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Fifteen shades of clay: distinct microbial community profiles obtained from bentonite samples by cultivation and direct nucleic acid extraction

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Characterizing the microbiology of swelling bentonite clays can help predict the long-term behaviour of deep geological repositories (DGRs), which are proposed as a solution for the management of used nuclear fuel worldwide. Such swelling clays represent an important component of several proposed engineered barrier system designs and, although cultivation-based assessments of bentonite clay are routinely conducted, direct nucleic acid detection from these materials has been difficult due to technical challenges. In this study, we generated direct comparisons of microbial abundance and diversity captured by cultivation and direct nucleic acid analyses using 15 reference bentonite clay samples. Regardless of clay starting material, the corresponding profiles from cultivation-based approaches were consistently associated with phylogenetically similar sulfate-reducing bacteria, denitrifiers, aerobic heterotrophs, and fermenters, demonstrating that any DGR-associated growth may be consistent, regardless of the specific bentonite clay starting material selected for its construction. Furthermore, dominant nucleic acid sequences in the as-received clay microbial profiles did not correspond with the bacteria that were enriched or isolated in culture. Few core taxa were shared among cultivation and direct nucleic acid analysis profiles, yet those in common were primarily affiliated with *Streptomyces*, *Micrococcaceae*, *Bacillus*, and *Desulfosporosinus* genera. These putative desiccation-resistant bacteria associated with diverse bentonite clay samples can serve as targets for experiments that evaluate microbial viability and growth within DGR-relevant conditions. Our data will be important for global nuclear waste management organizations, demonstrating that identifying appropriate design conditions with suitable clay swelling properties will prevent growth of the same subset of clay-associated bacteria, regardless of clay origin or processing conditions.

Numerous countries have amassed used nuclear fuel resulting from power generation over several decades. Although this spent fuel is held within temporary above-ground storage, it will remain radioactive for millennia and requires a permanent solution[1]. In accordance with international consensus on best-practices, many countries are in various stages of planning, designing, and constructing a deep geological repository (DGR) for sustainable isolation and management of used nuclear fuel[2,3]. Even though design features vary from country to country, all DGR design plans propose to bury used fuel within a stable geological formation, surrounded by multiple engineered barriers (e.g., Fig. S1). In Canada, Sweden, and Finland, for example, engineered barriers include storing used nuclear fuel bundles within carbon steel used fuel containers, for strength, with either an integrally bonded copper coating (e.g., Canada) or a self-supporting outer copper shell (e.g., Sweden and Finland) for corrosion resistance[3,4]. An additional engineered barrier involves surrounding used fuel containers with highly compacted bentonite clay, which swells when saturated. This swelling action serves to decrease water activity and microbial growth, while restricting transport of oxidants toward the used fuel container and migration of radionuclides in the unlikely event of container failure[5]. Bentonite clay thus serves as an important engineered

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barrier system component within the natural barrier of a stable host rock. Together, the combination of natural and engineered barrier components of a DGR are intended to isolate and contain nuclear waste through multiple glaciation cycles until relatively safe levels of radiation are reached within approximately one million years[3].

Because microorganisms are present within mined materials, such as bentonite clay, and are introduced during storage and processing, several studies have evaluated potential microbial impacts on engineered barrier system components related to copper corrosion, biofilm formation, radionuclide transport, transformation of clay minerals, and gas production[5,6]. One of the primary microbiological concerns relevant to long-term containment of nuclear waste is microbiologically influenced corrosion. Such corrosion might occur as active microorganisms release metabolites that directly or indirectly cause metal corrosion[7,8]. For example, sulfate-reducing bacteria (SRB) generate hydrogen sulfide (H_2S), which can be corrosive to metals such as copper and steel[7,10–12]. Heterotrophic bacteria are also relevant for DGR safety assessments because fermentation-associated hydrogen gas (H_2) and acetate production may promote sulfate-reduction by SRB through increased electron donor availability[11,13]. Production of gases by microorganisms like denitrifiers, methanogens, and methanotrophs is important to consider because the gases could lead to the formation of fissures in compacted clay, which may potentially allow transport of microorganisms, microbially produced compounds, or radionuclides in an improbable escape from used fuel containers[5,14]. Overall, evaluating the potential for microbial growth and activity within natural and engineered barrier components is an important priority for predicting DGR stability and identifying conditions that minimize or prevent microbial viability over geological timeframes.

Given that DGR-associated microorganisms will be derived from natural or engineered barrier components, an important precursor to modeling the potential impacts of microorganisms on DGR stability is developing an understanding of the microbiota naturally present in these components. Several studies have used cultivation-based approaches to enumerate SRB within bulk clay samples or clay subjected to experimental treatments under DGR-relevant conditions. In these studies, most probable number (MPN) tubes with sulfate-containing medium are used to estimate SRB abundances[12,16–23]. Heterotrophic bacteria from bentonite clay are typically grown on R2A medium[15,17,18,20,21,24–27] because of reduced nutrient concentrations[28], which may better mimic limited nutrient availability expected for clay samples. Detection of both aerobic and anaerobic heterotrophs are relevant for these studies because the DGR is likely to shift from oxic to anoxic conditions after a relatively short period of time[15,18,19,23]. Microorganisms capable of respiring nitrate, such as denitrifiers, have also been studied in the context of the DGR because of the potential impacts of nitrogen oxide and dinitrogen gas production[9,20,29].

Despite progress in quantifying and characterizing culturable microorganisms from bentonite clays, the extent to which cultivation represents all viable and relatively abundant clay microorganisms is unknown. Enumeration with traditional cultivation approaches limits detection to only those microorganisms that can grow under specific laboratory conditions. Furthermore, cultivation approaches overlook viable but non-culturable bacteria and fastidious or slow-growing microorganisms. Although community profiling methods, based on extracted nucleic acids, can help assess cultivation bias, sequencing of amplified 16S rRNA genes from bentonite clays has seldom been reported, presumably due to low nucleic acid yields that result from low biomass samples and the sorption of DNA onto charged montmorillonite clay layers[30]. Despite these limitations, a protocol for successful extraction of DNA from bentonite clay samples has recently been validated[21], allowing us to investigate whether dominant ASVs detected in as-received bentonite clays were the same as those identified through culture-dependent methods, and whether the taxa we culture differ based on clay composition, origin, and storage conditions. In addition to describing microbial community composition, clay microorganisms were quantified with cultivation, quantitative PCR (qPCR) of 16S rRNA genes, and phospholipid fatty acid (PLFA) analysis. Using cultivation-dependent and cultivation-independent methods, we aim to characterize a "core microbiome" and core culturable community subset from diverse clay samples and production lots. This represents an important step toward validating the choice of dry bentonite starting material in future experiments that will help identify suitable DGR conditions for preventing microbial activity and growth over geological timeframes.

Results and discussion

Microbial heterogeneity in bentonite clays. Assessing microbial heterogeneity within diverse commercially available bentonite clays is critical for predicting the microbial growth and activity that may occur within a DGR. In this study, 15 industrially processed bentonite clay samples were sourced from Canada, Greece, India, and the United States of America, with varying production dates, lot numbers, colours, and proportions of exchangeable cations (Table 1; Fig. 1). Although the manufacturing and storage process for each of our samples remain relatively unknown, studying a wide range of industrially mined and processed bentonites is essential to capture all possible variations that may be included in future large-scale DGR design and construction. The as-received clay samples were all relatively dry, with moisture contents ranging from 5 to 16% and water activities between 0.26 and 0.70 (Fig. S2), all well below the water activity threshold of 0.96 considered suitable for microbial growth in bentonite clay[23,31].

We compared culturable aerobic and anaerobic heterotrophs, SRB, and denitrifying bacteria from all samples. As well, quantification of biomarkers (i.e., 16S rRNA genes and PLFA) provided culture-independent enumerations and taxonomic profiles. Cultivation of microorganisms yielded lower abundance estimates than those obtained by DNA and PLFA quantification (Fig. 2), in most cases by orders of magnitude. Higher 16S rRNA gene copy numbers compared to culturable abundance estimations may be due to multiple 16S rRNA gene copies per genome or detection of "relic" DNA within samples. Most culturable aerobic heterotroph abundances ranged from 10^2 to 10^4 colony forming units per gram dry weight (CFU/gdw), which is within the range previously reported for bentonite clays (10^2–10^5 CFU/g;[15,17,24,26]). Eleven samples contained average aerobic heterotroph abundances below the limit of plate count quantification (i.e., 2500 CFU/gdw, based on 25 colonies per plate minimum). Anaerobic heterotroph abundance averages were lower than aerobic heterotroph averages for all

Sample	Clay type	Exchangeable cation	Origin	Manufacturer	Production date	Lot number
MX1	MX-80 bentonite	Na^+	Wyoming, USA	American Colloid Company	Jun 2015	65275772
MX2	MX-80 bentonite	Na^+	Wyoming, USA	American Colloid Company	Nov 2016	116315319
MX3	MX-80 bentonite	Na^+	Wyoming, USA	American Colloid Company	Mar 2017	37324182
MX4	MX-80 bentonite	Na^+	Wyoming, USA	American Colloid Company	Mar 2017	37324184
MX5	MX-80 bentonite	Na^+	Wyoming, USA	American Colloid Company	Mar 2017	37324190
MX6	MX-80 bentonite	Na^+	Wyoming, USA	American Colloid Company	NA	NA
MX7	MX-80 bentonite	Na^+	Wyoming, USA	American Colloid Company	NA	NA
MX8	MX-80 bentonite	Na^+	Wyoming, USA	American Colloid Company	Jun 2015	65275768
MX9	MX-80 bentonite	Na^+	Wyoming, USA	American Colloid Company	NA	NA
AB1	Asha bentonite	Na^+	Kutch, India	Ashapura Minechem Co	NA	NA
CC1	Sodium bentonite	Na^+	Saskatchewan, Canada	Canadian Clay Products Inc	NA	NA
CC2	Sodium bentonite	Na^+	Saskatchewan, Canada	Canadian Clay Products Inc	NA	NA
DC1	Deponit Ca–N	Ca^{2+}	Milos, Greece	S&B Industrial Minerals SA	NA	NA
IR1	IBECO-RWC	Ca^{2+}	Milos, Greece	S&B Industrial Minerals SA	NA	NA
NB1	National standard	Na^+	Wyoming, USA	Opta Minerals Inc	NA	NA

Table 1. Bentonite clay samples analyzed in this study. *NA* data not available.

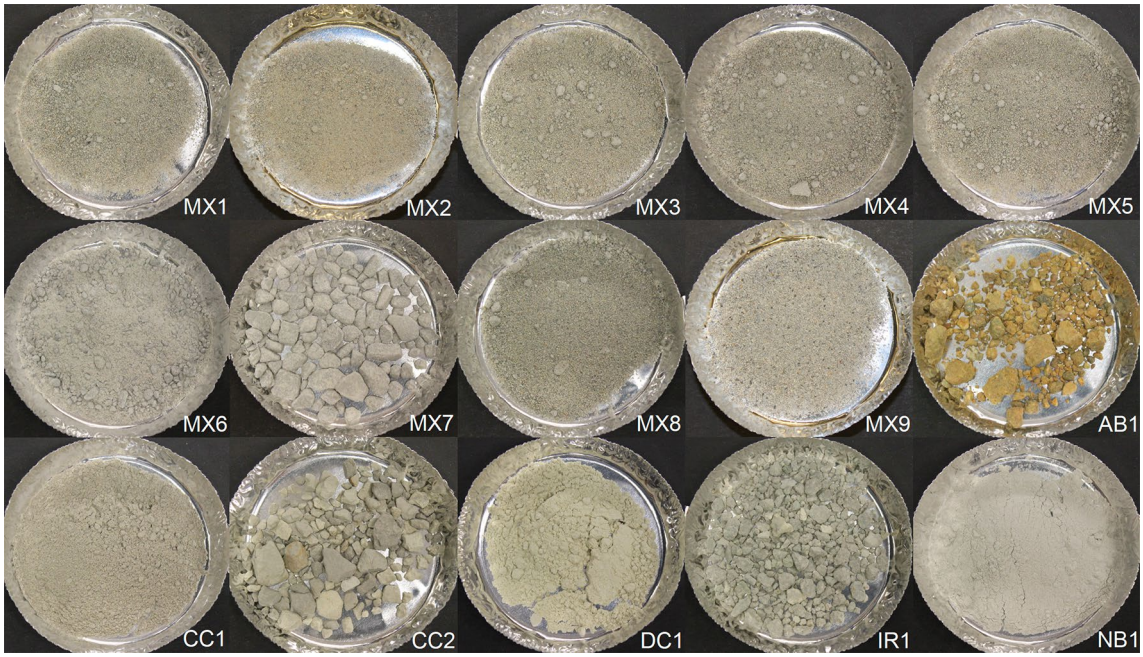

Figure 1. Photographs of the fifteen diverse samples of clay used in this study. Originally coarse samples (AB1, CC2, IR1, and MX7) were ground to smaller grain sizes before use in experiments.

samples (Fig. 2). All anaerobic heterotroph enumerations were below the lower limit of plate count quantification. Average most probable number estimates of SRB from bentonite samples was 63 ± 87 MPN/gdw, comparable to previously studied as-received bentonite samples with SRB abundances of up to 42 MPN/g[17,23,25]. The average estimated abundance of culturable denitrifying bacteria was 57 ± 36 MPN/gdw. Overall, sample AB1 had

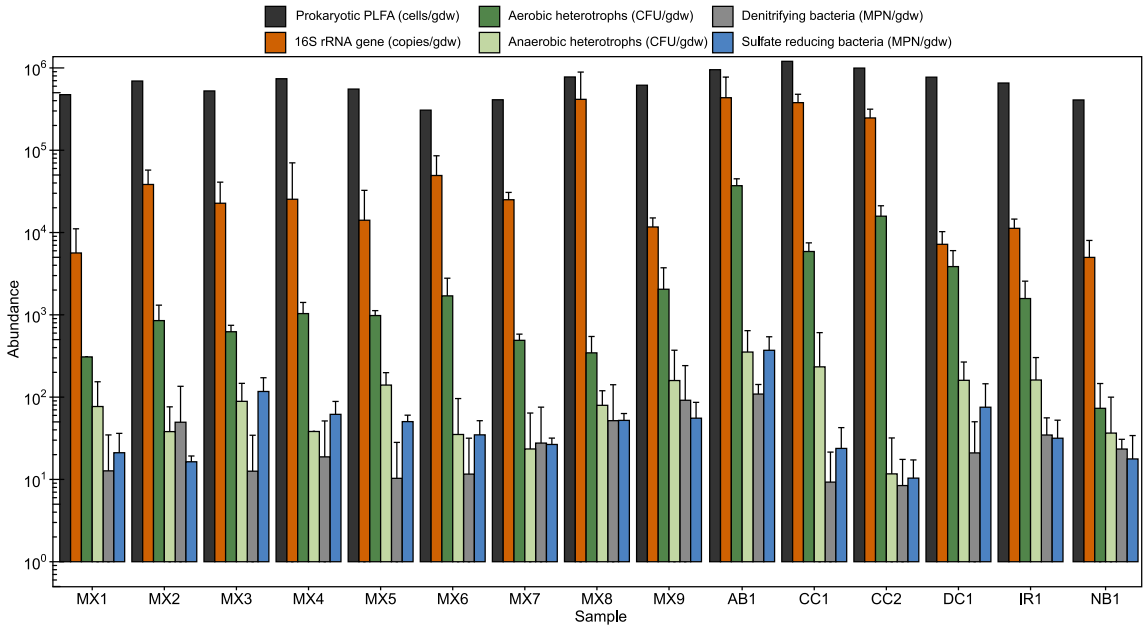

Figure 2. Microbial abundances estimated through culture-dependent and culture-independent methods, normalized to gram dry weight (gdw) using moisture content measurements. Error bars show the standard deviation of triplicate culture-based enumerations and the standard deviation of duplicate qPCR amplifications. Measurements for aerobic and anaerobic heterotrophs below 2.5×10^3 CFU/gdw are below the lower limit of plate count quantification (i.e., 25 colonies per plate) but are shown here for comparison nonetheless. The PLFA analyses were performed in singlicate (duplicate for AB1 and IR1) and only the predicted prokaryotic abundances are reported here for comparison.

the highest average abundance estimates determined by all enumeration methods, with the exception of PLFA analysis, for which it had the second highest abundance estimate (Fig. 2).

Quantification of PLFA from clay samples yielded the highest estimates of microbial cell abundance compared to the other enumeration methods (Fig. 2). Analysis of PLFA has been used previously to detect eukaryotic biomarkers in clay[32] and these biomarkers were also detected in the present study, ranging from 10^3 to 10^4 cells/gdw, and accounting for 1.6–11.0% of the PLFA-associated predicted cell abundances (Fig. S3). For all clay samples, the PLFA abundances ranged from 17–64 pmol/gdw, which corresponds to calculated estimates of 10^5–10^6 cells/gdw. The cell abundances based on PLFA are comparable to previous reports of PLFA in bentonite and Opalinus Clays that presented quantities of 10^6 cells/g[23,32,33]. These previous experiments also identified that the microbial cell abundance estimates based on PLFA exceeded those based on cultivation by ~ 1000-fold[23,32,33]. The reasons that PLFA analysis may be associated with relatively high biomass estimates may be related to preservation of PLFA within the clay matrix, as also occurs for DNA. In most environmental samples, PLFA are assumed to degrade within days to weeks of cell death due to biological recycling, thus the remaining PLFA represent viable biomass. However, in clay environments, it has been suggested that PLFA turn-over rates can vary due to environmental conditions like pH[34] and adsorption to clay surfaces[33,35], or by preservation within the clay matrix[32,33]. Overall, the quantity of background "noise" that may be detected relative to the abundance of living microorganisms remains unknown.

Microbial community profiles were generated for all clay samples using high-throughput sequencing of 16S rRNA genes. The microbial profiles generated from replicate extractions of the same clay sample usually grouped together within ordination space (Fig. 3). The duplicates of samples IR1, DC1, and NB1 exhibited higher dissimilarity compared to other duplicate extraction pairs. Apparent dissimilarity among duplicate clay samples may be due to clay sample heterogeneity or variable extraction efficiencies and detection limits of particular bentonite samples[21,36]. Of the microbial community profiles detected in the clay samples, those from MX-80 bentonite clays with the same production date grouped together despite different lot numbers (Fig. 3). Previous analysis of bentonite clays also revealed similarities between the microbial community profiles of clays from similar production dates[21]. Nonetheless, microbial community profiles of clay samples from March 2017 (MX3, MX4, and MX5) grouped closer together whereas samples from June 2015 (MX1 and MX8) showed more separation. Bentonite clay samples from Wyoming, USA also did not group separately from bentonite from different locations (e.g., Saskatchewan, Kutch, and Milos).

Distinct microbial 16S rRNA gene profiles were associated with each tested clay (Fig. 4). Many amplicon sequence variants (ASVs) were unique to a single clay sample, but several were more broadly detected across clay samples, such as those affiliated with *Streptomyces*, *Sphingomonas*, *Thiobacillus*, and *Xanthomonas*. Overall, most ASVs detected in bentonite clay samples were associated with members of the *Actinobacteria* and *Proteobacteria* phyla, similar to profiles previously generated from other MX-80 bentonite clay samples[21,37]. The potential

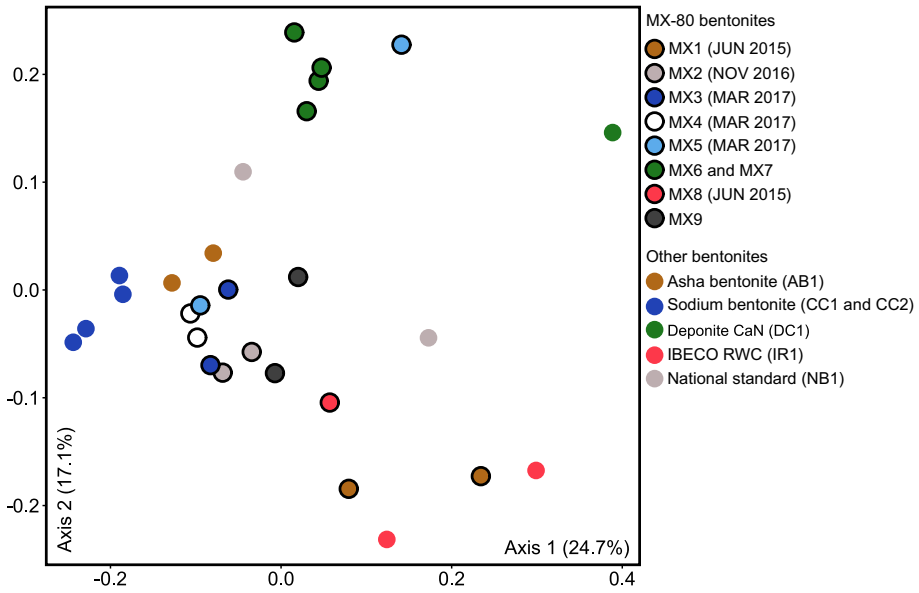

Figure 3. Principle-coordinate analysis (PCoA) ordination based on the weighted UniFrac distance metric of 16S rRNA gene sequences generated by direct DNA extraction from as-received clays. Replicates of DC1 and MX8 were removed during normalization due to low read counts.

contaminant ASVs that were flagged by Decontam were detected with low relative abundance withinin samples and were removed from the data (Fig. S4).

Comparison of cultivation and nucleic acid isolation approaches. By generating parallel cultivation-dependent and cultivation-independent microbial profiles, this study is the first to directly compare the microorganisms cultivated from industrially processed bentonite clay to those detected by direct DNA extraction, PCR, and 16S rRNA gene sequencing. Overall, the dominant taxa associated with 16S rRNA gene profiles generated from as-received bentonite clays do not reflect the microorganisms that responded to cultivation, and the taxa that were cultivated were very similar, regardless of the starting clay material.

The differences in microbial community profiles generated using cultivation-independent and cultivation-dependent methods are reflected by separate grouping of these two sample types in ordination space (Fig. 5), and can be attributed to the culture-based growth of only a subset of the taxa accounted for in the DNA extracted directly from the clay samples (Fig. 6). Notably, the absence of many culture ASVs in the dry clay may be due to adsorption of DNA to the clay matrix, incomplete lysis of viable spores during DNA extraction or insufficient sequencing depth. The microorganisms that were detected in cultures were commonly from the orders *Bacillales* and *Clostridiales*. Although similar orders were detected across all samples for the same cultivation method, cultures from each sample had unique microbial profiles at the ASV level (Figs. S5, S6, and S7). Here, and in previous research, several of the most frequently detected ASVs from cultures were affiliated with the *Bacillus* and *Clostridium* genera[12,17]. Dominant taxa detected in cultures were low in abundance or even absent in the microbial profiles of the corresponding clay samples (Fig. 4).

The microbial profiles generated from cultures of denitrifying bacteria, SRB, and aerobic heterotrophs included overlapping ASVs associated with bacteria belonging to the genera *Bacillus*, *Clostridium*, and *Paenibacillus* (Figs. S5, S6, and S7). Similarities between the ASVs in the 16S rRNA gene profiles of the denitrifying bacteria and SRB enrichments were likely due to the same culturable microorganisms from the clays responding positively to similar cultivation conditions that supported anaerobic heterotrophy. Compared to ASVs associated with *Bacillus* and *Clostridium*, ASVs associated with *Pseudomonas* were detected less frequently in denitrifying cultures (Fig. S6). *Pseudomonas* spp. are commonly reported in cultures of natural clay deposits or saturated and highly compacted clays[15,25,38–40], and have sometimes been reported in uncompacted as-received clay[17]. Several ASVs associated with the genera *Paracoccus* and *Thiobacillus*, potentially capable of denitrification and sulfur oxidation respectively[7,9,41], were detected in as-received clays but not in associated cultures, although they have also previously been detected in clay cultures[12,25].

The MPN tubes with sulfate-reducing bacteria promoted growth of bacteria from genera such as *Bacillus*, *Desulfosporosinus*, *Desulfitobacterium*, *Clostridium*, *Anaerosolibacter*, and *Sedimentibacter* (Fig. S7). *Desulfosporosinus,* a common genus of sulfate-reducing bacteria[41], was cultivated in SRB enrichments from every bentonite sample, but was rarely detected at a relative abundance greater than 2% in the initial clay samples (Fig. 4). In previous research, *Desulfosporosinus* spp. were also frequently detected in microcosms and cultures from multiple types of bentonite clay[12,17,38]. Although many studies suggest that SRB make up the largest group within the microbial communities of clays[10,11,42,43], and SRB were dominant in SRB enrichments here, microbial community profiles of initial clay DNA extracts were not dominated by SRB (Fig. 6). *Pseudomonas* and *Desulfosporosinus* ASVs detected in our cultures were identical to several of those from compacted bentonite exposed to natural

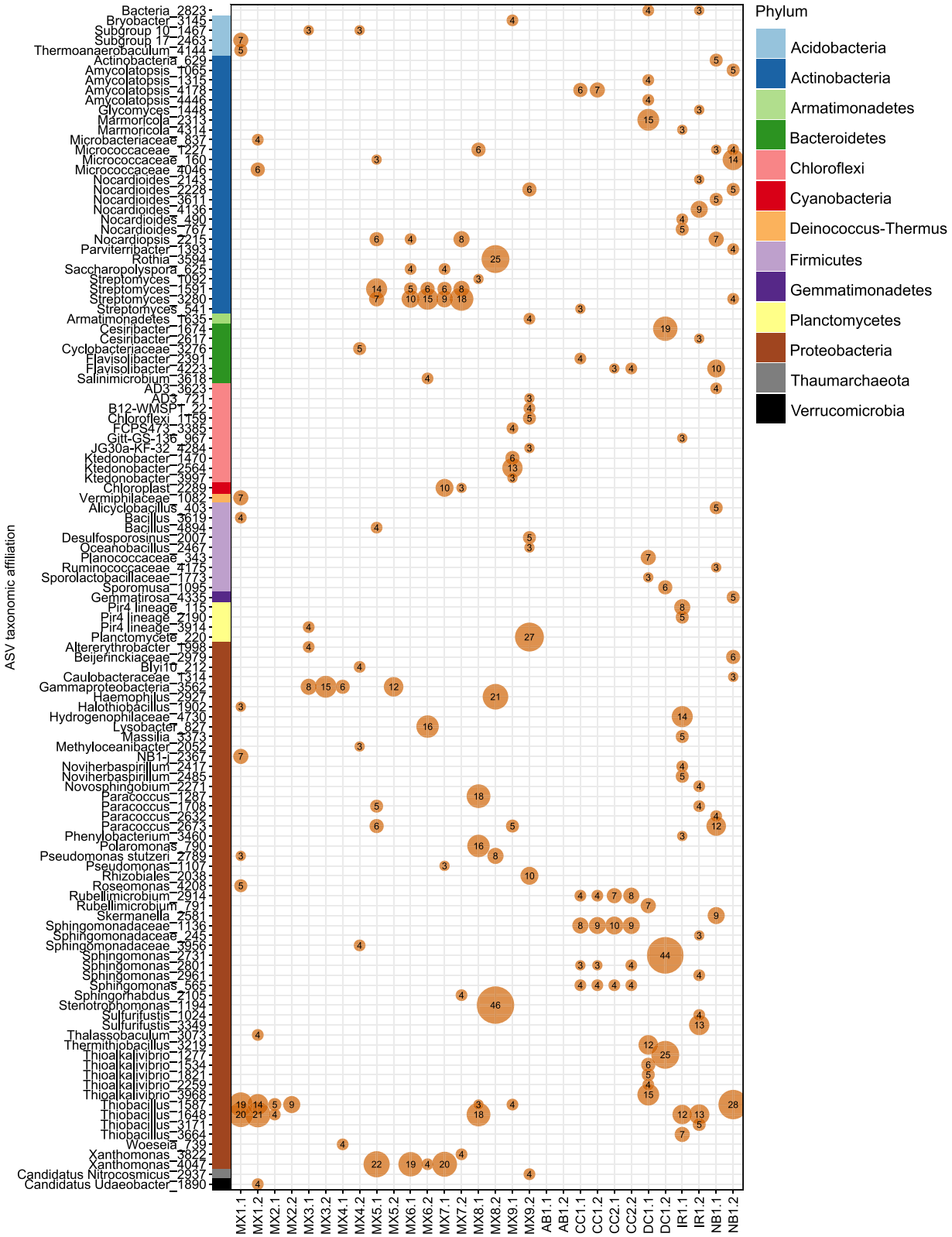

Figure 4. The ASV-level taxa affiliated with as-received clay samples. Phylum of each ASV is indicated with the coloured rectangular bar along the y-axis and the bubble sizes represent the relative abundances of ASVs observed in samples. Duplicates (denoted as "1" and "2") from direct DNA extractions of each clay sample are shown, and ASVs listed are ≥ 3% relative abundance in samples. For ASV labels, we report the lowest taxonomic ranks that have confidence values above the default 0.7 threshold.

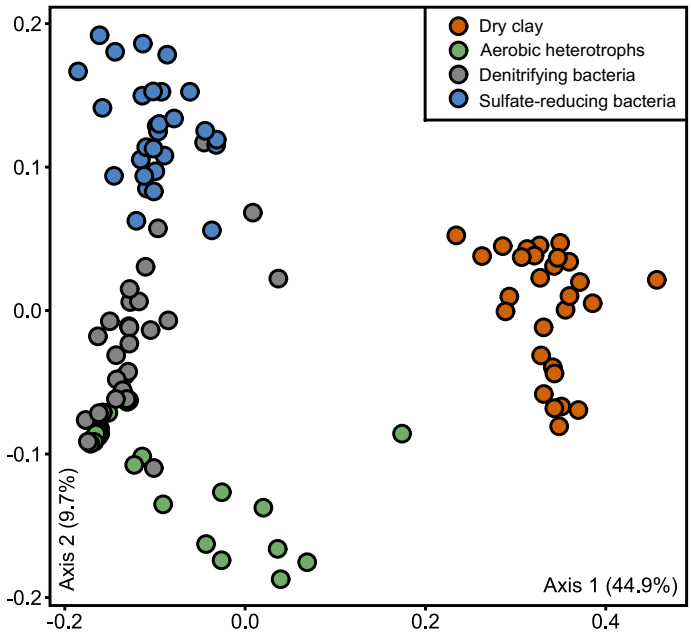

Figure 5. Weighted UniFrac principle-coordinate analysis (PCoA) ordination of 16S rRNA gene sequences generated from as-received clays and their associated cultures.

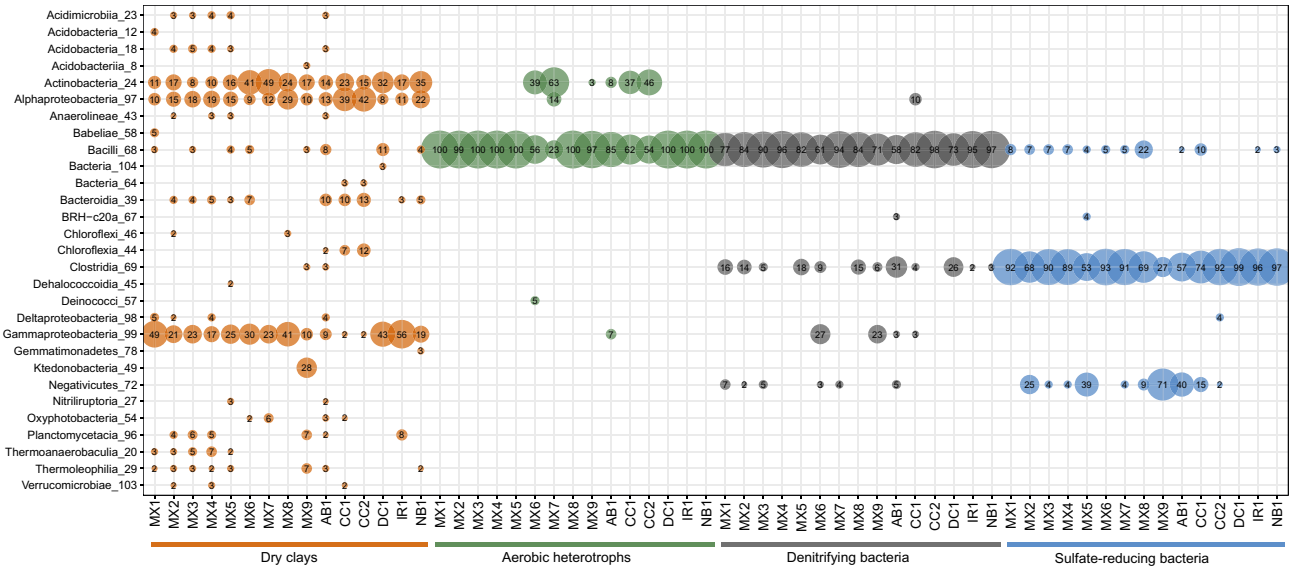

Figure 6. Bubble plot of ASV taxonomic affiliations collapsed to the class level for merged replicates of culture and as-received clay samples. All orders at or above 2% relative abundance for a merged replicate sample are shown.

groundwater in borehole modules[37], which indicates that the results presented here have direct "real world" implications for a proposed deep geological repository.

Our data imply that similar taxa to those cultured here may respond and grow in a nuclear repository environment if the appropriate engineered barrier component design conditions are not established. This also indicates that nuclear waste management organizations can effectively choose any mined bentonite clay material that achieves a sufficient swelling pressure when saturated, regardless of origin or initial molecular microbial profiles, because they all possess core taxa that respond similarly and opportunistically when conditions become suitable.

Core microbial community. Assessments of microbial profiles obtained from cultures and as-received clay DNA extracts can provide conflicting perspectives of the relative abundance and viability of clay microorganisms. Although sequencing of culture DNA revealed clay microorganisms that were viable, this approach did not measure the absolute abundance of microorganisms within clay samples. For direct comparisons of

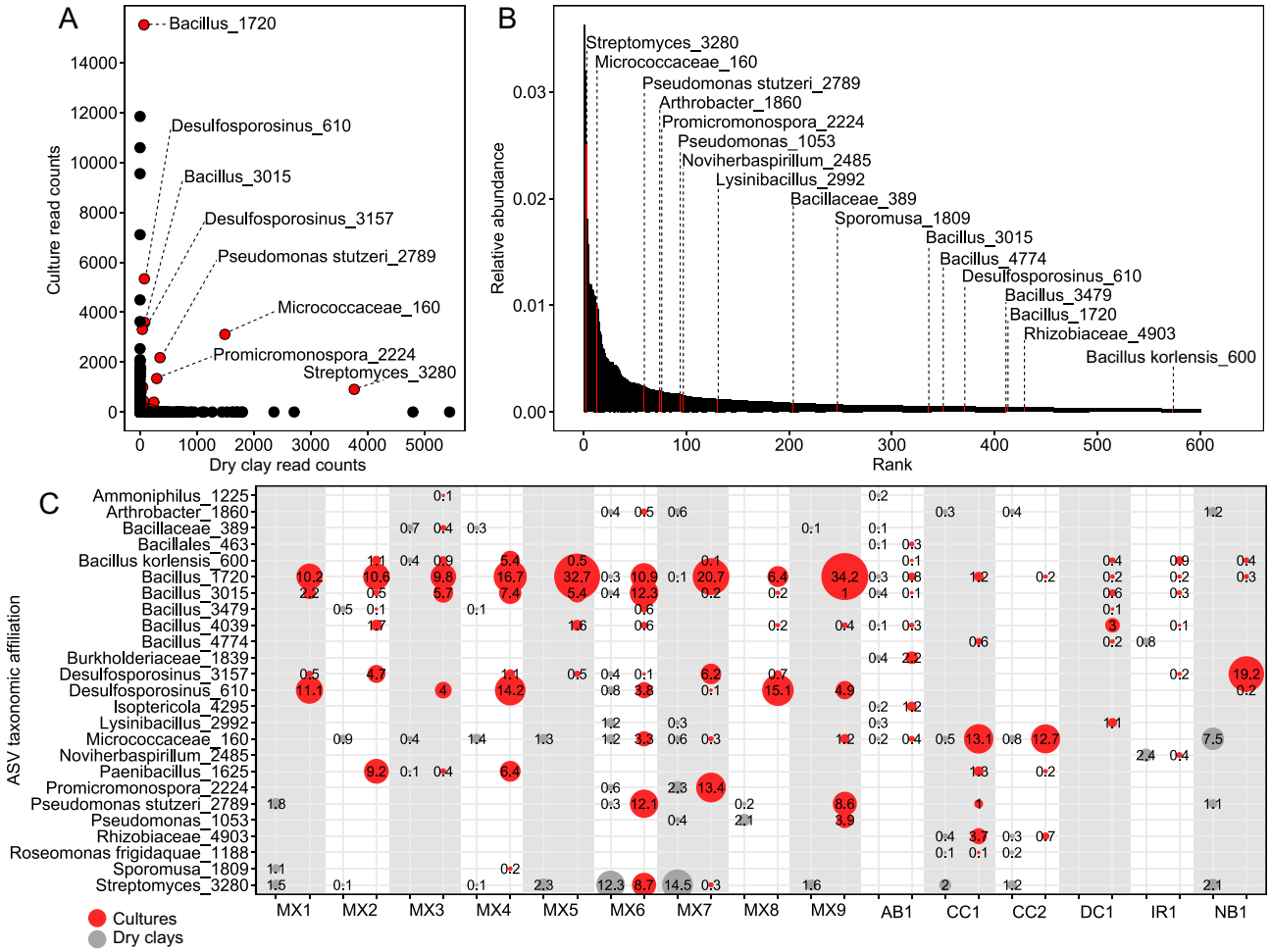

Figure 7. Core microbial community detected in bentonite clays after normalization to 10,000 reads. (**A**) Scatter plot of 16S rRNA gene sequence read counts from clays and cultures. Core ASVs, detected in both clays and cultures, are indicated with a red point. (**B**) Rank abundance curve of ASVs detected in clays, highlighting ASVs additionally detected in cultures in red. (**C**) Bubble plot of core clay ASVs present in clays and cultures. The bubbles represent ASVs present at ≥ 0.1% relative abundance in samples and the numbers contained within specify the percentages. For ASV labels, we report the lowest taxonomic ranks that have confidence values above the default 0.7 threshold. For each sample, sequences obtained from duplicates of all three cultures or from duplicate clay samples were merged into culture or as-received clay categories, respectively.

microorganisms detected from cultures and as-received clay DNA extracts, each ASV was categorized as being present only in cultures, only in clay, or present in both cultures and as-received clays. Of those ASVs, 81.9% (1604 ASVs) were only detected in clays, 16.8% (330 ASVs) only in cultures, and 1.3% (25 ASVs) were detected in both, highlighting that very few taxa were both cultivable and present in abundances sufficient for detection in direct DNA extracts from as-received clays (Fig. 7A). As resported by studies exploring soil and the human gut, cultivation preferentially recovers microorganisms of low relative abundance, often from the so-called "rare biosphere"[44,45].

Of the 600 most abundant ASVs directly detected within all dry clays, most were only detected in DNA extracts from as-received clay but not their corresponding cultures (Fig. 7B), indicating that they represent free DNA, slow-growing taxa, or microorganisms that did not respond to the culturing conditions used in this study. The 25 most abundant ASVs detected in both clay and cultures classified confidently to the following taxonomic ranks: *Streptomyces*, *Micrococcaceae*, *Promicromonospora*, *Bacillus, Rhizobiaceae, Pseudomonas, Burkholderiaceae, Desulfosporosinus, Noviherbaspirillum,* and *Isoptericola* (Fig. 7C). Detected in 10 of the 15 clay samples, *Streptomyces* was the most abundant ASV from all as-received clay DNA extracts combined (Fig. 7B), although five samples (AB1, IR1, DC1, MX3, and MX8) did not contain detectable *Streptomyces* with relative abundances greater than 0.1% (Fig. 7C). *Streptomyces* was also detected in cultures of two samples (MX6 and MX7; Fig. 7C), indicating the viability of these microorganisms in clay. Previous studies of microorganisms within compacted bentonite corroborate the presence[37,39] and viability[39] of *Streptomyces*. Species of *Micrococcaceae* and *Bacillus* were previously detected in compacted bentonite[37], natural bentonite formations[46], and clay cultures[17], and were also detected here in as-received dry bentonite samples and their corresponding cultures (Fig. 7). The large proportion of *Micrococcaceae* and *Bacillus* in many cultures, compared to their corresponding dry clay samples,

confirms that representatives were viable and cultureable bacteria, prevalent across samples, making them core members of diverse bentonite clays.

Conclusion

Microbial 16S rRNA gene profiles of as-received clay samples revealed distinct microbial community profiles, but dominant ASVs did not reflect the viable bacteria enumerated with cultivation-dependent approaches. Our cultivation methods routinely selected for the same taxa regardless of clay starting material. The ASVs that were detected in both as-received bentonite clay DNA and culture DNA profiles were primarily associated with desiccation-resistant taxa, potentially surviving in the dry clay through formation of spores or by preservation within the bentonite clay, including those affiliated with *Streptomyces*, *Micrococcaceae, Bacillus,* and *Desulfosporosinus*. Detection of ASVs in both culture and as-received clay implies that the associated bacteria were viable in clay, and therefore are key members of the overlapping core culturable community subset and core microbiome of bentonite clays. Identifying common microbial "targets" that can grow when conditions are suitable is important because this informs ongoing bentonite experiments that simulate saturated DGR barrier conditions. For example, detection of the same target microorganisms in all clay samples allows us to conclude that experimental results using one bentonite clay type may be extrapolated more generally to other clays as well. Future research should explore whether abundant taxa detected within 16S rRNA gene profiles that were not recovered in cultures are viable but uncultureable or instead reflect relic DNA that is adsorbed to the charged clay matrix. Given that microbiology is core to building a safety case for repository design, this study will be critical for nuclear waste management organizations globally as research continues investigating the microbiology of engineered barrier components for a deep geological repository.

Materials and methods

Clay sample selection. In order to obtain a diverse subset of bentonite clay, our samples were selected from four different countries, with five different manufacturers, and included bentonite clays dominated by either sodium or calcium exchangeable cations (Table 1). In general, following excavation of bentonite from a deposit, manufacturers crush and dry the crude ore to around 20–25% moisture content by mass[47]. Bentonite can either be air dried in stockpiles or dehydrated in a rotary dryer prior to storage. Screening and mixing of the stockpiles is also commonly conducted to achieve a specific grade (i.e., particle size distribution and chemistry) of material. After receiving the samples, all coarse as-received clay samples (i.e., MX7) were ground to a fine grain size using a DNA-free glass mortar and pestle. Two bentonite samples were provided with different initial granularities (CC1 and CC2, and MX6 and MX7) but, even though technically replicates, were treated as separate samples in this study. Wyoming MX-80 samples with different production dates and lot numbers were used to investigate possible influences of batch characteristics on microbial community profiles (Table 1).

Moisture content and water activity. Water potential was measured using a WP4 Dew Point Potentiometer (Meter Group, USA) with 2–5 g of as-received clay, following the manufacturer instructions for "fast mode" analysis at 25 °C. Water activity was calculated according to manufacturer instructions, using the potentiometer output of pressure (kPa) and temperature (°C). Moisture content was calculated by measuring the loss of water after heating clay at 110 °C for 24 h and weighing samples before and after drying using the following formula: $(g_{wet} - g_{dry})/ g_{wet}$.

Cultivation of bentonite clay bacterial communities. A dilution series was prepared in sterile phosphate-buffered saline solution (PBS; 0.01 M NaCl buffered to pH 7.6 with 9 mM Na_2HPO_4 and 1 mM NaH_2PO_4) with clay dilutions of 10^{-1}–10^{-3}. The 10^{-1} dilution was prepared in a 50-mL conical tube by slowly adding 2 g of clay to 18 mL of PBS while vortexing continuously, followed immediately by continuous gentle agitation for 30 min at room temperature. The agitation time was necessary to allow the clay to suspend and swell evenly, which has been shown to result in greater homogeneity of the clay-PBS solution and a higher efficiency of cell removal from clay interfaces[26]. Remaining dilutions were prepared by transferring 1 mL of the previous dilution into 9 mL of PBS. Aliquots from all dilutions were dispensed into most-probable number (MPN) test tubes (1 mL inoculum into 9 mL of medium) and onto R2A agar spread plates (100 µL inoculum) as described previously[23].

For enumeration of SRB, MPN test tubes were prepared with 9 mL of sterile sulfate-reducing medium (HiMedia Laboratories, India, M803). The medium contained ferrous iron (0.4 g/L) to react with sulfide, sodium chloride and sulfate salts that provided essential ions, sodium lactate (3.5 g/L) as the electron donor, and peptone and meat extract as sources of nitrogen and other nutrients. For denitrifiers, MPN tubes with liquid R2A medium (HiMedia Laboratories, M1687) were amended with 12 mM sodium nitrate and included an inverted Durham tube. For SRB and denitrifiers, each sample was analyzed using a five tube MPN method, with all test tubes placed into a stainless steel vacuum chamber (BVV, USA) containing a GasPak EZ Anaerobe Container System Sachet (BD, USA) and an anaerobic indicator strip (BD). Culture chambers were evacuated and flushed with N_2 3–4 times before incubation for 28 days at 30 °C. After incubation, positive MPN tubes were identified by a black precipitate for SRB or by a gas bubble in the inverted Durham tube for denitrifying bacteria activity. The MPN per gram dry weight (gdw) was calculated according to the moisture content measured for each sample. Mean MPN/gdw and standard deviation values were calculated based on triplicate MPN assays.

Aerobic and anaerobic heterotrophs were cultured on R2A medium with 1.5% agar[28]. Plates were incubated at 30 °C under oxic conditions for 5–7 days or under anoxic conditions for 28 days. Colony forming units (CFU)/gdw were calculated using the sample moisture contents, and standard deviation values were calculated based

on three replications of each plate count analysis. The lower limit of quantification for heterotrophic plate counts was 2500 CFU/g because plates with fewer than 25 colonies may exaggerate low cell counts[48].

Genomic DNA extraction from clays and cultures. Genomic DNA was extracted from 2 g powdered clay samples using the PowerMax Soil DNA Isolation Kit (Qiagen, Germany) and modifications to the manufacturer's instructions previously validated[21]; after addition of lysis solution, clay in PowerBead tubes was gently vortexted for 20 min to allow clay to fully suspend and swell, then PowerBead tubes were incubated at 65 °C for 30 min, immediately followed by bead beating at 30 Hz for 10 min using a mixer mill MM 400 (Retsch, Germany). Kit controls were included for each batch of DNA extractions.

For extraction of genomic DNA from aerobic heterotroph cultures, colonies on replicate agar plates for each sample were slurried by adding 1 mL of sterile DNA-free water and gently sweeping over the plate surface with a sterile disposable cell spreader. The slurry was then transferred to a DNA-free microcentrifuge tube. For genomic DNA extractions of SRB and nitrate-reducing bacteria cultures, the contents of positive MPN tubes were mixed for each sample, then 2 mL of the culture was transferred to a DNA-free microcentrifuge tube. Cells were pelleted by centrifuging all microcentrifuge tubes for 2 min at 10,000 × g. Genomic DNA was recovered from cell pellets following the protocol for the DNeasy Ultraclean Microbial Kit (Qiagen) using a bead beater (FastPrep-24 Instrument MP Biomedicals, USA) at 5.5 m/s for 45 s. The DNA from replicate MPN tubes of the same dilutions were pooled before amplification.

Quantitative polymerase chain reaction (qPCR). All qPCR mixtures were prepared in a PCR hood (AirClean Systems, Canada) that was cleaned with 70% ethanol and treated with UV light for 15 min. The qPCR standard curve template was generated from the V3-V5 16S rRNA gene fragment of *Thermus thermophilus* that was previously cloned into vector pUC57-Kan. The template was amplified through PCR with primers M13F (5'-TGTAAAACGACGGCCAGT-3') and M13R (5'-CAGGAAACAGCTATGAC-3') that flanked the 719 bp insert. The PCR product was separated on a 1% agarose gel and purified using the Wizard SV Gel and PCR Clean-Up System (Promega, USA).

Genomic DNA extracts from clay samples were amplified using universal 16S rRNA gene primers 341F and 518R[49]. All qPCR amplifications were performed in duplicate and each 15 µL reaction contained 1 × SsoAdvanced Universal SYBR Green Supermix (Bio-Rad, USA), 0.3 µM of each primer, 7.5 µg bovine serum albumin (BSA), and 4 µL of template DNA. The qPCR amplification was performed with a CFX96 Real-Time PCR detection system (Bio-Rad) beginning with 98 °C for 3 min followed by 40 cycles of 98 °C and 55 °C at 15 s and 30 s intervals, respectively. Initial 16S rRNA gene copy numbers were calculated for kit controls and clay samples from the linear regression equation produced from the standard curve with a 0.98 coefficient of determination (R^2). Average starting quantities of up to 1.5×10^3 copies/mL detected in kit controls were subtracted from the calculated starting quantities for respective samples. Final 16S rRNA gene copy numbers were corrected to per gram dry clay values using the moisture content measurements of each sample.

Amplification of 16S rRNA genes and high-throughput sequencing. All PCR mixtures were prepared in a PCR hood (AirClean Systems, Canada) that was cleaned with 70% ethanol and treated with UV light for 15 min. Each 25 µL PCR mixture contained 1 × ThermoPol Buffer, 0.2 µM forward primer, 0.2 µM reverse primer, 200 µM dNTPs, 15 µg BSA, 0.625 units of hot start *Taq* DNA polymerase (New England Biolabs, USA), and up to 10 ng of template DNA. The V4-V5 region of the 16S rRNA gene was amplified in triplicate using primers 515F-Y[50] and 926R[51], modified to contain unique 6 base indexes in addition to Illumina flow cell binding and sequencing sites[52]. Reaction conditions were an initial denaturation at 95 °C for 3 min, followed by 40 cycles of denaturation at 95 °C for 30 s, annealing at 50 °C for 30 s, and extension at 68 °C for 1 min, with a final extension at 68 °C for 7 min. Negative controls containing no template DNA (NTCs) were included in the 96 well PCR plates to test for cross contamination, and in tubes outside of the PCR plate to test for master mix contamination.

Based on agarose gel quantification, samples were normalized by pooling equimolar quantities into a single tube. The NTCs and DNA extraction kit controls were added to the pool with 5 µL each even if no visible band was detected on the agarose gel. The pooled amplicons were gel purified using the Wizard SV Gel and PCR Clean-Up System (Promega, USA) and the library was denatured and diluted following manufacturer's guidelines (Illumina document no. 15039740 v10). The 8 pM library containing 15% PhiX control v3 (Illumina Canada, Canada) was sequenced on a MiSeq instrument (Illumina Inc, USA) using a 2 × 250 cycle MiSeq Reagent Kit v2 (Illumina Canada). Samples were sequenced in two MiSeq runs and reads were merged in the post sequence analysis.

Sequencing analysis. Sequence reads were demultiplexed using MiSeq Reporter software version 2.5.0.5 (Illumina Inc) and analyzed using Quantitative Insights Into Microbial Ecology 2 (QIIME2; version 2019.10.0)[53] to denoise sequences, remove chimeras, and truncate sequences to 250 bases using default parameters with DADA2 (release 1.16)[54]. Samples were rarefied to 2,350 sequences for generating ordinations and collapsed ASV tables were generated using QIIME2 plugins. These analyses were managed by Automation, Extension, and Integration Of Microbial Ecology version 3 (AXIOME3; github.com/neufeld/AXIOME3)[55] and taxonomy was assigned to ASVs using SILVA 132 release[56–59]. Decontam (release 3.11) was used to identify contaminant ASVs using the prevalence method and an assigned score statistic threshold value of 0.5[57]. These ASVs were verified and removed manually from the sample ASV table and summarized in Fig. S4. Controls used for the Decontam analysis included a swab from sterile R2A agar plate, kit controls for DNA extraction (3 controls), PCR no-template controls (NTCs; 8 controls), and positive controls for sequencing (3 controls). Next, the microbial community profiles detected from each dry clay sample was compared to the profiles detected after culturing.

For this comparison, the ASVs detected in cultures (aerobic heterotrophs, SRB, and denitrifying bacteria) for each sample were added together as one culture category. All ASV read counts were normalized to 10,000 reads for each sample. From all of these normalized reads, ASVs representing ≥0.1% relative abundance in the dry clay microbial profile of ≥1 sample and in the culture microbial profile of ≥1 sample were labelled as core ASVs.

All DNA sequences were deposited in the European Nucleotide Archive with study accession number PRJEB39383.

Phospholipid fatty acid analysis. Phospholipid fatty acid (PLFA) analysis was carried out by Microbial Insights (Knoxville, USA). Lipids were recovered using a modified Bligh and Dyer method[60]. Estimates of prokaryotic cells per gram based on PLFA were calculated with the conversion of 20,000 cells/pmol of PLFA[61]. To allow cross comparison with other prokaryotic enumerations, eukaryote-associated PLFA quantities were removed and only prokaryotic PLFA quantities were reported for estimates of cell abundance. Each category of PLFA is generally associated with specific groups of microorganisms, except for Nsats which are found in all organisms. Although most PLFA analyses were performed without replication, duplicates were analyzed for samples AB1 and IR1.

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Author contributions

M.A.V., K.E., G.W.S., W.J.B., and J.D.N. conceived the work. M.A.V. completed the cultivation-based analyses. M.A.V., K.E., and R.C.B. performed the sequencing of 16S rRNA genes. M.A.V. analyzed the data and prepared the first manuscript draft and all authors reviewed and edited the manuscript.

Competing interests

The authors declare no competing interests.

Additional information

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