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Research Article

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Impact of dry density and incomplete saturation on microbial growth in bentonite clay for nuclear waste storage

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Abstract

Aims: Many countries are in the process of designing a deep geological repository (DGR) for long-term storage of used nuclear fuel. For several designs, used fuel containers will be placed belowground, with emplacement tunnels being backfilled using a combination of highly compacted powdered bentonite clay buffer boxes surrounded by a granulated "gapfill" bentonite. To limit the potential for microbiologically influenced corrosion of used fuel containers, identifying conditions that suppress microbial growth is critical for sustainable DGR design. This study investigated microbial communities in powdered and gapfill bentonite clay incubated in oxic pressure vessels at dry densities between 1.1 g cm^{-3} (i.e. below repository target) and 1.6 g cm^{-3} (i.e. at or above repository target) as a 1-year time series.

Results: Our results showed an initial (i.e. 1 month) increase in the abundance of culturable heterotrophs associated with all dry densities <1.6 g cm^{-3}, which reveals growth during transient low-pressure conditions associated with the bentonite saturation process. Following saturation, culturable heterotroph abundances decreased to those of starting material by the 6-month time point for all 1.4 and 1.6 g cm^{-3} pressure vessels, and the most probable numbers of culturable sulfate-reducing bacteria (SRB) remained constant for all vessels and time points. The 16S rRNA gene sequencing results showed a change in microbial community composition from the starting material to the 1-month time point, after which time most samples were dominated by sequences associated with *Pseudomonas*, *Bacillus*, *Cupriavidus*, and *Streptomyces*. Similar taxa were identified as dominant members of the culture-based community composition, demonstrating that the dominant members of the clay microbial communities are viable. Members of the spore-forming *Desulfosporosinus* genus were the dominant SRB for both clay and culture profiles.

Conclusions: After initial microbial growth while bentonite was below target pressure in the early phases of saturation, microbial growth in pressure vessels with dry densities of at least 1.4 g cm^{-3} was eventually suppressed as bentonite neared saturation.

Impact Statement

Our results offer insight into the process of DGR saturation, as it relates to microbial growth, that will assist with modelling corrosion dynamics over geological timeframes.

Keywords: bentonite clay; DNA extraction; 16S rRNA gene; sequencing; cultivation

Introduction

Nuclear power is an important global source of energy and an integral component of a fossil-fuel independent future. After a fuel bundle is removed from a nuclear reactor, it remains highly radioactive and must be stored safely until radiation levels return to those of naturally occurring uranium ore, essentially indefinitely (Weber et al. 2009). To ensure isolation of used nuclear fuel from the environment, several countries are in the process of designing a deep geological repository (DGR). While the details vary from country to country, generally DGR designs propose to seal fuel bundles within long-lived used fuel containers (UFCs), which will be surrounded in a buffer material, often bentonite clay, that has low hydraulic conductivity and is inhospitable to microorganisms. These UFCs will be stored in DGR placement rooms, which will be situated deep belowground in suitable host rock. In the Canadian DGR design, UFCs will be placed into highly compacted bentonite (HCB) buffer boxes, which will be placed underground as a package. After emplacement, the space between the HCB boxes and host rock will be packed with a granulated "gapfill material" (GFM) bentonite (Noronha 2016). Both the HCB and GFM are types of processed bentonite that are formed from the application of high isostatic pressure (~100 MPa) or a roll compacting/milling process, of powdered Wyoming MX-80 bentonite, respectively. Wyoming MX-80 bentonite is a sodium bentonite with a mineral composition of ~81% montmorillonite, 3.4% muscovite, 3.5% plagioclase, 0.6% pyrite, and 3.0% quartz (Bengtsson and Pedersen 2017).

To ensure the long-term sustainability of a DGR, there are several important safety considerations. Within the context of microbiology, an important concern is the potential for microbiologically influenced corrosion (MIC), which is a process where corrosion of a surface is initiated or propagated

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by corrosive compounds produced by microbial metabolism. The process of MIC is well studied for a wide range of environments where metals, such as pipes, tubes, and bridge foundations (Gaines 1910, Von Wolzogen Kühr and van der Vlugt 1934, Sarioğlu et al. 2002, Al-Jaroudi et al. 2010, Yang et al. 2017), or non-metals, including concrete sewer systems (Herisson et al. 2017), are placed underground or underwater. For a DGR, MIC is important because it could lead to corrosion of the copper-coated UFCs via sulfide induced corrosion. Sulfate-reducing bacteria (SRB) can contribute to MIC because sulfide reacts directly with copper and copper oxides to produce copper sulfides and hydrogen gas, potentially fuelling additional SRB activity. Previous studies show that SRB are dominant members of anoxic subsurface microbial communities when sulfate is available (Purkamo et al. 2014, 2016, Bagnoud et al. 2016a,b, Wu et al. 2016, Hernsdorf et al. 2017, Bell et al. 2018, 2020, Boylan et al. 2019), and they have additionally been identified in the commercially available MX-80 bentonite proposed for use in a future DGR design (Masurat et al. 2010, Vachon et al. 2021). Further, Canadian groundwaters relevant to DGR placement contain sulfate (Behazin et al. 2021). Although SRB are a focus of microbiology research related to long-term nuclear waste storage, due to their potential to cause MIC, other microorganisms could also potentially influence a DGR, for example, through the formation of gases that could lead to permeability fissures in clay or host rock (Boylan et al. 2019).

An important focus of microbiology research related to long-term nuclear waste storage has involved identifying conditions necessary to suppress microbial growth. In addition to nutrient availability, which is expected to be low in the subsurface and for engineered barrier components (e.g. clay, metal), factors expected to influence microbial growth in bentonite clay are water activity, dry density, and swelling pressure (Motamedi et al. 1996, Kieft et al. 1997, Pedersen et al. 2000b, Stroes-Gascoyne et al. 2010a). Previous research demonstrated that the abundance of culturable heterotrophs and SRB did not increase when powdered Wyoming MX-80 bentonite samples were packed to a dry density of 1.6 g cm^{-3} when pore water (i.e. water used to saturate the bentonite plugs) salinity was $\leq$50 g l^{-1}, which corresponded to a water activity of 0.96 and a swelling pressure of 2 MPa (Stroes-Gascoyne et al. 2010a). Stable phospholipid fatty acid abundances reported in this same study led to an interpretation that, rather than being killed, microorganisms persisted as dormant cells or inactive endospores (Stroes-Gascoyne et al. 2010a). Another study investigating powdered Wyoming MX-80 bentonite clay under pressure showed similar nonlethal suppression of microbial growth at a dry density of 1.7 g cm^{-3} (Jalique et al. 2016). An additional study made use of bentonite-containing modules placed in boreholes, showing that bentonite-associated microbial communities were stable within the first year of incubation (Engel et al. 2019b). However, the added variable of saturation with groundwater that had its own microbial community, separate from the bentonite, makes interpretations of these results more challenging.

Although dry densities necessary to suppress microbial growth in Wyoming MX-80 bentonite clay have been proposed, how transferrable these same dry densities are to other product forms (e.g. GFM) such that the same suppression of microbial growth is achieved is not as well known. Previous studies have also focused on longer term incubations, which miss the changes in microbial abundance and community composition during the process of saturation that can be observed using a temporal short-term assessment approach. Although the saturation process is relatively short in a small pressure vessel, it is expected to span for 50–5000 years in a DGR (King et al. 2017), and thus any increased microbial abundance and activity during this time could have important implications in DGR evolution. Because DNA is difficult to extract from bentonite clay in sufficient quantities for downstream analyses, DNA-based techniques were rarely used to directly profile and quantify clay-associated microbial communities until recently. However, applying DNA-based techniques directly to clay from pressure vessels is an important complement to culture-dependent analyses for comprehensive characterization of the types of microorganisms capable of growing in bentonite at various dry densities.

The objective of this study was to analyze the microbiology of both powdered and gapfill Wyoming MX-80 bentonite clay to identify dry density conditions necessary to suppress microbial growth. To accomplish this, powdered or gapfill bentonite clay was packed in pressure vessels to dry densities between 1.1 (below repository target dry density) and 1.6 g cm^{-3} (at or above repository target dry density), sampled at time points between 1 and 12 months, and analyzed with a combination of culture-dependent and independent techniques to estimate microbial abundances and community composition within each pressure vessel as they relate to the process of saturation (Fig. 1).

Materials and methods

Pressure vessel setup and disassembly

Pressure vessels were filled with bentonite clay that was supplied by the Nuclear Waste Management Organization and compacted to dry densities of 1.25 and 1.6 g cm^{-3} for gapfill bentonite and to dry densities of 1.1, 1.4, and 1.6 g cm^{-3} for powdered bentonite (Fig. 1). One batch of powdered bentonite was used for all powdered bentonite pressure vessels. Two different batches of GFM were used for gapfill pressure vessels; all 1.6 g cm^{-3} 1-, 3-, and 18-month vessels, one of the two 1.6 g cm^{-3} 6-month vessels, and one of the two 1.25 g cm^{-3} 6-month vessels were filled with GFM one, and the remaining vessels were filled with GFM two. The mineral composition of each bentonite sample was determined using XRD analysis performed by Activation Laboratories (Table 1). The diameter and height of the clay plugs were 5.3 and 14.8 cm, respectively. The water activity and moisture content of the gapfill bentonite prior to saturation in a pressure vessel were measured to be 0.38 and 6.1%, respectively. The water activity and moisture content of the powdered bentonite were 0.42 and 10.5%, respectively. The pressure vessel casings were fabricated from 316 stainless steel, with detachable stainless-steel filter stones and end caps. Each pressure vessel was lined with a polytetrafluoroethylene (PTFE) inner casing, and all vessels were sterilized by exposure to 99.5% (v/v) acetone and then to 70% (v/v) ethanol. Pressure vessels were assembled and disassembled in an oxic environment using a hydraulic press and sterilized bentonite sampling tools, and were sealed for the duration of incubation. To accelerate saturation of bentonite clay with water during the experiments, vessels were connected to an AZURA P6.1 L HPLC pump and pressurized to 10 MPa with naturally aerated Type 1 water (resis-

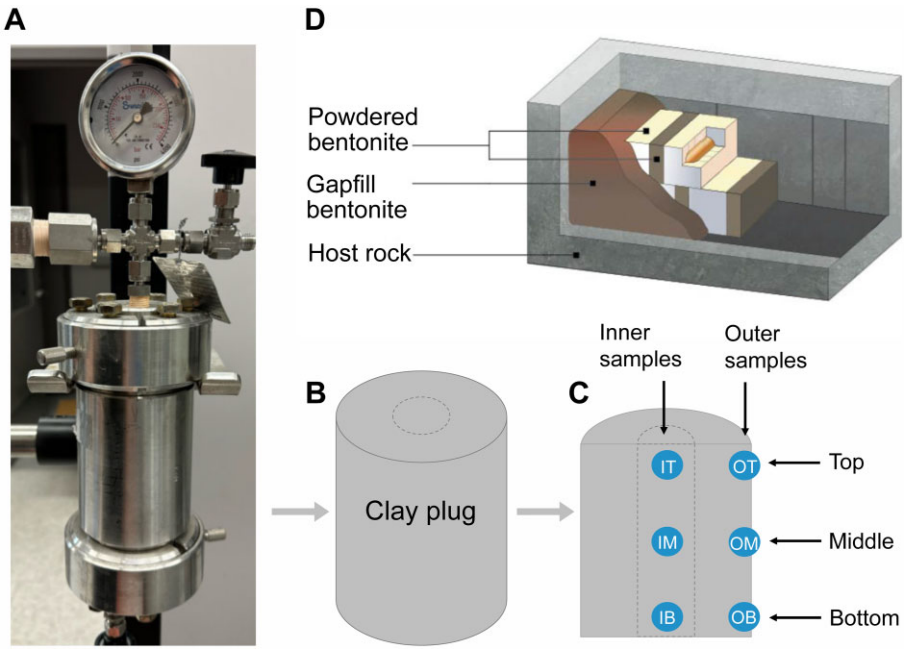

Figure 1. Photograph of a pressure vessel (A) and schematic of the clay plug within the pressure vessel (B) and the sample layers sampled: top, middle, and bottom of the inner core and the outer layer (C). Pressure vessels are filled with powdered or gapfill bentonite clay, the former being the raw material processed to produce HCB and GFM, which are both components of the theoretical design of a DGR (D; modified from King et al. 2017).

tivity = 18.2 MΩ cm^{-1}) from a purification system (Thermo Scientific). The bentonite was not amended with any nutrients for microorganisms.

Duplicate gapfill pressure vessels were incubated for 1, 3, and 6 months for each dry density. An additional single 1.6 g cm^{-3} dry density gapfill pressure vessel was incubated for 18 months. Duplicate powdered bentonite for 1.4 and 1.6 g cm^{-3} dry density pressure vessels were incubated for 1, 3–4, 6, and 12 months, and duplicate powdered bentonite 1.1 g cm^{-3} dry density pressure vessels for 1, 3, and 6 months. All pressure vessels were assembled, incubated, and disassembled at room temperature (21 ± 2°C).

Bentonite sample storage and preparation

After pressure vessel disassembly and bentonite coring at the University of Western Ontario (Fig. 1A, B), bentonite samples were shipped to the University of Waterloo on ice and transferred to 4°C storage upon arrival. Sampling of bentonite took place next to a Bunsen burner on sterile aluminum foil. The outer surfaces (i.e. surfaces in contact with the inside of the pressure vessel) of the three outer-layer bentonite samples (i.e. top, middle, and bottom, based on position in the pressure vessel) were removed with a sterile scalpel and are referred to throughout as "outer layer" samples (Fig. 1C). The outer surfaces of the inner core bentonite samples were removed with a sterile scalpel to avoid potential contamination introduced during the coring process. The inner core was then split into three separate samples (i.e. top, middle, and bottom, based on position in the pressure vessel). These core samples were cut into smaller pieces using a sterile scalpel and are referred to throughout as "inner layer" samples.

From each of six samples per pressure vessel, a 2-g aliquot was weighed into a sterile 50 ml conical tube and stored at 4°C for up to 24 h before cultivation. A second 2-g aliquot was added to a PowerBead Tube from the DNeasy PowerMax Soil Kit (Qiagen) and stored at −20°C prior to DNA extraction. A final portion of each sample was used to measure water activity and moisture content.

Water activity and moisture content

A WP4C Water Potential Meter (Decagon) was used to measure the water activity of each bentonite sample using the fast mode of the instrument. The same bentonite aliquots were then weighed and placed in an oven at 110°C for 24–48 h. Samples were weighed again when removed from the oven, and the difference in weight, before and after baking, was used to calculate sample moisture content (equation 1).

$$\text{Moisture content} = \frac{\text{mass before baking} - \text{mass after baking}}{\text{mass before baking}} \times 100\%. \quad (1)$$

Cultivation

A 2-g aliquot of each bentonite sample was added to 18 ml of phosphate-buffered saline (PBS; 8 g NaCl, 0.2 g KCl, 1.44 g Na_2HPO_4, and 0.24 g KH_2PO_4 in a final volume of 1 l of Type 1 water, with pH adjusted to 7.4 using HCl) solution in a 50-ml conical tube, then vortexed at low speed for 30 min at room temperature (~21°C). This clay suspension is referred to as "dilution A" throughout. After vortexing, two 10-fold serial dilutions were made in PBS from dilution A and are referred to as "dilution B" and "dilution C". Dilution A (100 μl) was added to six Reasoner's 2A (R2A; M1687, HiMedia Laboratories) agar plates and spread using a sterile glass spreader until dry. Dilutions B and C (100 μl) were added to three R2A agar plates each and spread using the same method as dilution A. Three dilution A, B, and C plates were incubated under oxic conditions in a 30°C incubator for 5–6 days. The remaining three dilution A plates were placed in a vacuum chamber (BVV3GSSW, BVV), which was flushed five times with a 90% N_2 and 10% CO_2 gas mixture. Each vacuum chamber contained a GasPak EZ anaerobe sachet (BD), to help remove

Table 1. Mineral composition of the three bentonites used.

	Concentration (wt%)									
	Montmorillonite	Quartz	Cristobalite	Plagioclase	K feldspar	Mica	Clinoptilolite	Kaolinite	Calcite	Gypsum	Anatase
GFM 1	83.4	1	n.d.	7.4	3.7	1.8	n.d.	n.d.	2.7	Trace	n.d.
GFM 2	73.7	1.6	2.7	10.4	2.9	2	6.7	n.d.	Trace	Trace	n.d.
Powdered bentonite	87	2.3	n.d.	5.3	2.8	1.2	n.d.	n.d.	1.4	Trace	n.d.

"n.d." indicates that the mineral was not detected.

residual oxygen present after flushing, and an anaerobic indicator strip (BD), to verify anoxic conditions throughout the incubation. Vacuum chambers were placed in a 30°C incubator for 28 days. After incubation, colonies were counted on each plate, and the number of colony-forming units per gram (CFU g^{-1}) was calculated for each sample. A sterile foam-tipped applicator (Puritan) was used to swab colonies from each plate, and swabs were stored at −20°C until DNA extraction. This culture biomass was collected for DNA sequencing for the purpose of identifying taxa that are viable in the clay samples. Not all viable microorganisms in the clay can necessarily grow in the cultures, but comparing the culture 16S rRNA gene profiles to the clay profiles can provide information about the viability of the microorganisms associated with the clay profiles, which would not otherwise be possible through sequencing of clay DNA alone.

One ml of dilution A, B, and C for each bentonite sample was added to each of 5 test tubes (i.e. 15 test tubes total for each bentonite sample) filled with 9 ml of SRB enrichment medium (M803, HiMedia Laboratories). Test tubes were placed in a vacuum chamber and flushed as described above and incubated under anoxic conditions at 30°C. After 28 days of incubation, positive tubes were identified by the presence of black ferrous sulfide precipitate, and the most probable number (MPN) method was used to estimate the number of SRB present per gram of original clay sample (U.S. Food and Drug Administration 2020). From each positive SRB tube, 1 ml was collected and centrifuged at 8000 × *g* for 10 min to pellet cells. Supernatants were discarded, and cell pellets stored at −20°C prior to DNA extraction.

Using the moisture content measured for each sample, each value of CFU g^{-1} and MPN g^{-1} was converted to a value of CFU or MPN per gram dry weight (gdw) (equation 2).

$$\text{abundance gdw}^{-1} = \frac{\text{abundance g}^{-1}}{(100 - \text{moisture content}) \times 0.01}. \quad (2)$$

Abundances of culturable microorganisms from the pressure vessels were compared to the abundances of culturable microorganisms in the dry clay starting material using the Mann–Whitney U Test. A non-parametric test was selected as the data was not normally distributed as determined by the Shapiro–Wilk test for normality. Both statistical tests were performed in R.

DNA extraction

Genomic DNA was extracted from a 2-g aliquot of each bentonite sample using the DNeasy PowerMax Soil Kit (Qiagen), from control swabs using the DNeasy PowerSoil Kit (Qiagen), and from culture biomass using the DNeasy UltraClean Kit (Qiagen). A "kit control" containing no sample was included for each extraction batch. The manufacturer's instructions were followed with the following changes. After addition of lysis solution, PowerBead Tubes were incubated at 70°C for 10 min for the PowserSoil and UltraClean extractions and at 65°C for 30 min for the PowerMax extractions. This was followed by beadbeating using a FastPrep-24 Classic Instrument (MP Biomedicals) at 5.5 m s^{-1} for 45 s for the PowerSoil and UltraClean Kit extractions and beadbeating using an MM 400 Mixer Mill (Retsch) at 30 Hz for 10 min for the PowerMax extractions. Final elution volumes for the PowerSoil, UltraClean, and PowerMax extractions were 60 μl, 50 μl, and 2 ml, re-

spectively. The PowerMax DNA extraction method used here was validated previously for clay samples (Engel et al. 2019a). DNA samples were stored at −20°C until further analysis.

Quantitative PCR

Bacterial 16S rRNA genes were quantified in duplicate for each bentonite sample using the CFX96 Real-Time PCR Detection System (Bio-Rad). The total volume of each PCR was 15 μl , which consisted of 1x Sso Advanced Universal SYBR Green Supermix (Bio-Rad), 0.3 μM each of forward primer 341F and reverse primer 518R (Muyzer et al. 1993), 7.5 μg of bovine serum albumin (BSA), and 2 μl of template DNA. The following thermocycler conditions were used: 98°C initial denaturation for 3 min, followed by 40 cycles of 98°C for 15 s and 55°C for 30 s. For the standard curve, the V3–V5 region of the Thermus thermophilus 16S rRNA gene was previously cloned into the pUC57-Kan Plasmid, flanked by M13F/R primer binding sites. The M13 primers were used to amplify the 16S rRNA gene fragment, which was then gel purified and stored at −20°C prior to use for the qPCR standard curve. Each aliquot of the standard DNA was thawed and quantified using the Qubit dsDNA High Sensitivity Assay Kit (Invitrogen) with 4 μl of sample per Qubit quantification immediately prior to preparing the qPCR standard curve.

Bacterial 16S rRNA gene copy numbers were calculated using linear regression of the standard curve based on the Qubit quantification of the standard. The starting quantity (SQ) results from the qPCR assay for each bentonite DNA sample were converted to values of 16S rRNA gene copies gdw^{-1}, and SQ values of no template controls (NTCs) on the same plate were subtracted (equation 3).

$$\text{16S rRNA gene copes gdw}^{-1} = \frac{(\text{average SQ sample} - \text{average SQ of NTCs}) \times 2000\ \mu\text{l elution in DNA extraction}}{2\ \mu\text{l template in qPCR reaction} \times 2\ \text{g clay in DNA exctraction}}. \quad (3)$$

Amplicon sequencing

The V4–V5 region of the 16S rRNA genes from each genomic DNA extract was amplified in triplicate in 96 well plates with universal primers 515F-Y (Parada et al. 2016) and 926R (Quince et al. 2011), modified to contain a unique six-base index sequence and flow cell binding and sequencing sites (Bartram et al. 2011). The PCR setup was performed in a PCR hood (AirClean Systems) with ISO 5-HEPA-filtered air and surfaces treated with UV light for 15 min before each use. All tubes, PCR water, and BSA were UV treated (302 nm) for 20 min on a transilluminator (ProteinSimple). The final volume of each PCR was 25 μl and consisted of 1x ThermoPol Buffer (New England BioLabs), 0.2 μM of each primer, 200 μM dNTPs, 15 μg BSA, 0.625 U hot start Taq DNA polymerase (New England BioLabs), and 1 μl of template DNA. The following thermocycler conditions were used: 95°C initial denaturation, followed by 35 or 50 cycles of 95°C denaturation for 30 s, 50°C annealing for 30 s, and 68°C extension for 1 min, followed by a final extension at 68°C for 7 min. Bentonite DNA samples were amplified for 50 cycles, and culture DNA samples were amplified for 35 cycles. Negative PCR controls containing no template DNA (NTCs) were included in each 96 well plate to test for cross-contamination between wells of the plate and NTCs were also prepared in tubes separate from the plate for each master mix to test for contamination.

Triplicate uniquely indexed PCR products were visualized on a 1% agarose gel containing ethidium bromide and were then pooled and quantified on a 1% agarose gel containing GelRed (Biotium). Based on gel quantification, equimolar volumes of each amplicon were pooled into a single tube. Negative PCR controls (in plate and out of plate NTCs) and PCR-amplified DNA kit controls were added to the pool (5 μl each), even if amplification was not detected on the agarose gel. Pooled 16S rRNA gene amplicons were excised from a 1.5% agarose gel and purified using the Wizard SV Gel and PCR Clean-Up System (Promega). The purified library was quantified using the Qubit dsDNA High Sensitivity Assay Kit (Invitrogen) and by qPCR. The library was denatured, diluted to 8 pM, and mixed with 15% PhiX control v3 (Illumina) following Illumina guidelines (Document No. 15039740v01). All samples were sequenced across five runs on a MiSeq System using a 2 × 250 cycle MiSeq Reagent Kit v2 (Illumina) with an average cluster density of 799 000 clusters mm^{-2} and average clusters passing filter of 83.8%.

Sequence analysis

Sequences were demultiplexed using Local Run Manager version 4.0.0.1769 and analyzed using Quantitative Insights Into Microbial Ecology 2 (Bolyen et al. 2019) and Divisive Amplicon Denoising Algorithm 2 (DADA2; Callahan et al. 2016), managed by Automation, Extension, and Integration of Microbial Ecology (AXIOME3; Min et al. 2021). Paired-end reads were imported into Quantitative Insights into Microbial Ecology 2 (QIIME2), and DADA2 was used to remove low quality reads and primer sequences and to truncate forward and reverse reads to 250 bases. The DADA2 pipeline was additionally used to denoise and dereplicate paired-end reads and generate amplicon sequence variants (ASVs). Taxonomy was assigned to ASVs using a naïve Bayes classifier pre-trained with SILVA database release 138 (Heidrich et al. 2021). Potential contaminant ASVs were identified using the Decontam R package (Davis et al. 2018) with a threshold of 0.5. For each MiSeq System run, clay samples and culture samples were processed through Decontam separately. The list of potential contaminants produced by Decontam was analyzed manually and likely contaminants were removed from the ASV table; this final ASV table was used for all subsequent analyses (Supplementary File S1). Samples were normalized to 5000 reads using the scaling with ranked subsampling (SRS) package in R (Heidrich et al. 2021), and this normalized ASV table was used to compute Bray–Curtis distances in Python (Fig. 5).

All sequence data were deposited in the European Nucleotide Archive with accession number PRJEB56656.

Results

Microbial abundance and growth

Prior to saturation in a pressure vessel, both gapfill and powdered bentonite had similar mineral compositions (Table 1), had relatively low abundances of culturable heterotrophs and SRB, and had 16S rRNA gene abundances greater than the combined numbers of culturable microorganisms detected (Fig. 2). The number of 16S rRNA gene copies derived from qPCR (P = .49), culturable aerobic heterotrophs derived from plate counts (P = .38), and culturable SRB derived from MPN tests (P = .11) were not significantly different between the powdered starting material and the gap-

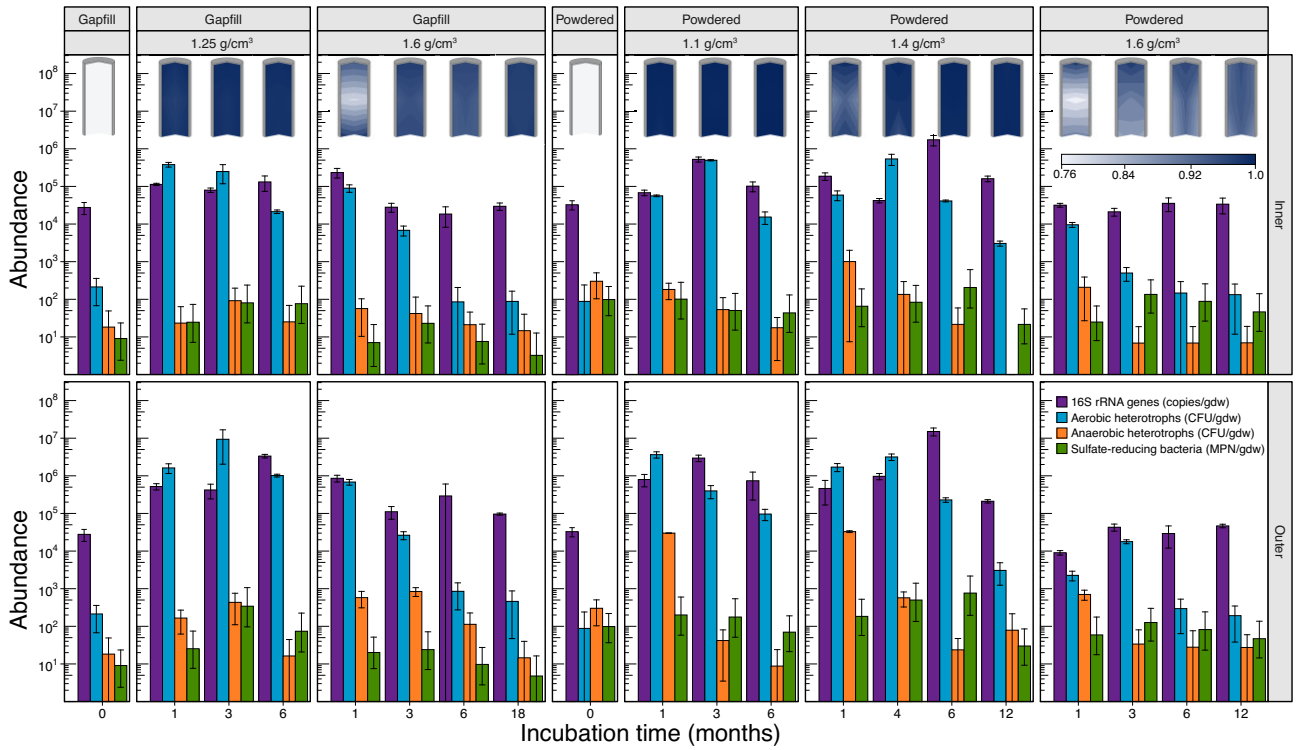

Figure 2. Abundance of 16S rRNA gene copies gdw^{-1}, aerobic and anaerobic heterotroph CFU gdw^{-1}, and SRB MPN gdw^{-1} for inner core (top) and outer layer (bottom) bentonite samples. Measures of CFU g^{-1} <2000 are below the limit of quantification for this method (as fewer than 20 colonies on a plate are considered statistically insignificant). Error bars represent the standard deviation of replicates for values of 16S rRNA gene copies gdw^{-1} and CFU gdw^{-1}, and represent average 95% confidence intervals specified by the MPN table for values of MPN gdw^{-1}. The contour plots (top of the figure) represent the water activity throughout the pressure vessel, generated using the average measured water activity values from the six sampled locations within each pressure vessel.

fill starting material. There were significantly more culturable anaerobic heterotrophs ($P = .04$) in the powdered starting material compared to the gapfill, but all abundance measurements were within the range of measurements of culturable anaerobic heterotrophs and SRB previously reported for similar powdered MX-80 bentonite clays (Vachon et al. 2021).

Within the first month of pressure vessel incubation, there was a significant increase in the average abundance of culturable aerobic heterotrophs in the six locations of each pressure vessel at all dry densities <1.6 g cm^{-3} (1.1 g cm^{-3} $P = .01$, 1.25 g cm^{-3} $P = .01$, 1.4 g cm^{-3} $P = .01$, 1.6 g cm^{-3} gapfill $P = .08$, 1.6 g cm^{-3} powdered $P = .13$). The number of culturable aerobic heterotrophs measured in each of the 1.6 g cm^{-3} dry density pressure vessels after 1 month of incubation varied. Although the average number of culturable aerobic heterotrophs across the six sampling locations of these 1.6 g cm^{-3} pressure vessels was not significantly greater than the abundance in the starting materials, several individual samples had significantly higher abundances of culturable aerobic heterotrophs than the starting material (data not shown). After the increase in abundance of culturable aerobic heterotrophs within the first month of pressure vessel incubation, the average abundance decreased to the level of the starting material in subsequent time points for the 1.4 and 1.6 g cm^{-3} dry density pressure vessels (Fig. 2). The average abundance of culturable aerobic heterotrophs in the 1.1 and 1.25 g cm^{-3} dry density pressure vessels remained significantly higher than that of the starting material at all time points.

Fewer anaerobic heterotrophs were cultured from the pressure vessel bentonite samples compared to aerobic heterotrophs, and most of these values were below the limit of quantification (2000 CFU g^{-1}, which corresponds to 20 colonies counted on each replicate agar plate). Although below the limit of quantification, the average number of anaerobic heterotrophs cultured from the 3-month 1.25 g cm^{-3} ($P = .04$) and from the 1-month 1.6 g cm^{-3} ($P = .02$) gapfill pressure vessels was significantly higher than starting material. The average increases in the abundance of anaerobic heterotrophs in the outer layer of both the 1-month 1.1 g cm^{-3} ($P = .01$) and 1.4 g cm^{-3} ($P = .03$) powdered bentonite pressure vessels were both above the limit of quantification of the method and significantly higher than the starting material. By the 6-month time point, the average numbers of culturable anaerobic heterotrophs in the gapfill pressure vessels returned to the same number as for starting material, and in the powdered bentonite pressure vessels the average numbers of culturable anaerobic heterotrophs were significantly less than for starting material.

For most samples, MPN estimates for SRB abundances were lower than culturable heterotroph abundances (Fig. 2). The average number of SRB cultured from the six sampled locations of each set of duplicate powdered bentonite pressure vessels, and from each set of duplicate 1.6 g cm^{-3} gapfill pressure vessels, never significantly exceeded the number of SRB cultured from the starting material. There was a significant increase in the average number of cultured SRB at the 3-month time point of the 1.25 g cm^{-3} gapfill pressure vessels com-

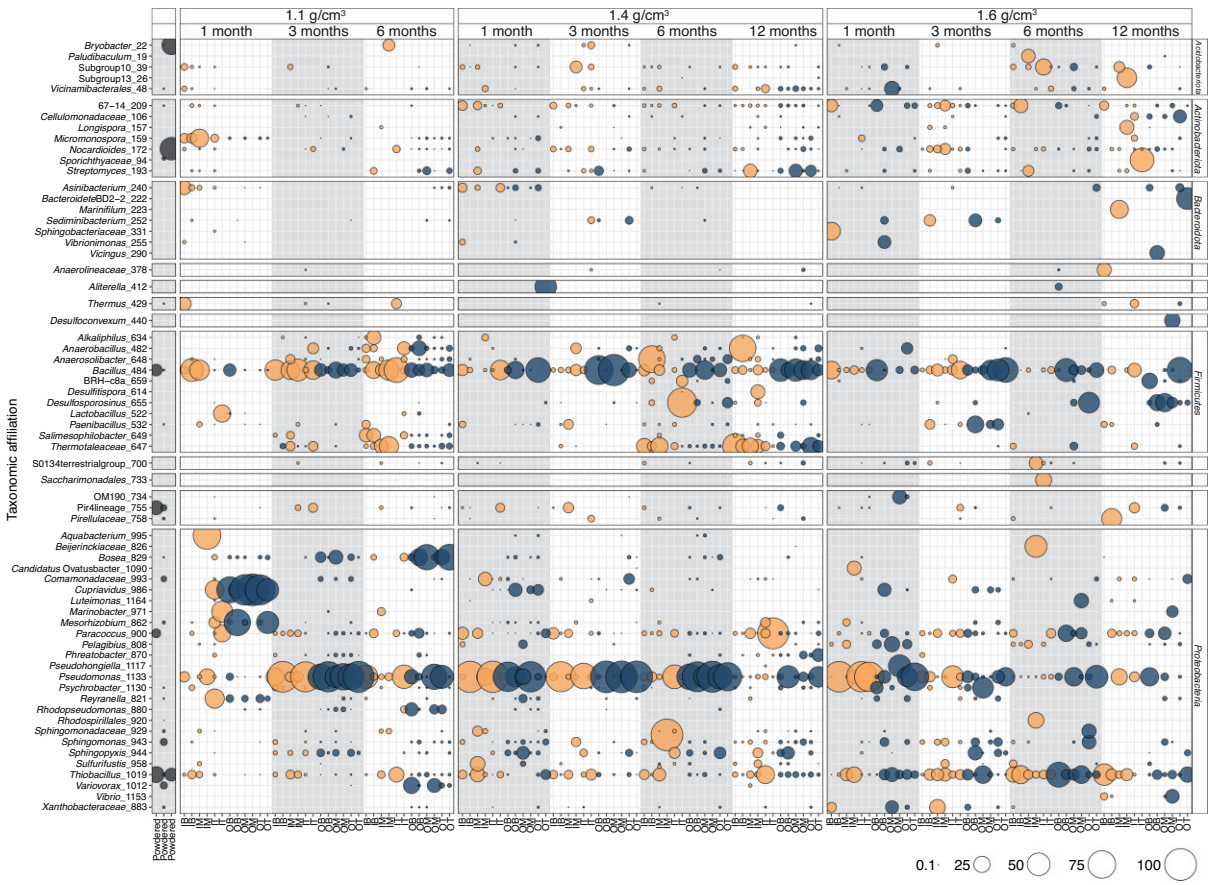

Figure 3. Bubble plot showing the 16S rRNA gene profiles of the powdered bentonite pressure vessel samples. Only genera at or above 15% relative abundance are shown. IB, IM, IT, OB, OM, and OT (*x*-axis) refer to the location in the pressure vessel (inner bottom, inner middle, inner top, outer bottom, outer middle, outer top; see Fig. 1). Bubbles are coloured by inner layer (orange) and outer layer (blue). Pressure vessel target dry density and incubation time are indicated at the top of the figure.

pared to the starting material ($P = .03$), but this returned to an abundance statistically the same as the starting material by the 6-month time point ($P = .13$).

Microbial characterization of pressure vessel clay samples

Very few taxa were detected in more than one replicate of the powdered bentonite starting material (Fig. 3), reflecting a low biomass and heterogeneous starting material. The genera detected in more than one replicate were *Bryobacter*, *Nocardioides*, *Bacillus*, PIR4 lineage, and *Thiobacillus*, with *Thiobacillus* being the only genus detected in all replicates. These genera have previously been detected in other powdered MX-80 bentonite samples (Engel et al. 2019a, Vachon et al. 2021). The gapfill bentonite starting material also had replicate 16S rRNA gene profiles with few overlapping taxa detected (Fig. 4). Genera detected in more than one replicate were *Amycolatopsis*, *Arthrobacter*, *Glycomyces*, *Micrococcacaea*, *Nocardioides*, *Streptomyces*, *Comamonadaceae*, and *Paracoccus*. Lack of consistency among replicates could be due to low read counts for certain replicates or could reflect heterogeneity in the distribution of microorganisms in the clay starting material. Microbial community profiles associated with the starting material were distinct from the 1-month time point samples for all dry densities and both clay types, after which similar dominant taxa were detected (Figs 3 and 4).

Microbial community profiles associated with the starting material were distinct from the 1-month time point samples for all dry densities and both clay types, after which similar dominant taxa were detected (Figs 3 and 4). Most pressure vessel samples had high relative abundances of ASVs associated with *Bacillus* and *Pseudomonas*, especially the powdered bentonite pressure vessel samples. Sequences associated with other genera of primarily aerobic heterotrophs, including *Cupriavidus* and *Streptomyces*, were also detected in several samples, especially from the 1.1 and 1.25 g cm^{-3} dry density pressure vessels at relative abundances greater than the starting material (Figs 3 and 4).

The only genera of SRB detected in powdered bentonite pressure vessel samples at a minimum relative abundance of 10% were *Desulfosporosinus*, *Desulfitispora*, and *Desulfoconvexum*, which were detected in 20, 1, and 1 sample, respectively (Fig. 3). *Desulfosporosinus* was the only genus of SRB detected in more than one gapfill pressure vessel sample and was the only putative SRB present at higher than 10% relative abundance in any sample (Fig. 4). The only other genus of putative SRB found in both gapfill and powdered bentonite was *Halodesulfovibrio*, which was detected in one gapfill and one powdered bentonite pressure vessel sample at 1.8% and 0.58% relative abundance, respectively (Supplementary File S1). The remaining genera

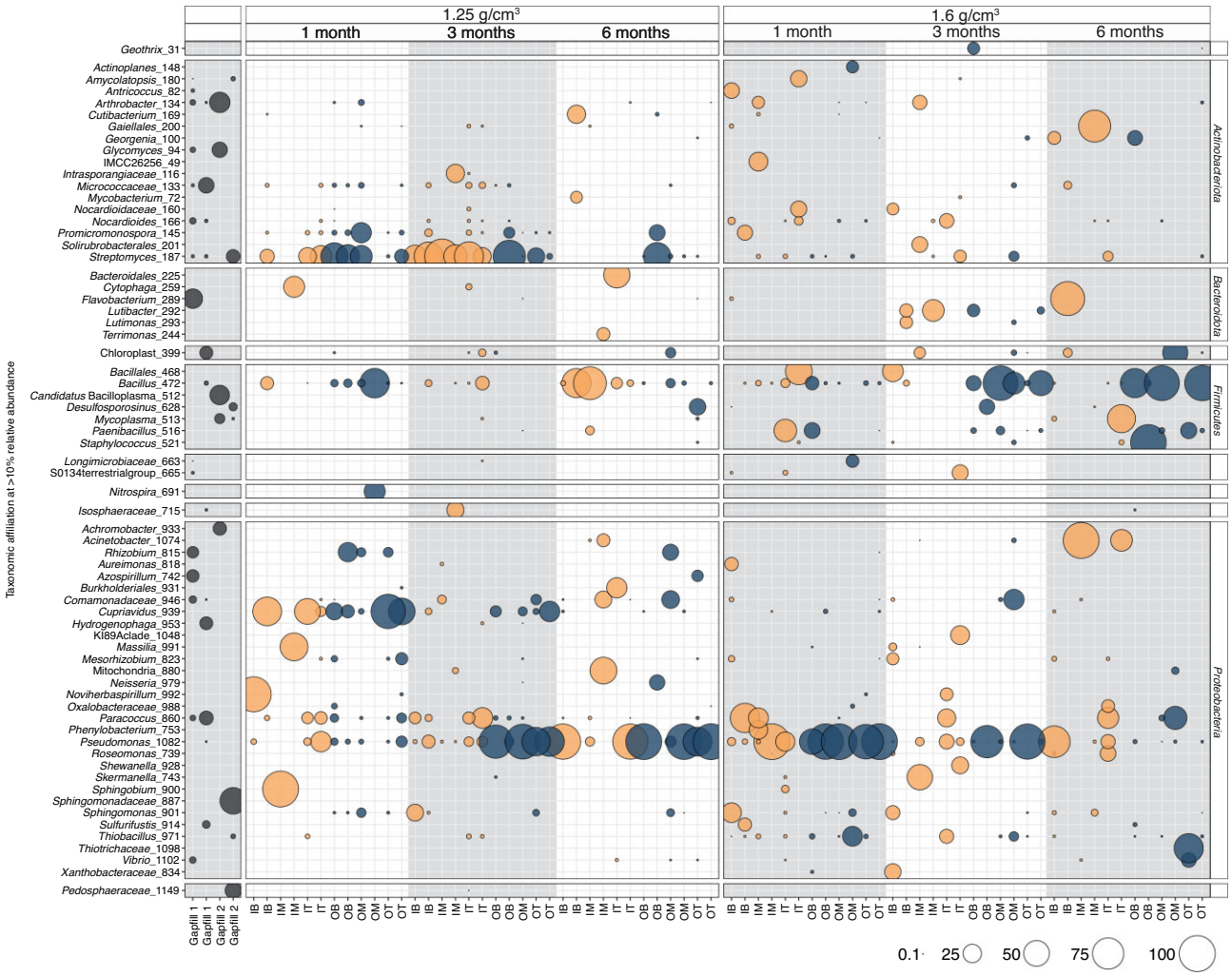

Figure 4. Bubble plot showing the 16S rRNA gene profiles of the gapfill bentonite pressure vessel samples. Only genera at or above 10% relative abundance are shown. IB, IM, IT, OB, OM, and OT (*x*-axis) refer to the location in the pressure vessel (inner bottom, inner middle, inner top, outer bottom, outer middle, outer top; see Fig. 1). Bubbles are coloured by inner layer (orange) and outer layer (blue). Pressure vessel target dry density and incubation time are indicated at the top of the figure.

of SRB, each detected in a single powdered pressure vessel sample, were affiliated with *Desulfobacca*, *Desulfoconvexum*, *Desulfuromonadaceae*, *Desulfuromonas*, *Desulfitobacterium*, *Desulfurispora*, and *Desulfitibacter*, each of which was detected at relative abundances <1%. Additional genera of SRB, each detected in a single gapfill pressure vessel sample, were *Desulfobacter*, *Desulfatitalea*, and *Desulfovibrio*, each detected at relative abundances between 0.39% and 8.82%.

Microbial characterization of pressure vessel culture samples

The 16S rRNA gene profiles for anaerobic heterotroph cultures were dominated by very few ASVs, mostly from the genera *Pseudomonas*, *Cupriavidus*, *Deftia*, and *Staphylococcus* (Fig. S2, Fig. 5). The 16S rRNA gene profiles of aerobic heterotroph and SRB cultures were dominated by sequences associated with genera similar to those detected in the clay samples from direct DNA extraction and sequencing. The most abundant genera detected in these cultures were *Pseudomonas* and *Bacillus* (Fig. S2, Fig. 5). Because of how SRB biomass was collected and that most tubes positive for SRB were from the lowest dilutions, it is likely that at least a portion of the DNA

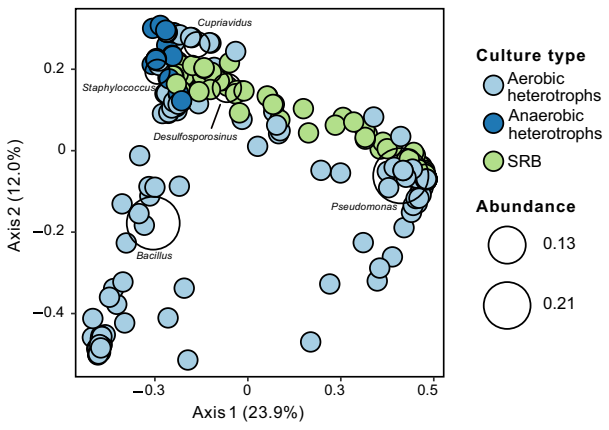

Figure 5. Principle coordinate analysis biplot based on Bray–Curtis distances of SRB, aerobic heterotrophs, and anaerobic heterotroph 16S rRNA gene profiles. Genera ≥5% relative abundance are displayed as clear circles. Samples were normalized to 5000 reads using the SRS package in R.

sequenced from these cultures came from the clay itself rather than the microorganisms that grew in the culture.

The dominant genus detected in SRB cultures was *Desulfosporosinus*, which was represented by 120 ASVs in the 16S rRNA gene profiles of 70% of the SRB cultures at an average combined relative abundance of 29% (Supplementary File S1). Three other genera of known SRB were detected in the SRB culture 16S rRNA gene profiles: *Desulfurispora*, which was represented by two ASVs and was detected in 11% of SRB cultures at an average relative abundance of 0.5%, *Desulfotomaculum*, which was represented by five ASVs and was detected in 5% of SRB cultures at an average relative abundance of 0.6%, and *Desulfallas*, which was represented by three ASVs and was detected in a single SRB culture at a relative abundance of 9% (Supplementary File S1). In total, 35 out of the 140 positive SRB culture tubes had no known SRB detected in the 16S rRNA gene profiles. Several of these samples had very few sequences due to low biomass, and likely represent culture tubes that had relatively small amounts of black precipitate and consequently low relative abundances of SRB.

Discussion

An increase in average abundance of culturable aerobic heterotrophs was observed in all pressure vessels with dry densities <1.6 g cm^{-3}, and in several individual samples from the 1.6 g cm^{-3} pressure vessels (Fig. 2). The variation in moisture content and water activities of the six sampled locations within the 1.6 and 1.4 g cm^{-3} density 1- and 3-month time points indicates that these high dry density pressure vessels were not fully saturated at the 1-, 3-, and possibly 6-month time points (Fig. 2 contour plots, Fig. S1). As such, the results indicate that the initial increase in abundance of culturable microorganisms for the high dry density samples likely occurred as water became available prior to complete pressure vessel saturation, when the pressure within the pressure vessel was still relatively low (Fig. 2). A previous study using powdered MX-80 bentonite clay in pressure vessels showed a suppression of microbial growth at measured dry densities of at least 1.6 g cm^{-3} and showed no increase in the abundance of culturable microorganisms throughout the 40–90 day experiment (Stroes-Gascoyne et al. 2010b). This difference may be explained by the smaller size of clay plugs in that study (2–2.5 × 1.6 cm) that would be expected to saturate more quickly than those used in the present study (5.3 × 14.8 cm). For the Canadian design, a DGR is expected to take 50–5000 years to fully saturate, depending on whether the host rock is crystalline or sedimentary (King et al. 2017), and so these results suggest that a similar increase in abundance of heterotrophs following the saturation front, in areas with available water and below-target swelling pressure, may be expected. By the final time point, the average abundance of culturable aerobic heterotrophs was statistically the same as the starting material, suggesting that microbial growth is eventually suppressed by a dry density of at least 1.4 g cm^{-3}.

Although an initial growth of aerobic heterotrophs was observed in several pressure vessel samples, the results of this study do not suggest an increase in the abundance of SRB throughout the saturation process if the target dry density is at least 1.4 g cm^{-3}. Oxygen in the DGR is predicted to be consumed within 1.5 years (King et al. 2017), well before the DGR is fully saturated. Because known SRB are strictly anaerobic, future pressure vessel experiments conducted under anoxic conditions will provide additional insight into potential pre-saturation increases in SRB abundance during the anoxic phase of the DGR.

Most pressure vessel clay samples had high relative abundances of ASVs associated with *Bacillus* and *Pseudomonas*, especially the powdered bentonite pressure vessel samples. Members of the *Pseudomonas* genus have previously been identified within bentonite profiles retrieved from borehole modules, although these were suggested to derive from the groundwater rather than clay itself (Engel et al. 2019b). Because the pressure vessels were saturated with pure water, the results of the present study suggest that viable *Pseudomonas* cells are present in both powdered and gapfill MX-80 bentonite clay and proliferate during bentonite saturation. In a previous study, *Bacillus* was the dominant genus cultured directly from dry clay samples on both oxic R2A, as well as a denitrifying bacteria enrichment medium (Vachon et al. 2021). Most *Pseudomonas* and *Bacillus* representatives are putative aerobic chemoheterotrophic bacteria, and a previous study demonstrated that spore-forming species of *Bacillus* survived better than other types of bacteria in spiked and compacted bentonite samples (Pedersen et al. 2000a). Others have demonstrated that specific *Pseudomonas* representatives can withstand desiccation by producing internal solutes to increase cell turgor pressure, and montmorillonite within bentonite has additionally been shown to increase the survivability of desiccation-resistant microorganisms in low water content environments (Dandurand et al. 1994). Sequences associated with other genera of primarily aerobic heterotrophs, including *Cupriavidus* and *Streptomyces*, were detected in several samples, especially from the 1.1 and 1.25 g cm^{-3} dry density pressure vessels at relative abundances greater than the starting material. These results suggest that *Pseudomonas*, *Bacillus*, and to a lesser degree *Cupriavidus* and *Streptomyces* may be amongst the taxa expected to grow initially in a DGR as saturation occurs and while oxygen remains available.

Previous work demonstrated that most microorganisms that grew in cultures from dry as-received clay did not overlap with the dominant microorganisms detected in the 16S rRNA gene profiles of the clays (Vachon et al. 2021). However, in the present study, the dominant members of the 16S rRNA gene profiles of the culture and clay samples often did overlap. The overlap of dominant genera detected in the 16S rRNA gene profiles of the clays and the cultures suggests that the culturing methods applied are targeting the dominant microorganisms present, and that the dominant microorganisms detected in the clay 16S rRNA gene profiles are viable.

Although SRB were not dominant members of the pressure vessel clay 16S rRNA gene profiles, the genera *Desulfosporosinus*, *Desulfitispora*, and *Desulfoconvexum* were all detected at relative abundances of >10% in at least one clay sample. *Desulfosoporosinus* was the dominant genus of SRB detected in the SRB cultures, although genera *Desulfurispora*, *Desulfotomaculum*, and *Desulfallas* were all also detected in some SRB cultures at low relative abundance. These four genera of SRB are all spore-forming members of the *Firmicutes* phylum (Watanabe et al. 2018), and all but *Desulfosporosinus* are known to be thermophilic or moderately thermophilic (Kaksonen et al. 2007, Nandasena et al. 2014, Watanabe et

al. 2018). The dominance of *Desulfosporosinus* in the cultures and the clays compared to the other genera could be attributed to the pressure vessel incubation (i.e. room temperature) and culture (i.e. 30°C) conditions that were more suited to its growth than to that of the other SRB detected. Taxa affiliated with *Desulfosporosinus* have also previously been identified as dominant SRB representatives in as-received dry bentonite and bentonite clay exposed to high swelling pressures (Haynes et al., 2018; Engel et al., 2019b; Gilmour et al., 2021; Vachon et al., 2021). Because the temperature of a DGR is expected to change, temporally and spatially, there is potential for different types of SRB to occupy different temperature niches. For example, in a study looking at the response of bentonite-associated microbial communities to heat, the dominant SRB switched from *Desulfosporosinus* to *Desulfotomaculum* before and after a 24-h heat incubation at 90°C (Haynes et al. 2018). The four genera of SRB detected in the cultures were also detected in the 16S rRNA gene profiles of bentonite in a study where bentonite was incubated in an excess of sterile groundwater, sometimes as dominant members of the 16S rRNA gene profiles (Miettinen et al. 2022). Future research should explore the viable SRB in MX-80 hydrated bentonite clays incubated at a range of DGR-relevant temperatures.

Conclusions

Studying the suppression of microbial growth in bentonite clay exposed to varying dry densities is necessary for informing DGR design and for assisting with modelling corrosion dynamics over geological timeframes. A key goal of this study was to determine the minimum dry density necessary to suppress microbial growth in powdered and gapfill bentonite clay, as well as to study the types of microorganisms present and viable under the different dry density conditions. The results of this study demonstrate that, under oxic conditions, microbial growth is suppressed by a dry density of 1.6 g cm^{-3} in both powdered and gapfill bentonite, and likely by a dry density of 1.4 g cm^{-3} as well once saturation is complete. Both the culturing and DNA-based results presented here show an initial increase in the number of aerobic heterotrophs in pressure vessels with dry densities $<$1.6 g cm^{-3}, and in several individual samples from the 1.6 g cm^{-3} dry density pressure vessels. This is proposed to be due to the slow and uneven saturation of high dry density pressure vessels allowing for growth of microorganisms in areas with available water and below-target pressures. A similar trend is possible in a DGR, where saturation is expected to take many years. The 16S rRNA gene profiles showed a switch from starting material with low abundance ASVs to pressure vessel bentonite samples dominated by sequences associated with *Bacillus*, *Pseudomonas*, *Cupriavidus*, and *Streptomyces* genera. These genera could be amongst those expected to grow in a DGR environment prior to complete saturation if the elevated temperature surrounding the UFCs does not preclude their growth. *Desulfosporosinus* was the dominant genus of SRB detected in culture 16S rRNA gene profiles from most samples. The experiments in the present study were conducted under oxic conditions, which would be expected to preclude the growth of strictly anaerobic SRB while the oxygen persisted in the pressure vessels. The next step of this research will be to conduct the experiments under anoxic conditions to investigate if SRB do increase in abundance in bentonite at high dry densities when their preferred oxygen requirements are met.

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Supplementary data

Supplementary data is available at *JAMBIO Journal* online.

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Author contributions

Rachel C. Beaver (Conceptualization, Formal analysis, Investigation, Methodology, Validation, Visualization, Writing – original draft, Writing – review & editing), Melody A. Vachon (Investigation, Methodology, Writing – review & editing), Claire S. Tully (Conceptualization, Investigation, Methodology, Writing – review & editing), Katja Engel (Conceptualization, Investigation, Methodology, Project administration, Supervision, Writing – review & editing), Emilie Spasov (Investigation, Methodology, Writing – review & editing), W. Jeffrey Binns (Conceptualization, Funding acquisition, Project administration, Resources, Writing – review & editing), James J. Noël (Conceptualization, Funding acquisition, Methodology, Project administration, Resources, Supervision, Writing – review & editing), and Josh D. Neufeld (Conceptualization, Formal analysis, Funding acquisition, Investigation, Project administration, Resources, Supervision, Visualization, Writing – review & editing)

Data availability

The DNA sequencing data are available in the European Nucleotide Archive (ENA) with project accession number PRJEB56656. The ASV table used for analysis is included as Supplemental_file_S1.

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