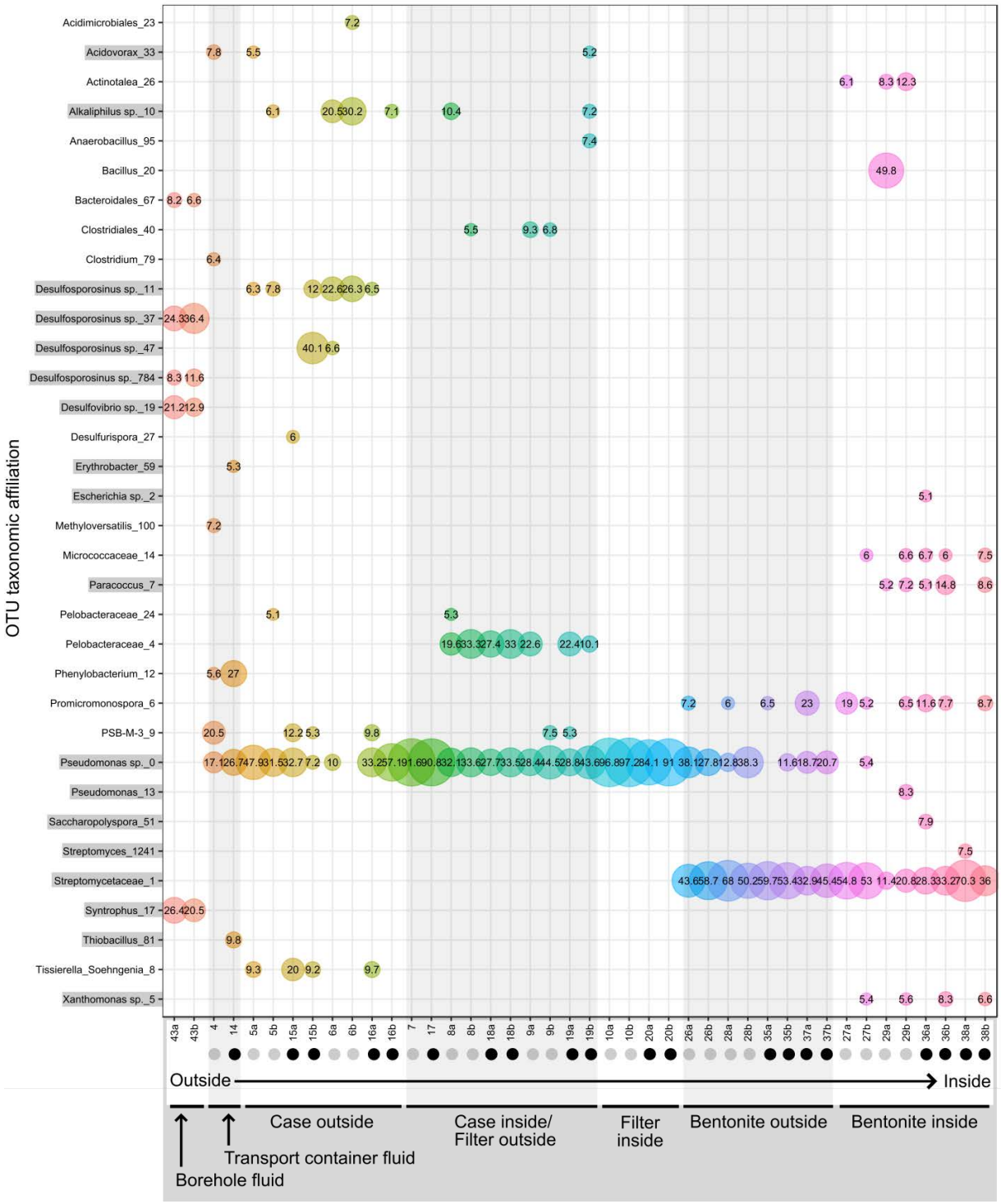

Figure 18. Bubble plot showing 16S rRNA gene profiles of borehole modules. Samples taken from module 1a (●) and 2a (●) are sorted from the most outer to the most inner location within the module. Results of two replicate extractions using the PowerSoil or PowerMax DNA Isolation Kit are shown. OTUs highlighted in grey were assigned to a potential function in the FAPROTAX analysis below. OTU taxonomic affiliation is shown on the y-axis followed by an OTU number. Only OTUs at and above 5% relative abundance are shown.

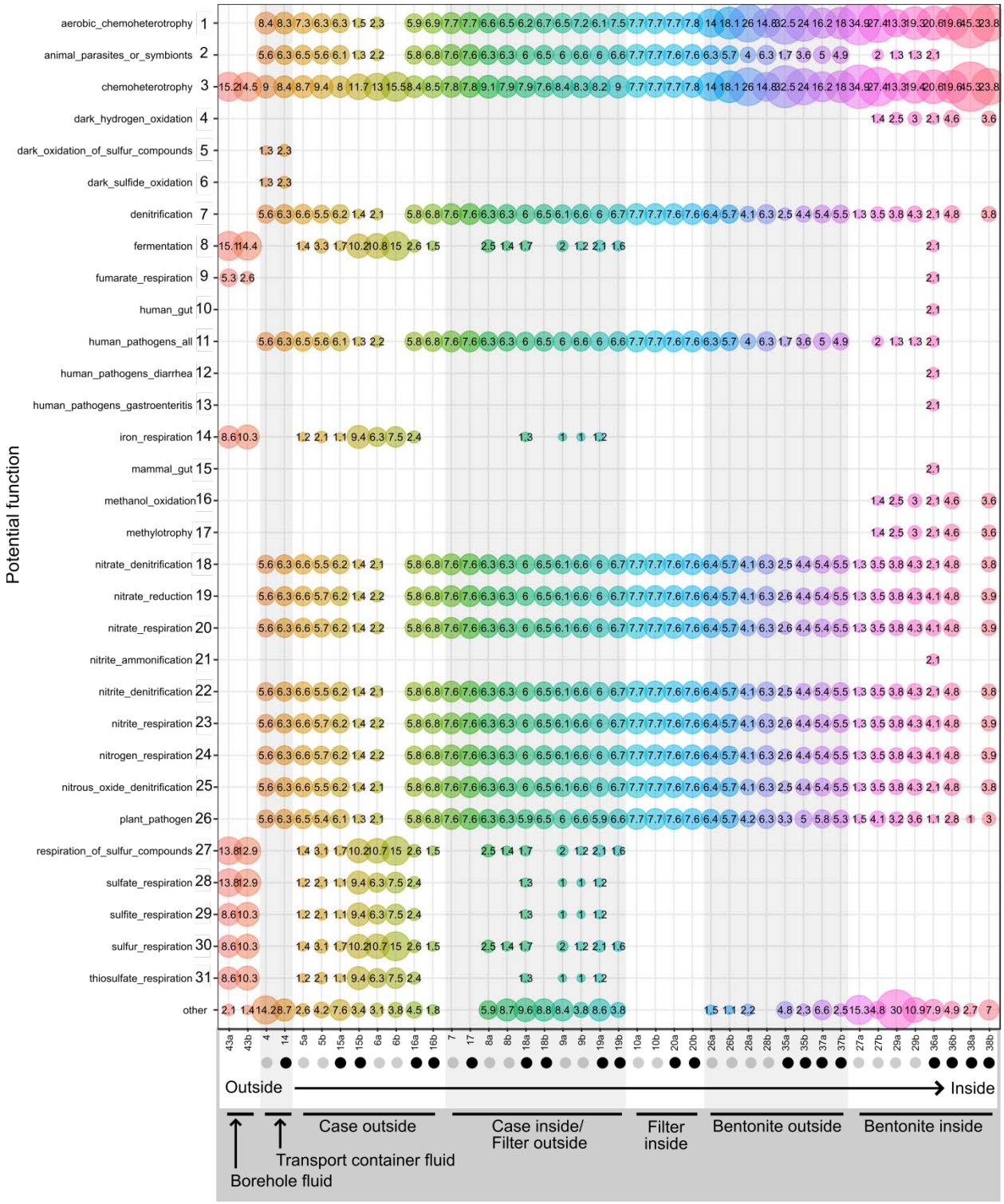

Figure 19. Bubble plot showing functional profiles of borehole modules. Samples taken from module 1a (●) and 2a (●) are sorted from the most outer to the most inner location within the module. Results of two replicate extractions using the PowerSoil or PowerMax DNA Isolation Kit are shown.

3.3 PLFA RESULTS

Bentonite samples from module 1a and module 2a were analyzed for PLFA abundances and distributions (Table 6). Concurrently, two replicates of Volclay MX-80 were analyzed. Note that this Volclay MX-80 sample is not from the same manufacturing date as the bentonite used in the borehole module.

3.3.1 PLFA CONCENTRATIONS AND CELL ABUNDANCE ESTIMATES

Total PLFA concentrations for all samples were similar (Table 6). PLFA concentrations for borehole module samples ranged from 19 to 38 ng/gm, with a mean of 24 +/- 7 ng/gm. These PLFA concentrations resulted in estimates of cell abundances of 1 to 3 × 10^6 cells/gm (Table 6) based on generic conversion factors (Green and Scow, 2000). These PLFA concentrations were above the level observed for combusted bentonite process blank analyses (4.9 +/- 1 ng/gm: n=2). All PLFA concentrations were below the 280 ng/gm concentrations of C14 to C20 alkanoic acids observed in MX-80 analyzed by Marshall *et al.* 2015.

Small variations in the cell abundances were observed within the module 1a or 2a samples. For module 1a, the highest cell abundance was in the outer layer bentonite. The two inner layer bentonite samples showed very similar cell abundances. For module 2a, the inner layer S5 sample had the highest cell abundance; however, variations within the module were very minor.

Table 6. PLFA concentrations and estimated cellular abundances based on generic conversion factor (Green and Scow, 2000). NA = not applicable.

	Mass of sample	[PLFA] ng/gm	Total pmol	[PLFA] pmol/gm	Cells/gm
Process Blank	NA	NA	463	NA	NA
Combusted MX-80 (Lot 06525768)	51 +/- 0.7	4.9 +/- 1	1016	20 +/- 4	4E+05
MX-80 (Lot 06525768)	51 +/- 0.6	78 +/- 21	16493	323 +/- 62	6E+06
Module 1a					
Outer layer	39.8	37.9	1.51	140	3E+06
Inner layer	50.1	21.9	1.10	81	2E+06
Inner layer Section 5	51.8	23.5	1.22	86	2E+06
Module 2a					
Outer layer	50.1	17.1	0.86	63	1E+06
Inner layer	51.5	19.4	1.00	70	1E+06
Inner layer Section 5	50.8	22.2	1.13	80	2E+06

3.3.2 PLFA DISTRIBUTIONS

PLFA distributions were very distinct between the Volclay MX-80 and the bentonite from module 1a and 2a. (Figure 20). The aliquots of Volclay MX-80 had 20 PLFA identified with a wide diversity of chemical structures. Many of these PLFA were present at relatively low (<5%) molar percentages. Such a diversity of PLFA would often be assumed to indicate a relatively diverse microbial community. Combusting the MX-80 sample for four hours at 450°C resulted in the detection of saturated PLFAs only. The PLFA are the most recalcitrant and often the most abundant which may account for their presence at low concentrations in these samples.

In contrast, there were approximately half as many individual PLFA present in the borehole bentonite samples. This decreased diversity of PLFA would be consistent with a reduced diversity of the microbial community in the borehole samples. This is consistent with the relatively simple community structures observed based on the DNA analysis. The PLFA present in the borehole samples were generally similar (Figure 20). PLFA distributions for all six samples were dominated by saturated (54 +/- 10%) and branched (25 +/- 3%) PLFA, followed by monounsaturated (14 +/- 4%) and one polyunsaturated PLFA (C18:2ω9,12: 8 +/- 1%). Cyclopropyl PLFA were also observed in the Volclay MX-80 sample and were only identified in one of the borehole samples (module 1a, outer layer). There was some variation in PLFA distributions between both modules because two PLFA (C15:0 and isoC:17:0) were only present in samples from module 1a but were absent from module 2a samples. These differences in PLFA distributions are indicative of differences in the microbial community between these two sample sets. PLFA distributions were consistent across sample sets for a given module with the exception of the presence of cycloC17:0, which was only present in the outer layer of the module 1a sample.

Notably, iso- and anteiso- C15:0 PLFA were present in bentonite core samples, which are often associated with sulfate-reducing bacteria (Chang *et al.*, 2001). OTUs associated with sulfate-reducing organisms were identified in borehole liquid using 16S rRNA gene analysis; however, they were almost absent from the inner layer of bentonite. The presence of C18:2ω9,12 was indicative of the presence of fungi (Frostegård *et al.*, 1991) although the presence of fungi should be confirmed via future DNA-based approaches.

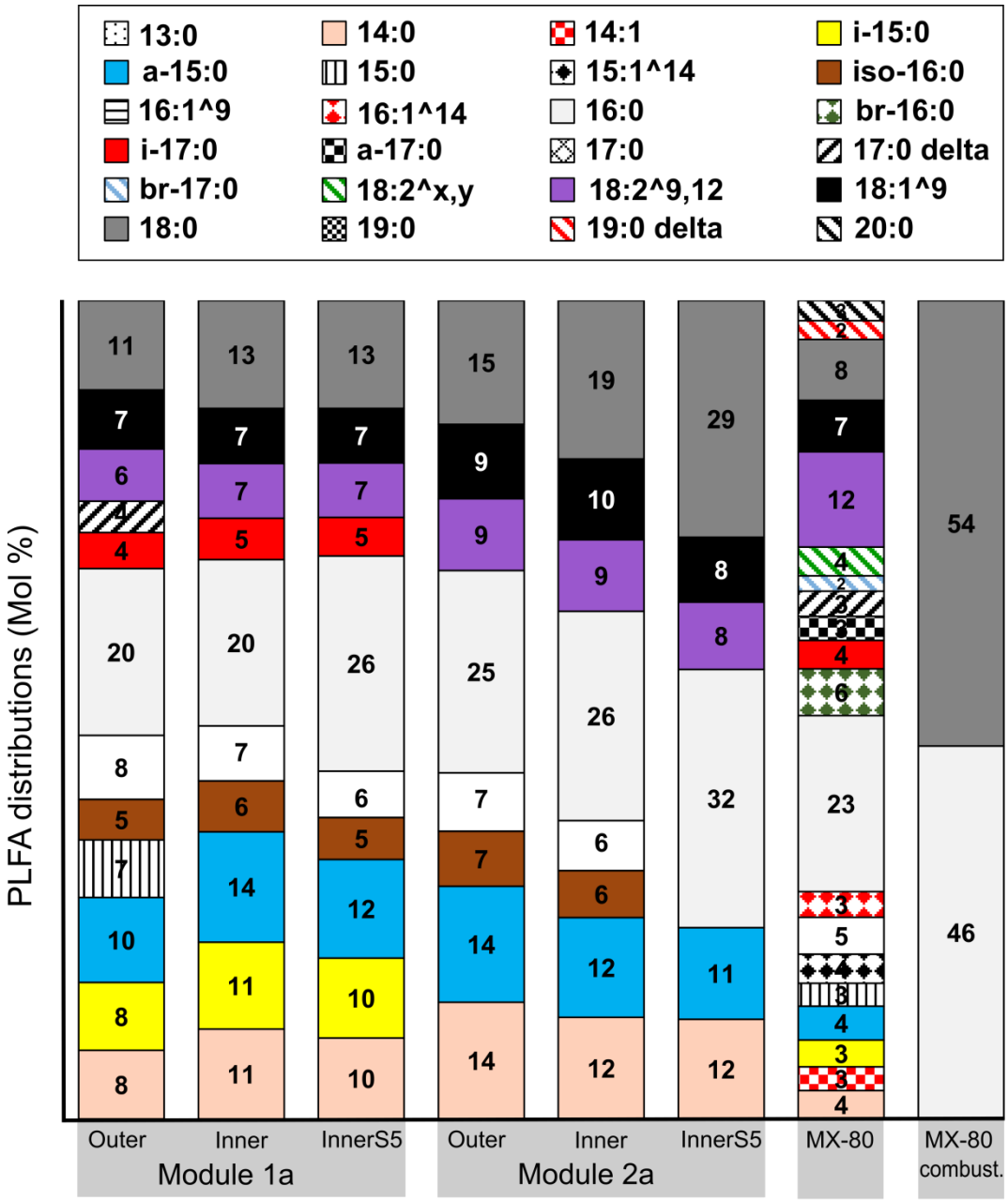

Figure 20. PLFA distributions as mole percentages for borehole module samples. Bentonite from the outside (Outer) and inside (Inner) of the bentonite core of borehole module 1a and 2a were analyzed. Section 5 of the bentonite core (InnerS5) did not contain metal coupons and was sampled and analyzed separately. A Volclay MX-80 sample was used to validate the method, but it was not from the same bentonite lot used for the MaCoTe borehole modules. A combusted bentonite sample (MX-80 combust.) was analyzed as well.

3.4 EFFECT OF BENTONITE DENSITY ON THE MICROBIAL COMMUNITY

Bentonite was compacted in borehole modules 1a and 2a, targeting 1.25 and 1.50 gm/cm^3 dry density, respectively. As mentioned previously, the number of OTUs present in the inner layer of the high density bentonite module did not differ significantly from the low density module (Table 5). The 16S rRNA gene sequencing showed tight grouping of replicate samples, especially for the outer layer bentonite samples (Figure 21). Inner layer bentonite samples from both borehole modules did not form distinct groups from each other. However, there was some variation in PLFA distributions between modules 1a and 2a that are indicative of differences in the microbial community between these two borehole modules (Figure 20).

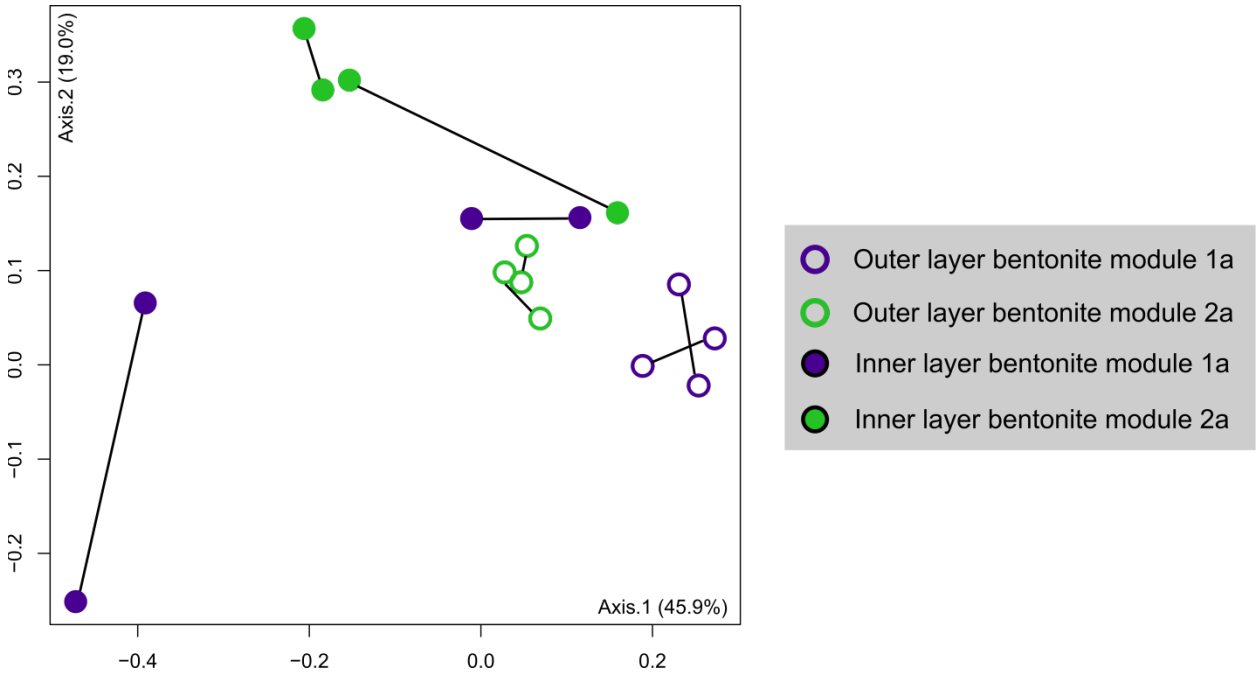

Figure 21. Grouping of inner and outer layer bentonite samples from the borehole modules 1a and 2a in a PCoA ordination based on Bray-Curtis metrics. Bentonite was compacted targeting 1.25 (1a) and 1.50 (2a) gm/cm^3 dry density. Samples were rarefied to 4,490 reads per sample. Replicate extractions of the same bentonite sample are connected with black lines.

4. DISCUSSION

4.1 DNA EXTRACTION AND CONTROLS

The results suggest very low microbial biomass in borehole module samples after one year of storage in the borehole. DNA concentrations for the majority of samples were below detection limits of the sensitive fluorescence-based Qubit assay (Table 3). Reagent and laboratory contaminant sequences can contribute a large proportion of detectable DNA in samples associated with low biomass. As a result, careful reagent and workspace decontamination was performed prior to PCR analysis. By PCR, we amplified 16S rRNA genes in borehole module samples but their low biomass required vigilant examination of the sequencing results to differentiate sample-specific signal from "noise". We started with the hypothesis that if samples did not separate well from controls in a PCoA ordination (Figure 8 and Figure 17) the sequence data likely contain contamination. Furthermore, if replicate samples did not yield similar sequencing results we rejected the data from the analysis. Coupon-clay swab samples did not separate well from controls in a PCoA ordination and no common OTUs were identified in replicates (Figure 16, Figure 17). The samples are considered below detection limits, preventing confident conclusions about microbial communities in these samples. 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 (Figure 9, Figure 12, and Figure 13). In five independent PowerSoil extraction controls, we could not identify common taxa (Figure 7). OTUs affiliated with *Staphylococcus* and *Actinobacter* were present in both PowerMax extraction controls (Figure 7) and might be contaminants from kit reagents. However, both OTUs are below 0.1% relative abundance in bentonite samples (data not shown). Borehole fluid samples grouped closely with controls in the PCoA ordination (Figure 17). However, both replicate extractions yielded similar taxonomic profiles and dominant OTUs were absent from corresponding controls (Figure 12). Outer and inner layer bentonite separated well from the controls as well as samples from case and filter (Figure 8).

The 16S rRNA gene sequencing showed very different taxonomic profiles for bentonite core samples extracted with PowerSoil and PowerMax kits (Figure 13). Insufficient amounts of DNA were recovered with the PowerSoil kit preventing a representative 16S rRNA gene profile. Therefore, we do not recommend using the PowerSoil DNA Isolation kit for the DNA extraction from the bentonite core. Instead, we recommend that DNA is extracted from at least 2 gm of bentonite using the PowerMax DNA Isolation kit. Randomization, duplicate DNA extractions, and sequencing of controls are essential to identify potential contamination, especially for low biomass samples (Salter *et al.*, 2014; Kim *et al.*, 2017).

4.2 MICROBIAL DIVERSITY IN BOREHOLE MODULE SAMPLES

In this proof-of-principle research, we demonstrated that high-throughput sequencing can determine microbial community composition and relative abundances of bacteria in samples associated with MaCoTe borehole modules. *Pseudomonas stutzeri,* a denitrifying bacterium commonly found in MX-80 bentonite (Stroes-Gascoyne *et al.*, 1997; Pedersen *et al.*, 2000; Chi Fru and Athar, 2008; Persson *et al.*, 2011) was the dominant organism in case and filter samples (Figure 18). It was shown previously that *Pseudomonas* spp. can comprise up to 26-35% of the microbial community in borehole water that was maintained anoxic for 10 months, presumably growing fermentatively on organic matter from clay and releasing organic acids and hydrogen (Bagnoud *et al.*, 2016). The highly compacted bentonite core is thought to prevent

microbial growth (Stroes-Gascoyne and West, 1997) and *Pseudomonas stutzeri* is 13× less abundant in the inner layer bentonite of module 1a as compared to the outer layer. In module 2a, *Pseudomonas stutzeri* was 82.5× less abundant in the inner layer of module 2a (Figure 14). Because our analyses only measured relative abundances, the observed decrease of *Pseudomonas* in the bentonite core can also be due to an increase in the numbers of sequences from other genera in the inner bentonite layer. However, the distribution of abundant OTUs between inner and outer layer bentonite are relatively similar and only *Pseudomonas* can be identified with a strong shift in relative abundance.

Black deposits and circular spots were visible at several locations in the borehole modules. DNA was extracted from black deposits (samples #7 and #17 in Table 2) and analyzed using 16S rRNA gene sequencing. *Pseudomonas stutzeri* was affiliated with 91.6% and 90.7% of the reads in both samples (Figure 9). Strains of *Pseudomonas stutzeri* are capable of producing an extracellular black-coloured pigment (Ganesh Kumar *et al.*, 2013), which might be the cause for the black spots visible in the borehole module. However, sulfate-reducing bacteria are known to be present in MX-80 bentonite (Masurat *et al.*, 2010a) and black sulfide deposits on bentonite or copper surfaces were seen previously (Johansson *et al.*, 2017; Bengtsson and Pedersen, 2017; Pedersen, 2010). Circular black spots, similar to what we observed on filter and bentonite of both modules (Figure 10) have been reported previously and were identified as ferrous sulfide due to its disappearance when exposed to oxygen (Bengtsson *et al.*, 2017; Pedersen, 2017). We did not perform a chemical analysis of the black material but it is recommended for future samplings.

OTUs affiliated with *Streptomycetaceae* and *Xanthomonadaceae* were the most abundant in the MX-80 bentonite lot which was used to pack the borehole modules (Figure 15). The presence of *Streptomyces* in the inner layer bentonite (Figure 14, Figure 15) indicates their resistance to high swelling pressure, presumably in the form of metabolically inactive spores or extracellular "relic" DNA. Before starting DNA isolation from inner layer bentonite samples, we removed several millimeters of bentonite from the outside using a sterile, DNA-free single-use scalpel to remove potential contamination from the outer layer of bentonite, filter, or case. Therefore, the dominance of OTUs affiliated with *Streptomyces* in the bentonite core is unlikely due to contamination. *Streptomyces* is the most abundant organism in our study but is seldom reported in MX-80 bentonite. However, Persson *et al.* 2011 have identified *Streptomyces chungwhensis* and *Streptomyces monomycini* in MX-80 bentonite, with a density of 1,850 kg/m^3, and Chi Fru and Athar 2008 identified *Streptomyces albidoflavus* in bentonite with a density of 2,000 kg/m^3. *Streptomyces* are considered aerobic organisms and thus unlikely to actively grow in an anoxic repository.

The shipping flask liquid of both modules contained a Gram-negative, strictly aerobic organism affiliated with *Phenylobacterium* with 5.6 or 27.0% relative abundance (Figure 9). Modules 1a and 2a were accidentally exposed to oxygen during the transport from the Grimsel test facility in Switzerland to Harwell, UK, which could explain the growth of a strictly aerobic organism. However, no reads associated with this OTU were identified in the borehole fluid (data not shown). The origin of *Phenylobacterium* is unclear; an introduction during packing of the modules is possible.

OTUs affiliated with *Pelobacteraceae* were found in samples from the case and filter (Figure 9) but were absent from the highly compacted bentonite core (Figure 14). *Pelobacteraceae* is an anaerobic organism from the order of *Desulfuromonadales* and an Fe(III)-reducer (Lovley *et al.*, 1995).

OTUs affiliated with *Desulfosporosinus meridiei*, *Syntrophus*, and *Desulfovibrio mexicanus* were most abundant in the borehole fluid of borehole 13.001. *Desulfosporosinus* and *Desulfovibrio* are anaerobic sulfate-reducing bacteria. Members of the genera *Syntrophus* are strictly anaerobic, can use crotonate as electron acceptor, or are fermenters. Some members grow only in the presence of hydrogen/formate-utilizing partners in a syntrophic association i.e. with *Desulfovibrio sp.* (Kuever, 2014). Metagenomic sequencing of DNA from borehole 13.001 identified *Syntrophus aciditrophicus* as the dominant organism (data not shown). *S. aciditrophicus* is an anaerobic bacterium that degrades fatty- and aromatic acids in syntrophic association with methanogens or sulfate reducers (McInerney *et al.*, 2007). Less than 0.04% of reads in the inner layer bentonite samples were affiliated with *Desulfosporosinus meridiei*, *Syntrophus*, or *Desulfovibrio mexicanus* (data not shown).

Microbial activity and cell survival is known to decrease with higher bentonite compaction and higher swelling pressures (Masurat *et al.*, 2010b; Bengtsson and Pedersen, 2017; Pedersen, 2010; Motamedi *et al.*, 1996). Using OTU counts as a proxy of how many species are present in the bentonite, we expected lower counts in the high density bentonite core. However, OTU counts in the inner layer bentonite of module 2a did not differ significantly from module 1a (Table 5), nor did the community composition (Figure 14). Although 16S rRNA gene sequencing determined the total microbial community in the bentonite samples, their presence does not necessarily reflect activity. RNA levels can be used as a proxy for the active microbial community but RNA test extractions on module 1 inner layer bentonite (#27) was not successful.

PLFA abundances, and commensurate cellular abundance estimates, were similar for modules 1a and 2a bentonite samples, indicating 1 to 3 × 10^6 cells/gm. Due to low microbial biomass in borehole module samples, we were not able to determine cell numbers using a DNA-based method, highlighting one advantage of PLFA. The PLFA concentrations in the inner layer bentonite for both modules were very similar, with 19.4 to 23.5 ng/gm indicating no changes in microbial biomass. As mentioned previously, OTU counts in the inner layer bentonite did also not differ significantly between modules, nor did the community composition. However, there were minor variations in the lipid profiles between bentonite core samples, suggesting minor variations in the microbial community present based on PLFA. The presence of iso- and anteiso- C15:0 PLFA is often associated with sulfate-reducing bacteria. However, OTUs affiliated with sulfate-reducers were almost absent from bentonite cores based on 16S rRNA gene analysis. The presence of relic PLFA potentially stabilized by the clay may have affected the PLFA results.

5. CONCLUSIONS

We developed protocols for PLFA analysis, DNA extraction, 16S rRNA gene sequencing and data analysis with an emphasis on determination of sample signal to background noise for low biomass samples. We demonstrated that PLFA and high-throughput sequencing can determine the microbial community structure and (relative) abundances in borehole module samples. Due to the low biomass in samples and the high risk for contaminants to outcompete sample related signal, the importance of randomization, replication, and controls was highlighted. Extraction blanks have to be prepared with each batch of PLFA and DNA extraction. No template controls have to be prepared with each batch of 16S rRNA gene amplification and need to be included in the Illumina MiSeq sequencing, even if amplicons are not detected. A careful analysis of sample

signal and background noise is prudent to determine real signal for low biomass samples. Samples not differentiating well from controls need likely be discarded from the analysis.

6. RECOMMENDATIONS

Due to the low biomass in the bentonite core it is important to prevent contamination at every step during the sampling procedure. In our sampling protocol, we have outlined many steps for contamination prevention but we recommend the use of bleach and UV light irradiation for cleaning of surfaces and equipment in the anaerobic glovebox before commencing work.

The presence or absence of microorganisms in the inner bentonite core is of great interest in this study. We would like to emphasize the importance of removing several millimeters of bentonite from the outside using a sterile, DNA-free single-use scalpel to remove potential contamination from the outer layer bentonite, filter/case surfaces or sampling equipment. Also, the direct swabbing of the metal coupons might need to be reconsidered for better insight about microorganisms on the coupon surface.

With respect to high-throughput DNA extraction, we tested the use of the PowerSoil DNA Isolation kit for bentonite core samples. However, we do not recommend using the PowerSoil DNA Isolation kit, rather DNA has to be extracted from at least 2 gm of bentonite using the PowerMax DNA Isolation kit.

PCR with 35 cycles of amplification did not yield a PCR product for the majority of borehole module samples. However, nested PCR on low biomass samples is prone to contamination. Instead, we recommend testing a PCR with 45 cycles of amplification. Randomization and replicate extraction for low biomass samples is recommended for future investigations.

In this proof-of-principle research, we demonstrated that high-throughput sequencing can determine the relative abundance of bacteria in borehole modules. As a next step, quantification of the total number of 16S rRNA gene copies by quantitative PCR (qPCR) with or without synthetic DNA standards should be considered to determine absolute abundances of microbial DNA in the borehole modules.

Pseudomonas stutzeri and *Streptomyces* were the dominant organisms in the case and bentonite samples. Cultivation and activity assays could confirm their viability in the borehole or bentonite cores.

The detection of the C18:2ω9,12 PLFA indicates the presence of fungi in the bentonite samples. The analysis of 18S rRNA genes could identify eukaryotes present in the samples in future work.

7. ACKNOWLEDGEMENTS

Thanks are due to Karsten Pedersen, Microbial Analytics Sweden, for his thoughtful peer-review of this report.

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APPENDIX A: PROTOCOL FOR BOREHOLE MODULE SAMPLING

A.1 INTRODUCTION

In 2015, two modules (1.25 gm/cm^3 and 1.50 gm/cm^3) were removed from the borehole at the Grimsel Test Facility in Switzerland after one year of underground storage and were transported to AMEC Foster Wheeler (Harwell, UK) for analysis. Metal coupons were extracted from the modules for corrosion testing (i.e., microscopy and weight loss analysis) to be conducted by AMEC. Samples which contained coupons partially embedded in bentonite were also issued to AMEC for corrosion analysis and therefore were unavailable for swabbing. Only samples of bentonite which contained the imprint of the coupon (i.e., were adjacent to the coupon but do not contain the coupon itself) were made available for microbiological analysis (i.e. swabbing). Canadian researchers (University of Waterloo and the University of Saskatchewan) obtained swab, bentonite, and water samples for microbial and genomic assessment. Bentonite samples were also taken for Greg Slater from McMaster University for PFLA analysis.

Modules arrived at AMEC in individual anoxic shipping flasks to prevent exposure to oxygen. Shipping flasks were moved into the anaerobic glovebox before opening. Modules were dismantled under anoxic conditions to preserve the corrosion products on the surface of the coupons. Work in the glovebox was very restricted in space and flexibility. It was critical to make work as efficient as possible by prior planning and preparation. Consideration was also given to the time required to shuttle material into the glovebox. Once bentonite was separated from the coupons it was removed from the glovebox.

Please see Appendices for further explanation of sampling locations and procedures:

- Appendix B: Sampling Pictures
- Appendix C: Sampling Drawings

A.2 SAMPLING PROCEDURE

Materials

- Wear clean, single-use, gloves (if available, sterile and DNA-free)
- Use sterile, DNA-free foam swabs (Puritan, ME, USA, cat. no. 25-1506 1PF BT)
- Use clean Ziploc bags (if available, DNA free i.e. use UV treatment)
- Use 70% ethanol prepared in ultrapure water to flame sterilize equipment
- Bleach and UV treat equipment, if possible

Swab the shipping flask

- Wear clean gloves
- Take 2 swabs from side and top of shipping flask after its removal from the shipping box
- Swabs act as background control
- Clean the outside of the shipping flask with 70% ethanol
- Shuttle shipping flask into the glovebox

Swabbing of modules

- Label all swabs before sampling
- Always wear clean, disposable gloves on top of the black rubber gloves of the glovebox
- Change gloves regularly, especially when changing sampling areas
- Arrange labelled swabs in order and upright (i.e. in a rack) for easy access with gloves
- Rotate swab when sampling and place back into sterile plastic shipping flask
- Swab approximately 25 cm^2 but make note of actual area swabbed
- Mark the location of the actual sampling sites (Figure A1), if appropriate
- Collect all swabs in a Ziploc bag and store at −20°C
- Collect University of Waterloo and University of Saskatchewan samples in a separate bag
- Obtain following swab samples for each module (also see Table A1)
    - Control1: unused swab →background control of swab material
    - Control2: swabs from different zones in the glovebox on work day 1 →background control i.e. working surface, walls, non-disposable black rubber gloves
    - Control3: swabs from different zones in the glovebox on work day 2
    - Swabs from holes in the metal casing →Use one swab for all 5 holes that are aligned longitudinally on the module; see Figure A1a
    - Swabs from outer metal surface of the module
        a) Swab the top of the module before taking it out of the shipping flask
        b) Swab ca. 25 cm^2 in all 5 areas that correspond to the 5 sections of the core
    - Swabs from inner lining of module →Use one swab to swab ca. 25 cm^2 in top, middle, and bottom area
    - Swabs from outer filter surface →Use one swab to swab ca. 25 cm^2 in top, middle, and bottom area
    - Swabs from inner filter surface →Use one swab to swab ca. 25 cm^2 in top, middle, and bottom area

Table A1. List of swab samples from modules 1 and 2. Swabs were collected separately for the University of Waterloo (UofW) and the University of Saskatchewan (UofS) researchers.

Module	Location	Quantity UofW	Quantity UofS	Label (add date, time and initials)
-	Control1 (empty swab)	3	3	Control1 (a-c)
1	Control2 (working surface day 1)	3	3	Control2 (a-c)
1	Shipping flask (outside)	2	2	Mod1 Shipping flask (a,b)
1	Hole (5 per swab)	2	2	Mod1 Hole (a,b)
1	Metal case outside	2	2	Mod1 MCase Outs (a,b)
1	Metal case inside	2	2	Mod1 MCase Ins (a,b)
1	Filter outside	2	2	Mod1 Filter Outs (a,b)
1	Filter inside	2	2	Mod1 Filter Ins (a,b)
Module	**Location**	**Quantity UofW**	**Quantity UofS**	**Label (add date, time and initials)**
2	Control3 (working surface day 2)	3	3	Control3 (a-c)
2	Shipping flask (outside)	2	2	Mod2 Shipping flask (a,b)
2	Hole (5 per swab)	2	2	Mod2 Hole (a,b)
2	Metal case outside	2	2	Mod2 MCase Outs (a,b)
2	Metal case inside	2	2	Mod2 MCase Ins (a,b)
2	Filter outside	2	2	Mod2 Filter Outs (a,b)
2	Filter inside	2	2	Mod2 Filter Ins (a,b)

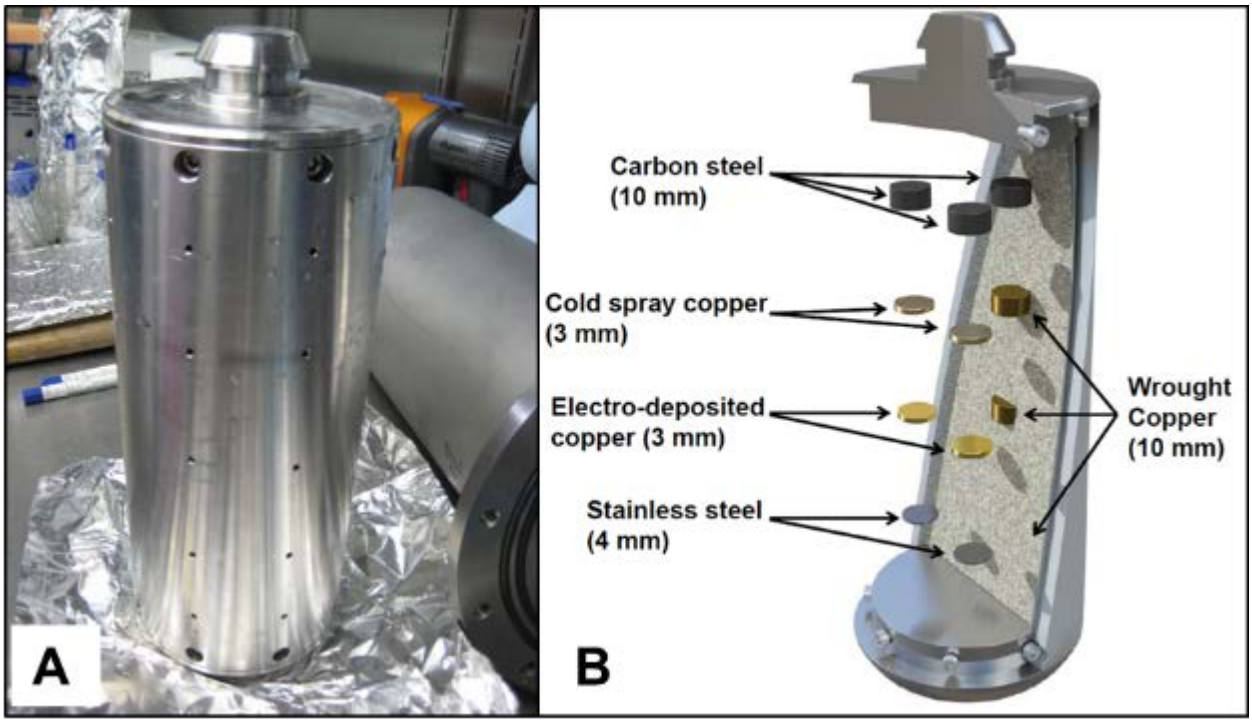


Figure A1. Borehole module design for the Grimsel corrosion test project. A) Borehole module showing permeable holes (arranged longitudinally in a set of 5 holes); B) Schematic showing metal coupons surrounded by bentonite (picture B provided by Nagra). The module is 308 mm in height with a diameter of 126 mm.

Water samples

- The shipping flask may contain small amounts of liquid, which potentially drained from the moist module during transport
- Use a sterile syringe to aspirate water sample and collect in sterile, DNA-free 50 ml centrifuge tube. Alternatively, filter 50 ml of liquid through a Sterivex-GV 0.22 µm filter (Millipore, MA, USA, cat. no. SVGV010RS). Retrieve a duplicate sample, if possible. Freeze the filter at −20°C.
- If filtration is not possible, freeze the water sample at −20°C.

Bentonite Sampling

- Samples will be taken from 2 modules with different densities of bentonite.
  - Module 1: Dry density of MX-80 of 1.25 gm/cm^3
  - Module 2: Dry density 1.50 gm/cm^3
- Minimize potential microbial contamination by wearing clean disposable sterile gloves, change gloves between different steps and between different sampling sites. Use ethanol (70%) to clean tools, flame sterilize when applicable.
- Minimize potential DNA contamination by using sterile and DNA free tubes. Use ethanol (70%) to flame sterilize equipment and expose metal equipment to a flame, apply flame well after the ethanol has burned off to destroy DNA. Change gloves regularly.
- Take bentonite from outer and inner regions of the module and bentonite surrounding the coupons (coupon-clay). Refer Appendix C “Sampling Drawings” for further explanation of sampling steps and sites.

- If possible, break bentonite up into smaller fractions before placing into Ziploc bags and before freezing.
- Label bags with date, time of collection, location in module (see Table 2 for labels).
- Place sample in −20°C freezer (once frozen, do not thaw and refreeze).
- Collect up to 300 gm bentonite for PLFA analysis at McMaster University (Greg Slater).
    - Freeze bentonite and store at −20°C.
    - Ship samples to the University of Waterloo and forward to G. Slater from there.
    - Try to get the same locations as the University of Waterloo but, if only one sample per module is available that is acceptable.

Table A2. List of bentonite samples from modules 1 and 2. Bentonite was collected separately for the University of Waterloo (UofW), the University of Saskatchewan (UofS) and McMaster University (McMaster) researchers.

Module	Location	Quantity UofW	Quantity UofS	Quantity McMaster	Label (always add date, time and initials)
Coupon-clay is an imprint of a metal coupon in surrounding bentonite. Imprint is split between UofW and UofS at −20°C. One sliver of the imprint will be stored at 4°C for UofS.					
1	Coupon-Clay CSteel	1× −20°C	1× −20°C 1× 4°C	-	Mod1_CC_CSteel
1	Coupon-Clay Wcopper	1× −20°C	1× −20°C 1× 4°C	-	Mod1_CC_Wcopper
1	Coupon-Clay CScopper	1× −20°C	1× −20°C 1× 4°C	-	Mod1_CC_CScopper
1	Coupon-Clay EDcopper	1× −20°C	1× −20°C 1× 4°C	-	Mod1_CC_EDcopper
1	Coupon-Clay SSteel	1× −20°C	1× −20°C 1× 4°C	-	Mod1_CC_SSteel
Outer layer and inner layer are composite samples of sections 1 to 4.					
1	Outer layer	1× −20°C ca. 50 gm	1× −20°C ca. 50 gm 1× 4°C ca. 5 gm	1× −20°C ca. 50 gm	Mod1_OuterL
1	Inner layer	1× −20°C ca. 200 gm	1× −20°C ca. 200 gm 1× 4°C ca. 100 gm	1× −20°C ca. 200 gm	Mod1_InnerL
Section 5 (S5) is sampled separately from section 1 to 4.					
1	S5-outer layer	1× −20°C ca. 50 gm	1× −20°C ca. 50 gm 1× 4°C ca. 5 gm	-	Mod1_S5_OuterL
1	S5-inner layer	1× −20°C ca. 50 gm	1× −20°C ca. 50 gm 1× 4°C ca. 5 gm	1× −20°C ca. 200 gm	Mod1_S5_InnerL
Module	**Location**	**Quantity UofW**	**Quantity UofS**	**Quantity McMaster**	**Label (always add date, time and initials)**
2	Coupon-Clay CSteel	1× −20°C	1× −20°C 1× 4°C	-	Mod2_CC_CSteel
2	Coupon-Clay Wcopper	1× −20°C	1× −20°C 1× 4°C	-	Mod2_CC_Wcopper
2	Coupon-Clay CScopper	1× −20°C	1× −20°C 1× 4°C	-	Mod2_CC_CScopper
2	Coupon-Clay EDcopper	1× −20°C	1× −20°C 1× 4°C	-	Mod2_CC_EDcopper
2	Coupon-Clay SSteel	1× −20°C	1× −20°C 1× 4°C	-	Mod2_CC_SSteel
2	Outer layer	1× −20°C ca. 50 gm	1× −20°C ca. 50 gm 1× 4°C ca. 5 gm	1× −20°C ca. 50g	Mod2_OuterL
2	Inner layer	1× −20°C ca. 200 gm	1× −20°C ca. 200 gm 1× 4°C ca. 100 gm	1× −20°C ca. 200 gm	Mod2_InnerL
2	S5-outer layer	1× −20°C ca. 50 gm	1× −20°C ca. 50 gm 1× 4°C ca. 5 gm	-	Mod2_S5_ OuterL
2	S5-inner layer	1× −20°C ca. 50 gm	1× −20°C ca. 50 gm 1× 4°C ca. 5 gm	1× −20°C ca. 200 gm	Mod2_S5_ InnerL

Instructions for Sample Shipment:
For customs clearance, please indicate that the samples are for research purposes only and have ZERO commercial value. Bentonite clay is not toxic and is not classified as dangerous goods. Ship as "rock samples for destructive analysis" or "bentonite clay, a naturally occurring mineral".

Parcel 1 – On dry ice

Insert shipping address

Indicate on the package: "Dry Ice. Deliver Immediately."

Parcel 2 – On dry ice

Insert shipping address

Indicate on the package: "Dry Ice. Deliver Immediately."

Parcel 3 – Cold but not frozen

Insert shipping address

APPENDIX B: PICTURES OF THE BOREHOLE MODULE SAMPLING

B.1 INTRODUCTION

The Switzerland National Technical Competence Centre in the field of deep geological disposal of radioactive waste (Nagra) has set up *in situ* corrosion experiments at the Grimsel Test Facility in Switzerland. AMEC Foster Wheeler prepared borehole modules for material corrosion testing (MaCoTe) containing corrosion test pieces (metal coupons) embedded in bentonite. In 2015, two modules, 1a and 2a with different bentonite dry densities (1a = 1.25 gm/cm^3 and 2a = 1.50 gm/cm^3) were removed from the borehole in Grimsel after one year of storage and transported to AMEC Foster Wheeler (Harwell, UK) for analysis. Sampling of the modules took place from November 8, 2015 to November 13, 2015. Modules were dismantled under anoxic conditions to preserve potential corrosion products on the surface of the coupons. All work was carried out in an anoxic (argon-purged) glovebox. Coupons were sampled by AMEC for corrosion testing. Bentonite and swab samples were taken for the Universities of Waterloo and Saskatchewan for microbial and genomic assessment. Bentonite samples were also taken for Greg Slater from McMaster University for PLFA analysis.

Please see appendices for further explanation of sampling location and procedures:

- Appendix A: Sampling Protocol
- Appendix C: Sampling Drawings

In the following we document each step of the module sampling with images and comments.

- Part 1. Preparation of glovebox (anaerobic chamber) and equipment.
- Part 2. Dismantling and sampling of modules.
- Part 3. Changes and adjustments for module 2 (high density) sampling.

1. Preparation of glovebox (anaerobic chamber) and equipment.

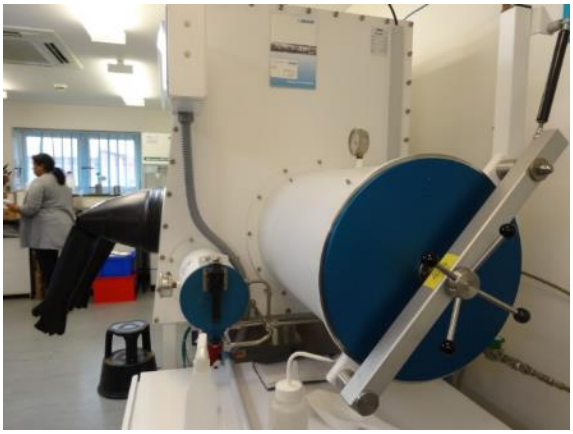
All work was done in an anaerobic chamber (glovebox). Coupons must stay in an anaerobic environment at all times to avoid oxygen contamination after removal from borehole.

Glovebox was cleaned with a vacuum and wiped with 70% ethanol several times before work was started.

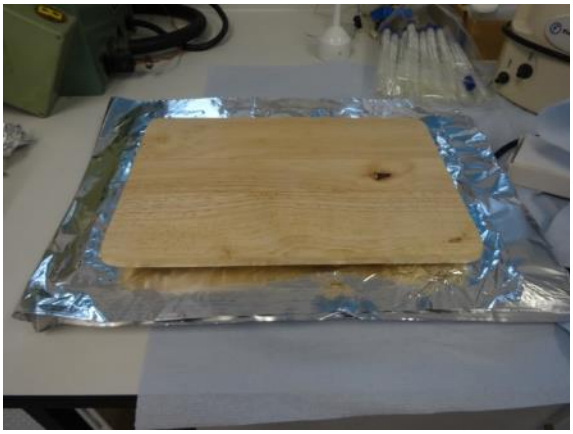
Before the work on the second module was started, the glovebox was carefully cleaned again.

For future sampling: Cleaning of the chamber with a UV lamp (removal of DNA) is recommended prior to beginning work.

Oxygen monitor in the glovebox to verify anoxic environment.

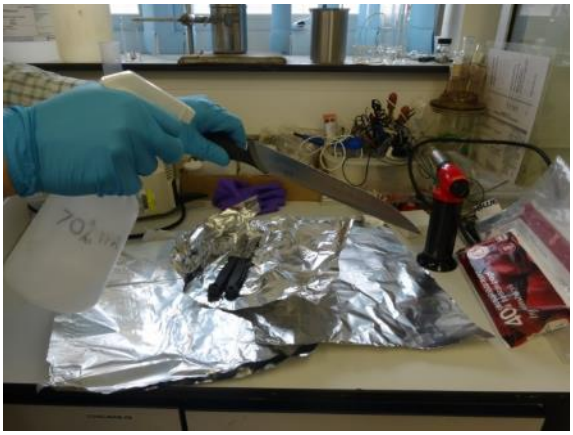


Two ports are available to shuttle equipment into the glovebox. Each port was evacuated and filled with argon gas 3 times before opening to the inside. The large port takes ca. 10 min for 3 flushings; the small port takes less than 2 min.



Cutting boards were covered with layers of sterile and DNA free aluminum foil.

Bentonite cores were cut on the boards.



Outside of the glovebox, all equipment was cleaned with 70% ethanol. All metal equipment was flame sterilized with 70% ethanol and also heated with a Bunsen burner to destroy any DNA.

Cleaned and flame sterilized equipment was wrapped in aluminum foil and shuttled into the glovebox.

2. Dismantling and sampling of modules

Shipping flask was taken out of shipping box.

Shipping flask kept the module in an anoxic environment after removal from the borehole until sampling in Harwell, UK.

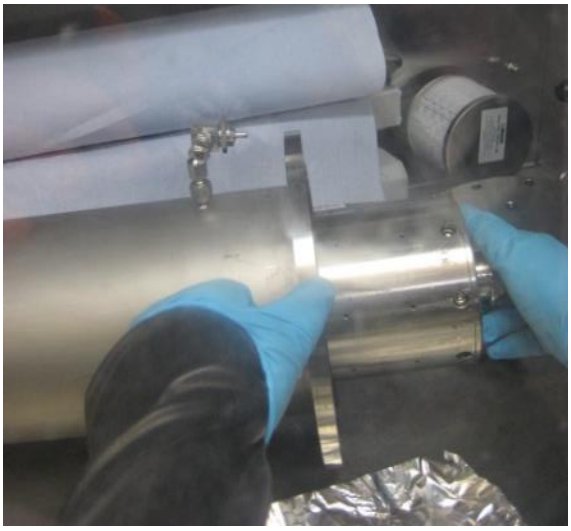
Shipping flask was swabbed on the top and sides.

After swabbing, the outside of the shipping flask was cleaned with 70% ethanol.

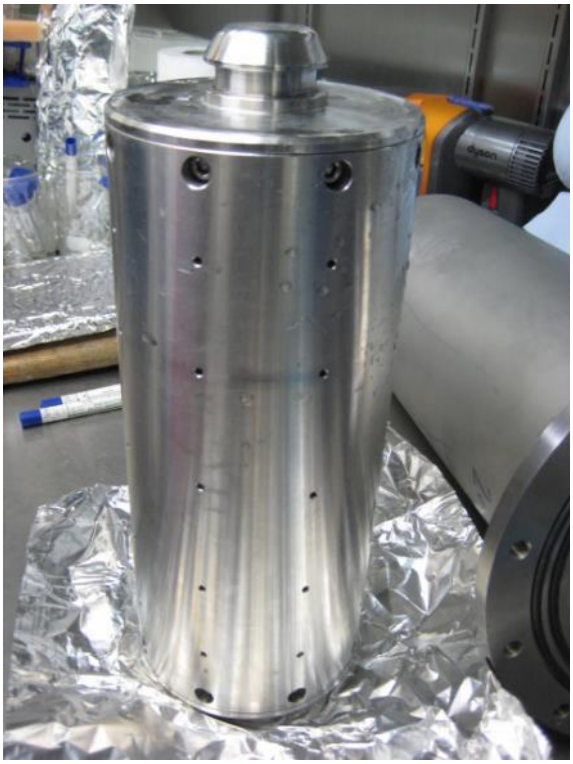
Shipping flask was moved into the anaerobic glovebox via the large port.

Shipping flask was opened.

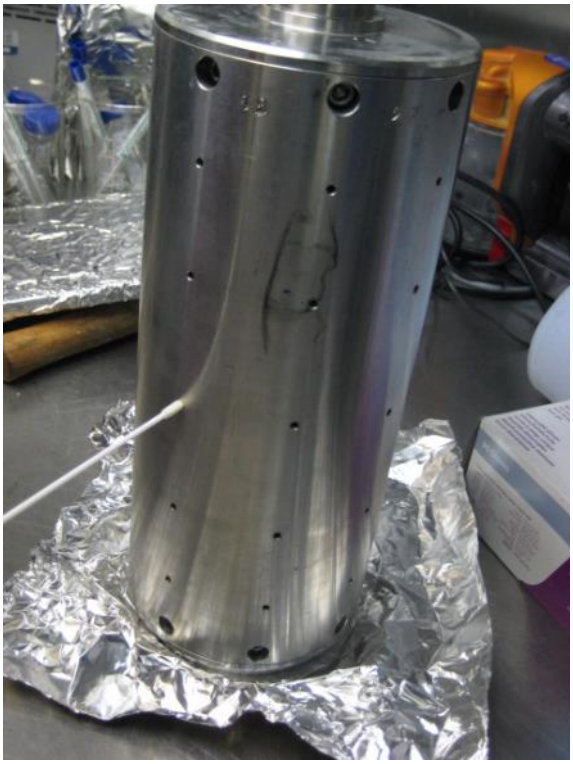
Top of module was swabbed before removing from shipping flask.



Module was removed from shipping flask and placed on clean surface i.e. aluminum foil.



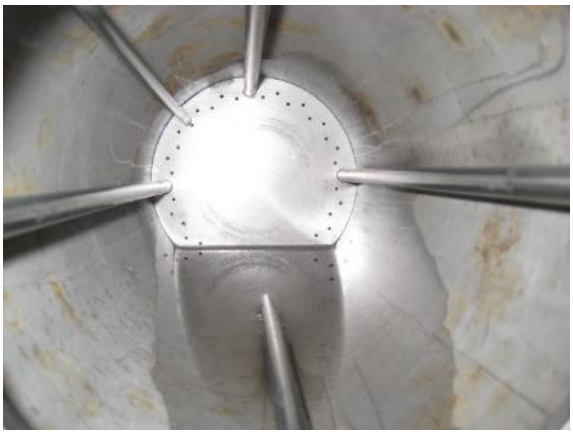
Outside of the module was swabbed.



Holes of the module were swabbed.

One swab was used for all 5 longitudinal holes.

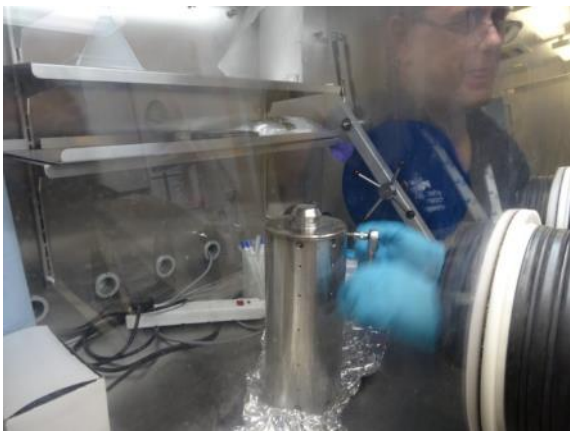
Caution: Swab carefully because the opening is smaller than the swab and cuts the swab surface when applying too much pressure.



Liquid was sampled from the shipping flask.

Sterile syringe was used to aspirate liquid and eject into sterile 50 ml tubes.

For future sampling: filter the water through 0.22-µm Sterivex filter (Millipore) and freeze the filter for subsequent DNA extraction.



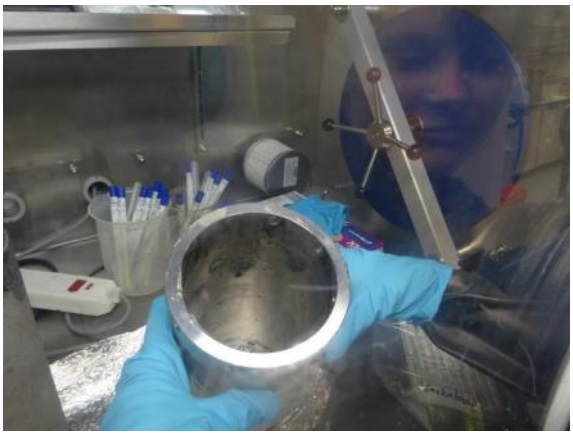
Module was opened.

Screws were removed on the top and bottom using an Allen key.

Hammer and screw driver were used to pry lid open.

Lid of the module was removed.

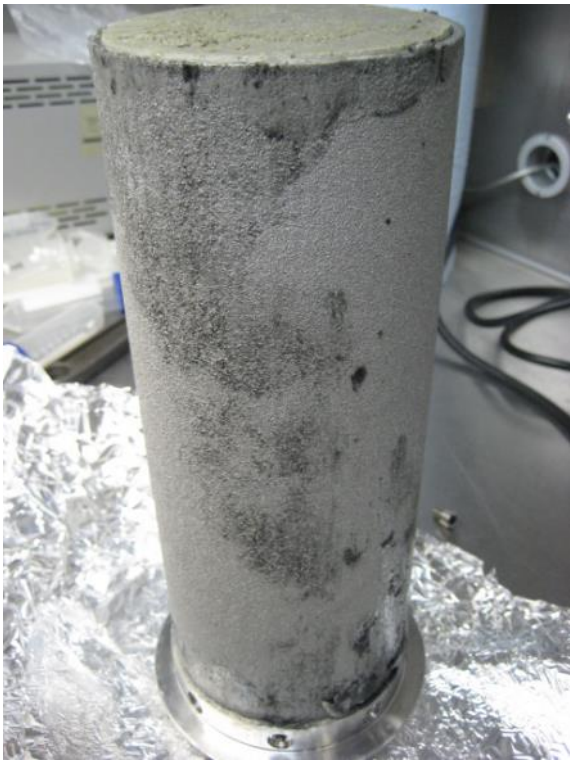
The filter (containing the bentonite core) was removed from the module and placed on clean surface i.e. aluminum foil.



Inside of the module was swabbed.

View of the inside of module 1.

Black deposit was mostly found close to the top and bottom of the module. Black deposit was not found around the longitudinal holes.



The outside of the filter was swabbed. The filter surface was very rough and difficult to swab without losing foam material. Swab lightly only.

Black deposit was found more concentrated at the top and bottom of module.

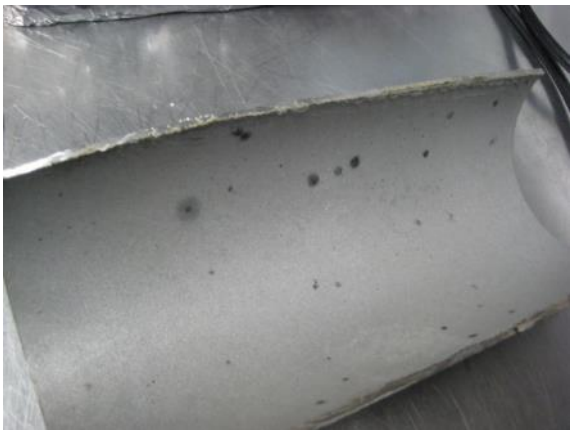
Black deposit was also found in the middle area of the filter but was not co-localized with the longitudinal holes in the module.

Filter was cut longitudinally using a hand-held metal cutter (flex).

Cutting was performed inside a plastic bag to avoid dusting the glovebox.

One half of the filter was lifted from the bentonite core.

Black deposit was found on the inside of the filter as well as in the bentonite (only shallow i.e. 3-6 mm). Black deposit was always circular but not co-localized with longitudinal holes in module.



The inside of the filter was swabbed in areas without and with visible black spots.

The inside of the filter was very smooth and easy to swab.

Black spots could be removed from the filter completely with the swab.

Caution: Always locate and label the side of the core with the ball bearing. The ball bearing is a reference for the location of metal coupons.

Refer to the appendices for further information on sectioning and coupon locations:

- Appendix A: Sampling Protocol
- Appendix B: Sampling Drawings

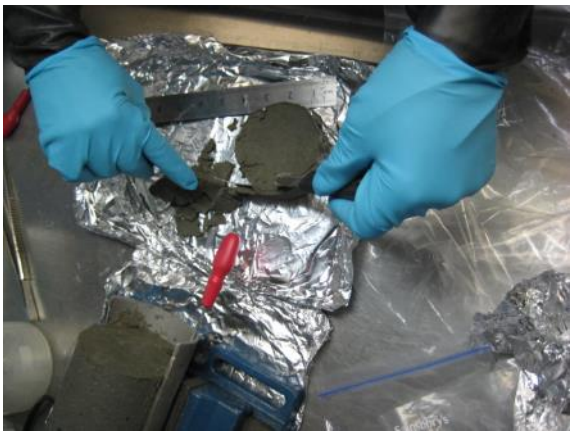
The core was cut into 5 sections. Top and bottom sections were labelled as well as the location of the ball bearing in each section.

Try to cut as straight as possible.

First section was cut.

Outer layer of bentonite was removed from sections 1 to 4 and collected as a composite in one bag.

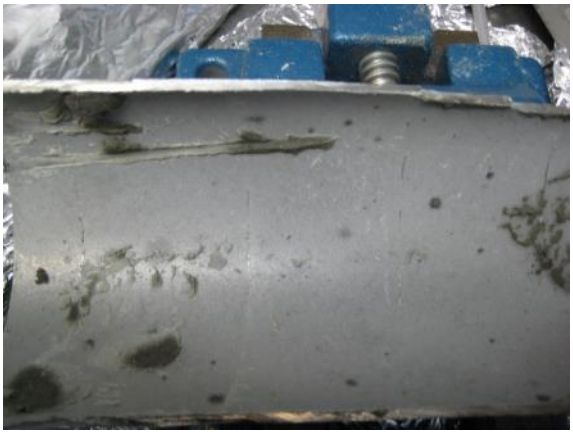
Sections were placed into a plastic bag and heat sealed to prevent drying until further sampling.



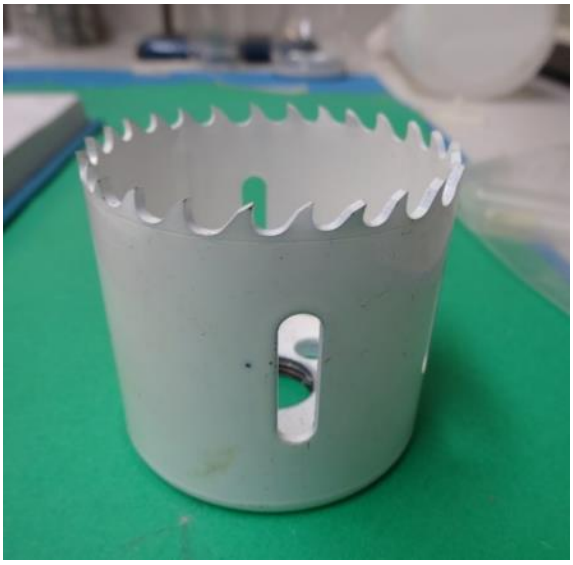
Outer layer of section 5 was removed and collected in one bag.

Inner layer of section 5 was collected separately in one bag as well.

Further cutting and separation of bentonite between universities was performed outside of the glovebox.



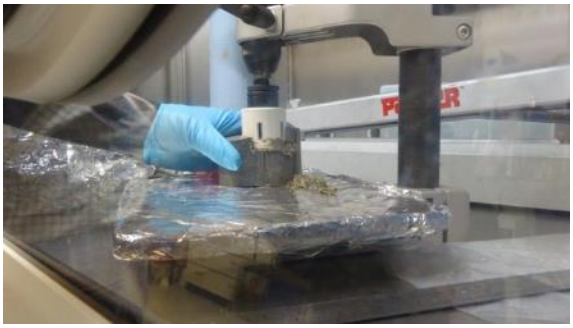
Once all sections were removed the second half of the filter was swabbed.



Coring device to remove the coupons from the bentonite sections.

Coring device attached to electric drill inside the glovebox.

Location of the coupons was marked using a template.



Bentonite surrounding the coupon was cored.

This method was used successfully for module 1 (low density bentonite).

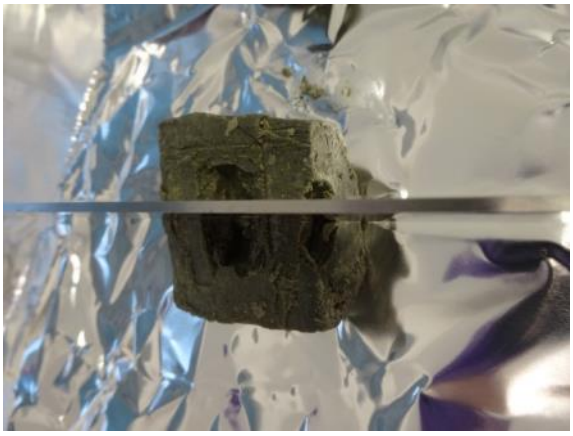
Methodological change for module 2 will be described in section C of this document.

Bentonite core containing coupon.

Bentonite from approximately 1 cm away from the coupon was removed. Trimmings were collected in “inner layer” bag (composite of section 1 to 4).

Bentonite surrounding the coupon was scored very carefully, without touching the coupon surface. Scored coupon-clay was pried open. One half contained the coupon, the other half was an imprint of the coupon.
AMEC kept the coupon for weight loss measurement and microscopy (corrosion analysis). AMEC also needed the coupon-bentonite interface for analysis and therefore the coupon could not be removed from the second half.

Imprint of coupon was collected for each metal type in one bag.



Coupon imprint was cut in half for analysis at Universities of Waterloo and Saskatchewan.

3. Changes/ adjustments for module 2 (high density) sampling.

When cutting the first section of the high density bentonite we realized that coupons had shifted down.

When coring the first coupon, we accidentally cut into the coupon. High density bentonite and its higher swelling pressure must have caused the shift of coupons upwards and outwards. Heterogeneity of bentonite will have contributed.

As a result, the location of the coupons was unpredictable in module 2 and we needed to change the sampling plan.

Instead of coring with the electrical drill, we cut the sections with a knife to avoid damage to the coupons.

Sections were cut into V-sections around the estimated location of the coupon.

Cutting with the knife was very difficult because the high density bentonite was very solid. Work carefully, ensuring no damage to the coupon.

The V-sections were scored with a knife and broken apart as described previously.

Imprint was collected for Universities of Waterloo and Saskatchewan.

Excess bentonite was removed around imprint.

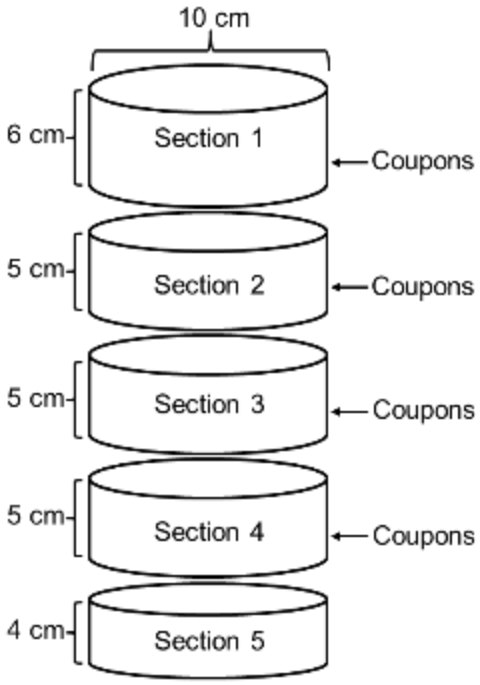
Imprint was cut into half for analysis at the Universities of Waterloo and Saskatchewan.

A sliver of the imprint was cut for 4°C storage and analysis at University of Saskatchewan.

When the imprint was not clearly visible due to the direction or angle of the bentonite samples were broken apart and the bentonite adjacent to the imprint (i.e. within 5 mm) was removed before freezing.

See Appendix A: Sampling Protocol and Appendix C: Sampling Drawings for further information.

APPENDIX C: DRAWINGS OF THE BOREHOLE MODULE SAMPLING



Inner dimension of the module
= 10 cm (diameter) x 25 cm (height)

Approx. volume and weight of the module
V_{Mod} = 1,963 cm^3 = 2,454 g (1.25 g/cm^3)

Aprox. volume and weight of one section
V_{Sec} =392 cm^3 = 490 g (1.25 g/cm^3)

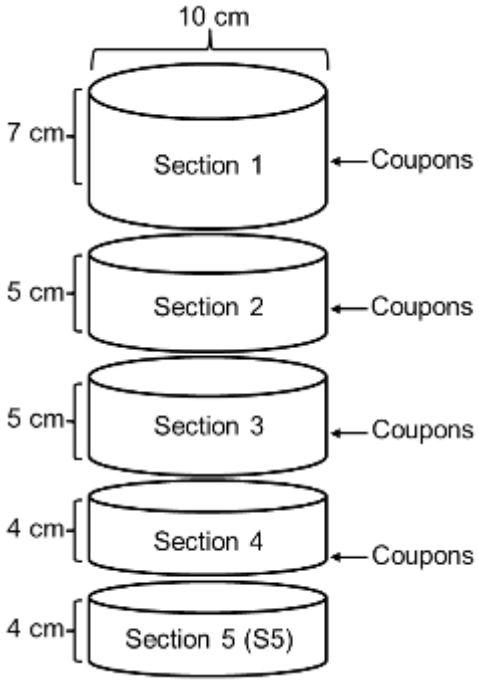
Sections 1 to 4 contain 3 coupons each.

The coupons are located at 1cm away from bottom of each section.

Location and orientation of the coupons is mapped by a ball bearing in each section.

Section 5 contains no coupons.

Figure C1. Bentonite core sections for module 1a (1.25 gm/cm^3). In each section the coupons are 1 cm away from the bottom of the section.



Inner dimension of the module
= 10 cm (diameter) x 25 cm (height)

Approx. volume and weight of the module
V_{Mod} = 1,963 cm^3 = 2,945 g (1.5 g/cm^3)

Aprox. volume and weight of one section
V_{Sec} =392 cm^3 = 589 g (1.5 g/cm^3)

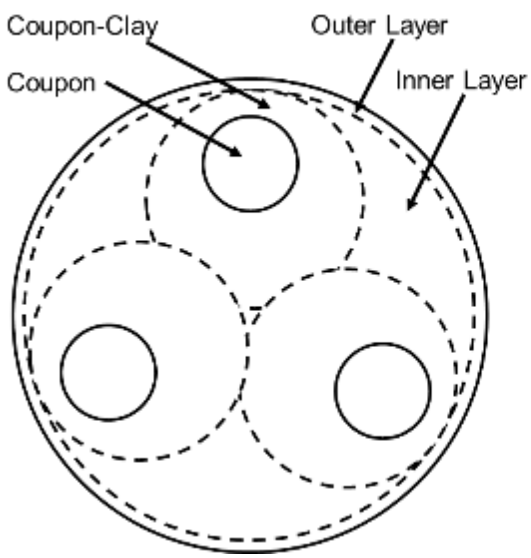
Sections 1 to 4 contain 3 coupons each.

The coupons are located more than 1 cm away from bottom of each section.

Location and orientation of the coupons is mapped by a ball bearing in each section.

Section 5 contains no coupons.

Figure C2. Bentonite core sections for module 2a (1.50 gm/cm^3). The cutting of Section 5 revealed that coupons had moved in the high density bentonite most likely due to swelling pressure and heterogeneity of bentonite. To avoid cutting into coupons we shifted the sections 1 cm further down (compared to module 1a) to ensure coupons are well embedded within the bentonite.



Outer Layer (OL) = 5 mm ring (cylinder) on the outer edge of the bentonite section: V = 74 cm^3

- Sample a composite of Outer Layers of Section 1 to 4 = 4 x 74 cm^3 = 296c m^3

Coupon-Clay (CC) = 1 cm around coupon in each dimension:
V = 34 cm^3 (1 cm high coupon)
V = 28 cm^3 (0.3 cm high coupon)

- Sample a composite CC for the same metal type (max. 2 coupons each).

Inner Layer (IL) = left over bentonite
$V_{IL} = V_{total} - V_{CC} - V_{OL}$ = ca. 800 cm^3

Section 5 does not contain coupons and OL and IL will be sampled separately.

Figure C3. Terminology of samples retrieved from the bentonite core. Each section is ca. 4 to 7 cm in height. For module 1a, we cored the CC before removing the Outer Layer and therefore lost some Outer Layer for sampling. For module 2, we first removed the Outer Layer around each section before starting to core. This later procedure is recommended for future sampling. Module 2a: In the first coring attempt we cut into a metal coupon because the coupons shifted unexpectedly in the high density bentonite. We therefore had to stop coring but needed to cut with a knife as described in the next image.

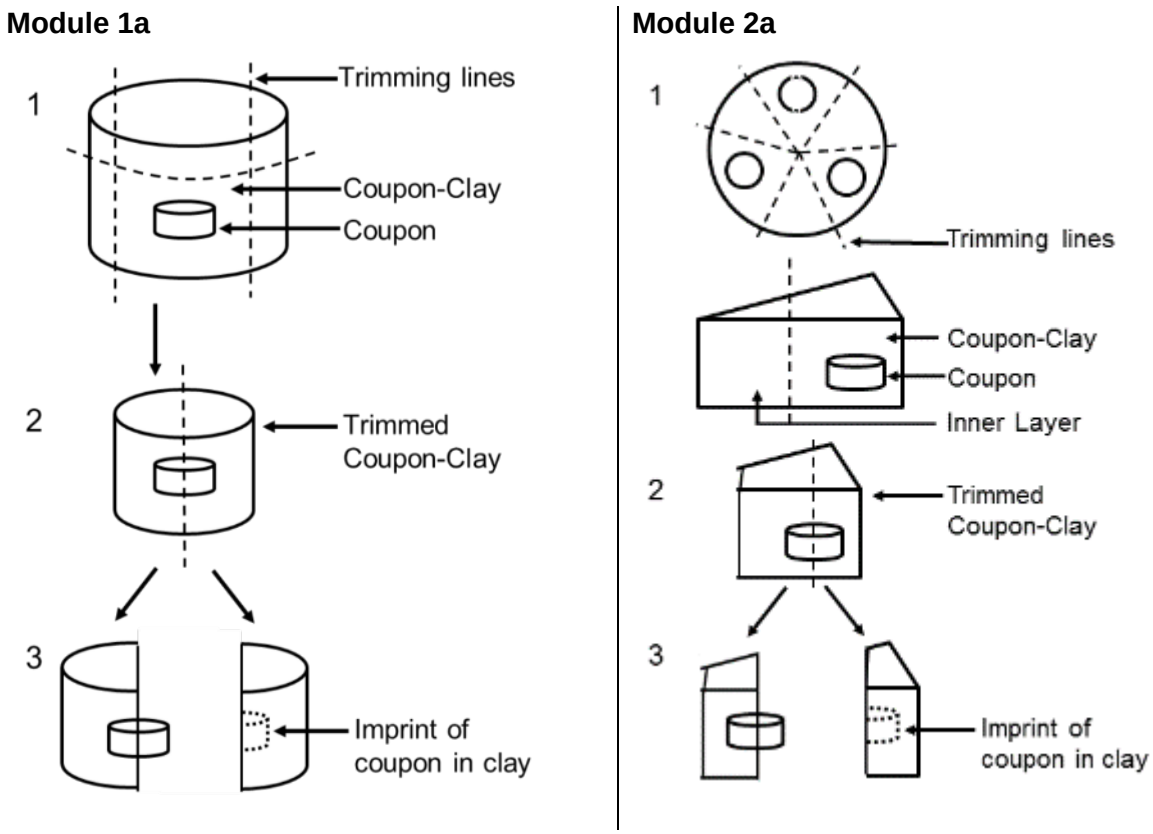


Figure C4. Sampling of coupon-clay (CC) for module 1a and 2a. For module 1a, we cored all coupons but due to time constraints we were only able to trim one coupon per metal type. The second metal coupon was trimmed ca. 1 week later by colleagues from AMEC and imprint was shipped to Canadian universities. Step 1: CC was trimmed ca. 1 cm in each dimension around the coupon. Step 2: The CC was carefully scored around the coupon with a knife avoiding scoring the coupon itself. Step 3: CC was carefully pried open using a knife and CC broke in two pieces leaving the coupon attached to one side and an imprint in the other half. AMEC kept the part with the coupon and the Universities of Waterloo and Saskatchewan took the imprinted half. The Imprint was cut in half, one for each university. A thin sliver of the imprint was cut for University of Saskatchewan and stored at 4°C for cultivation experiments.

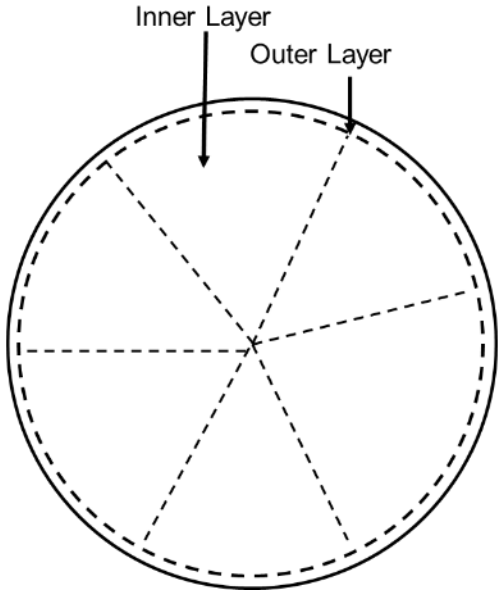


Figure C5. Section 5 does not contain coupons and was sampled separately. The outer layer was shaved off from the core and collected in one bag. The inner layer was cut into triangles, like a pie.

Recommendations for sampling

A UV-lamp and bleach should be used to remove DNA contaminations in the glovebox before starting work.

Coupons can move in the bentonite due to swelling pressure and therefore sections should be cut 1 cm away from the anticipated coupon position (as shown for module 2a).

After the sectioning is completed, always remove the outer layer from all sections before retrieving coupons.

Coring coupons from each section is not recommended, rather, cut wedges with a knife, as described for module 2a.

Cutting the bentonite with a knife is more difficult than coring but is a “cleaner” way to work because the rotating coring unit mixes inside and outside regions of bentonite on the contact surface.

Having a wedge-shaped inner layer section makes it easier to identify regions that are actually from the center of the core or are relatively close to the outer layer.