Microbiology of Bentonite Clay

by

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A thesis
presented to the University of Waterloo
in fulfillment of the
thesis requirement for the degree of
Master of Science
in
Biology

Waterloo, Ontario, Canada, 2020

Author's Declaration

This thesis consists of material all of which I authored or co-authored: see Statement of Contributions included in the thesis. This is a true copy of the thesis, including any required final revisions, as accepted by my examiners.

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Statement of contributions

Chapter 2

Katja Engel extracted DNA from bentonite clay samples. Rachel Beaver performed PCR and library preparations for high-throughput sequencing.

Chapter 3

Dr. Jamie Noel, Dr. Dmitrij Zagidulin, and Claire Tully at the University of Western Ontario designed pressure vessels and managed the setup, maintenance, and disassembly of clay-containing pressure vessels. Rachel Beaver performed PCR and library preparations for high-throughput sequencing.

Abstract

Highly compacted bentonite clay has been proposed as an important engineered barrier component of a deep geological repository for Canada's high-level nuclear fuel waste. As a result, the microbiology of bentonite clay is relevant for the long-term stability of such a repository. Understanding microbial communities in "time-zero" clay samples and detection of temporal changes to clay microbial communities in experiments that mimic repository conditions, are essential for predicting how microbial activity within the barrier system may influence corrosion of other barrier components. The microbial communities contained within bentonite clay have traditionally been studied using culture-dependent detection methods. The difficulty associated with adsorption of DNA to the clay matrix has resulted in relatively few reports involving extraction and high-throughput sequencing of nucleic acids directly from mined bentonite clay.

Circumventing longstanding barriers to cultivation-independent analysis of nucleic acids with a recently validated method, this research used DNA extraction and high-throughput sequencing to explore nucleic acid profiles in multiple representative bentonite clay samples. Assessment of 16S rRNA gene profiles from clay samples reveals heterogeneity among bentonite clay sources and dates. Microbial 16S rRNA gene profiles for clay samples and corresponding cultures selecting for sulfate-reducing bacteria, denitrifying bacteria, and heterotrophic bacteria revealed distinct microbial community profiles. The ASVs detected in both bentonite clay DNA and enrichment culture DNA profiles were primarily associated with desiccation-resistant taxa, including those affiliated with *Streptomyces*, *Micrococcaceae, Bacillus,* and *Desulfosporosinus*.

Using oxic pressure cells with saturated bentonite clay, this research used cultivation-based approaches and nucleic acid profiling to assess the microbiology of pressure vessels containing compacted Wyoming MX-80 bentonite at dry densities of 1.1, 1.4, and 1.6 g/cm^3 for durations of 1, 3, and 6 months. The data show that some outer layers of bentonite clay provided more favourable conditions for growth of clay microorganisms than inner layers. In addition, low dry density conditions (i.e., 1.1 and 1.4 g/cm^3) increased the average abundances of culturable heterotrophic aerobes without a corresponding increase in culturable sulfate-reducing bacteria. Sequencing of 16S rRNA genes agreed with cultivation data and confirmed that putative heterotrophic bacteria (associated primarily with *Bacillus* and *Pseudomonas*) proliferated in clay pressure vessels at the lowest dry density, but not for those in high dry density (1.6 g/cm^3). These preliminary results

reinforce that microbial growth is prevented, at least over relatively short incubation times, in dry density bentonite clay conditions similar to those proposed for use in a deep geological repository. In addition, the use of cultivation-dependent and cultivation-independent approaches provides an effective and complementary methodological combination for monitoring future experiments that mimic engineered barrier conditions to assess potential microbiology implications for a deep geological repository.

Acknowledgements

First and foremost, thank you to my supervisor Josh Neufeld for his enthusiasm, encouragement, and for providing a supportive learning environment throughout my degree. Also thank you to my committee, Barb Butler and Andrew Doxey, for their continuous guidance and support.

Thank you to my collaborators at the University of Western Ontario, Jamie Noel, Dmitrij Zagidulin, and Claire Tully. Their perseverance and patience with pressure vessels, clay, microorganisms, and copious emails is greatly appreciated. As well, thank you to Jeff Binns and the Nuclear Waste Management Organization (NWMO) for providing funding and resources for this research. Specifically, this research was supported by an Ontario Research Fund: Research Excellence (ORF-RE) grant and a Collaborative Research and Development (CRD) grant from the Natural Sciences and Engineering Research Council of Canada (NSERC).

To my friends in the Neufeld lab, who share my determination and feed my passion for microbiology, thank you for the endless inspiration and reassurance as we each tackled successes and failures. A special thanks to Katja for being a great mentor and for always “having a moment” to run through ideas. Thanks to Zinedine Cobourne for assistance with cultivation.

Finally, thank you to my family and friends for constant support, especially to Mom, Dad, Grandpa, and Jake for being there through thick and thin.

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List of Abbreviations

ASV	Amplicon sequence variant
BSA	Bovine serum albumin
CFU	Colony forming unit
COx	Callovo-Oxfirdian clay
Cu	Copper
Cu_2S	Copper (I) sulfide
DGR	Deep geological repository
DNA	Deoxyribonucleic acid
Fe^{2+}	Ferrous iron
Fe^{3+}	Ferric iron
FeS	Ferrous sulfide
FeS_2	Pyrite
gdw	Gram dry weight
H^+	Hydrogen
H_2	Dihydrogen
H_2S	Hydrogen sulfide
HCO_3^-	Bicarbonate
HS^-	Bisulfide
MIC	Microbiologically influenced corrosion
MPN	Most probable number
N_2	Dinitrogen
NO_2^-	Nitrite

NO_3^-	Nitrate
NTC	No-template control
NWMO	Nuclear waste management organization
OH^-	Hydroxide
OTU	Operational taxonomic unit
PCR	Polymerase chain reaction
PLFA	Phospholipid fatty acid
qPCR	Quantitative polymerase chain reaction
R2A	Reasoner's 2A agar
rRNA	Ribosomal RNA
S^{2-}	Sulfide
SO_4^{2-}	Sulfate
SRB	Sulfate-reducing bacteria
$SrSO_4$	Celestite
VBNC	Viable but non-culturable

Chapter 1
Introduction and literature review

1.1 Microorganisms and nuclear waste storage

1.1.1 Canada's storage of nuclear waste

Many countries that use nuclear power have developed plans for safe and isolated storage for the radioactive by-products of nuclear power generation. The Nuclear Waste Management Organization (NWMO) is responsible for the development and implementation of a storage solution for Canada's spent nuclear fuel in a deep geological repository (DGR). Storage and containment of spent nuclear fuel is required for about one million years for the radioactivity to decay to levels that approach those of natural uranium (Figure 1.1) [1].

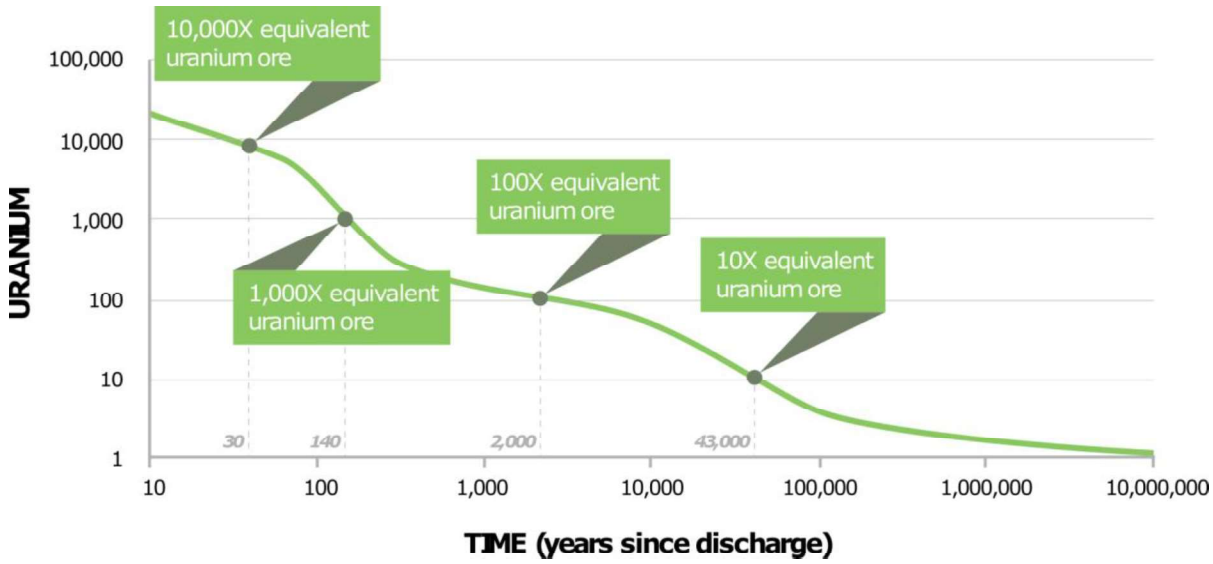

Figure 0.1 Used fuel radioactivity (relative to uranium ore) over time. The green trendline represents the used fuel radioactivity relative to equivalent quantities of uranium. This figure was reproduced from a previous publication [1].

Radioactive waste is a by-product of nuclear power generation. Before placement into nuclear reactors, small and durable fuel pellets of uranium dioxide are formed. Nuclear fuel pellets (2.0 cm × 1.2 cm) are packed within 0.5-m long rods of corrosion resistant metal called Zircaloy, creating what

are known as fuel pencils [2]. Fuel pencils are welded together within 0.1 m diameter cylindrical fuel bundles. Once depleted, used bundles are stored in pools of water for initial cooling, followed by storage in concrete tanks prior to long term DGR storage. In Canada, there are approximately 2.9 million bundles of spent nuclear fuel and there will be another 2.6 million bundles created over the remaining lifetime of the current nuclear reactors in operation [3].

The plan for Canada's spent nuclear fuel involves an engineered barrier system that will safely store and prevent escape of radionuclides for more than one million years (Figure 1.2). The innermost component of the engineered barrier system is the nuclear fuel pellets themselves. The high-density uranium dioxide is a hard and durable ceramic material, resistant to wear and high temperatures [4, 5]. A copper-coated vessel of carbon steel will further secure 48 fuel bundles [6]. The 3 mm cold-spray copper coating will act as a corrosion-resistant layer and the thick steel and spherical head will serve as the strength for the container [7]. The final 0.6 m × 2.5 m used fuel container design anticipates pressures that may occur due to multiple ice ages involving 3-km thick glaciers passing over the storage site [8].

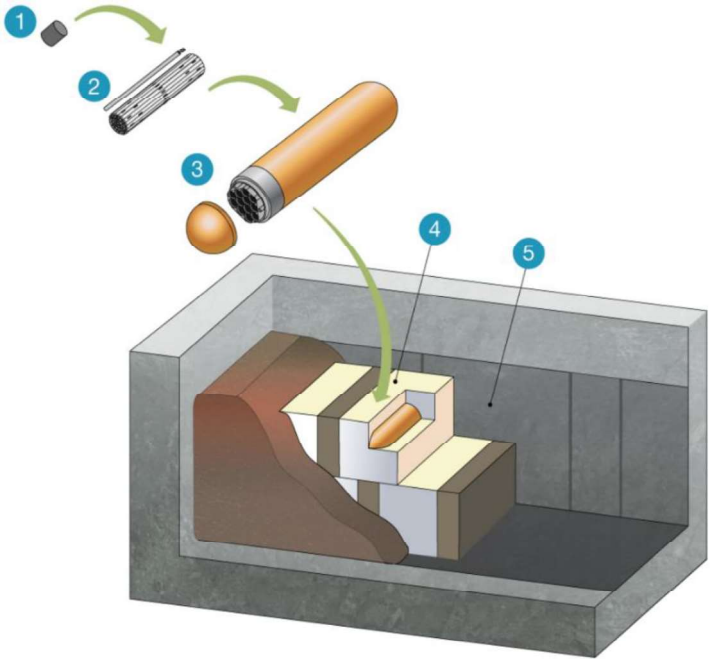

Figure 0.2 The multiple-barrier system that will contain and isolate spent nuclear fuel. The uranium pellets (1) are assembled into fuel bundles composed of fuel pencils (2). Copper-coated, carbon steel used fuel containers (3) containing 48 fuel bundles, will be encased in highly compacted bentonite clay (4), and surrounded by host rock (5), approximately 500 m below ground. This figure was reproduced from a previous publication [9].

Bentonite clay represents an important engineered barrier component of the DGR design. Swelling bentonite clay will seal the system and prevent the passage of water, microorganisms, and radionuclides from the used fuel container [10, 11]. According to NWMO design specifications, the copper-coated used fuel container will be encased in a highly compacted bentonite clay buffer box (Figure 1.2). The buffer box containing the spent fuel will be placed into the proposed DGR located approximately 500 m deep in Canadian host rock. A DGR will be a system of underground tunnels, with a total area of approximately 2.0 km^2, designed to safely isolate Canadian spent nuclear fuel [12]. The clay buffer boxes placed in the tunnels will be separated from adjacent buffer boxes with a layer of clay spacer blocks. Bentonite clay is an important component of a DGR because it acts as the buffer between natural and engineered components of the multiple-barrier system [11]. Following sealing of DGR tunnels, an anoxic environment will persist, and the performance of this repository will be continually monitored. The final barrier in the storage system will be the host rock. By exhibiting low permeability and little groundwater movement, the host rock will protect the repository from water flow and destructive natural events [12].

Clays are common components of soil and sediments and are categorized based on their mineral composition [13]. At the molecular level, clay minerals are composed of interlayers of tetrahedral and octahedral silicate sheets containing relatively low cationic charges [14]. Clays can differ based on elemental makeup of the tetrahedrons and octahedrons [13]. For example, compared to the illite clays, minerals in smectite clays result in higher sensitivity to changes in water that leads to greater sorption and swelling [13]. Depending on underlying geology and the resources available, each country has employed different models for safe spent nuclear fuel storage. Engineered (compacted) or natural clays will be used around the world in the multi-barrier framework for storage of high-level radioactive waste. For example, clay barriers will be composed of Boom clay in Belgium, Callovo-Oxfirdian (COx) clay in France, Opalinus clay in Switzerland, and bentonite clay in Canada, Sweden, Switzerland (along with Opalinus clay), Finland, Japan, and Korea [15, 16]. These clay barriers differ in swelling capacity and porosity because they contain different proportions of clay minerals, such as illite, smectite, and kaolinite [17, 18].

Characteristics of bentonite clay make it well suited for use in Canada's DGR. Bentonite clay is an iron-poor mineral created through naturally occurring alterations to volcanic ash [13]. It is also primarily composed of montmorillonite (within the smectite group) and usually contains other minerals like quartz, feldspar, pyrite, and either sodium or calcium exchangeable ions [19]. Clays,

like Wyoming MX-80 bentonite, with exchangeable sodium cations, rather than calcium cations, continually permit uptake of water within the interlayers, resulting in higher swelling pressures [13]. Bentonite is also a nutrient limited environment that makes it difficult for potentially dangerous microorganisms to grow. For example, organic carbon, required by heterotrophic microbes, can make up only about 0.4% of the oven-dried weight of Wyoming MX-80 bentonite clay [19]. Highly compacted clay also has low water activity and small pore sizes that minimize microbial activity. Bentonite clay is capable of radionuclide retention, transportation of heat away from the high-level radioactive waste, maintenance of swelling pressure after drying, and prevention of porewater influxes [11]. The high swelling capacity and stability of bentonite will allow for effective long-term separation of engineered and natural components in the multi-barrier system.

1.1.2 Potential impacts of clay microorganisms

The microbiology of bentonite clay is relevant for long term DGR stability. Minimizing microbial activity in bentonite can help prevent corrosion of copper-coated used fuel containers. Corrosion is the result of a metal interacting with its environment, leading to changes in the physiochemical properties of that metal [20]. Corrosion of the copper and steel (iron) components of the used fuel container would pose risks for release of radionuclides from the used fuel container. Microbiologically influenced corrosion (MIC) is an electrochemical process where the presence of microorganisms initiates, facilitates, or accelerates corrosion of metals [20]. Sulfate-reducing bacteria (SRB) are common examples of microorganisms capable of promoting MIC and *Desulfosporosinus, Desulfobulbaceae, Rhodospirillaceae,* and *Desulfotomaculum* are examples of sulfate-reducing microorganisms commonly associated with cultures originating from clay samples [21–24].

Using sulfate as a terminal electron acceptor, SRB reduce sulfate to sulfide through dissimilatory sulfate reduction. Energy for sulfate reduction is provided by dihydrogen (H_2) for hydrogen-oxidizing autotrophic SRB (Equation 1.1), and acetate or lactate for heterotrophic SRB (Equation 1.2) [22, 25–27]. In clay, high pH can favour sulfide (S^{2-}) and bisulfide (HS^-) reactions leading to the formation of ferrous sulfide (FeS) (Equation 1.3) [25, 28]. Hydrogen sulfide can additionally react with copper, resulting in copper corrosion (Equation 1.4) [29].

$4H_2 + SO_4^{2-} \rightarrow S^{2-} + 4H_2O$ (Equation 1.1)

$CH_3COO^- + SO_4^{2-} \rightarrow 2\ HCO_3^- + HS^-$ (Equation 1.2)

$HS^- + Fe^{2+} \rightleftarrows FeS + H^+$ (Equation 1.3)

$2\ Cu + HS^- + H_2O \rightarrow Cu_2S + H_2 + OH^-$ (Equation 1.4)

Despite bentonite clay having high sealing properties, formation of fissures or fracture zones in the clay barrier is feasible [30]. Imperfections in clay may arise due to external pressures or microbial activities [30]. Gas-producing microorganisms, such as denitrifying bacteria, are naturally present in clay and their generation of gases might result in formation of fissures in compacted clay. Denitrification is the process where a microorganism or microbial consortium anaerobically reduces nitrate (NO_3^-) or nitrite (NO_2^-) to nitrogenous gases. Many denitrifying bacteria, like those affiliated with *Pseudomonas, Bacillus,* and *Parococcus* genera are heterotrophic*,* but autotrophic denitrifiers like *Thiobacillus denitrificans* also exist [18, 31]. Denitrifying bacteria may also have a positive impact in a DGR. Because they can compete with SRB for the same electron donors, like acetate, lactate, and H_2, thus preventing metabolism and the production of corrosive hydrogen sulfide by SRB [32].

Microorganisms inducing physical and chemical alterations to bentonite clay might also impact its performance as a barrier. Microbial activity can lead to alterations to the clay matrix, potentially compromising the clay's ability to swell. Some clay microbial communities are capable of causing smectite to illite reactions by reducing structural iron (Fe^{3+}) in the octahedral sheets of smectite [14, 28]. Changes to the iron redox state in smectite can impact factors such as charge, surface area, and swelling capacity [28]. For example, mesophilic *Shewanella oneidensis* MR-1, thermophilic metal-reducing bacteria, SRB, and methanogens can promote the reduction of Fe^{3+} to Fe^{2+} [33]. Modification of the clay matrix can also occur through *Desulfitobacterium fappieri* coupling the oxidation of Fe^{2+} with the reduction of nitrate [14]. Because smectite and illite minerals are similar in structure, a smectite to illite reaction can occur over time due to specific conditions or microbial activity [14]. In a DGR, this unfavourable change in mineral composition of smectite could compromise some ability for the clay to swell [13].

Bentonite clay inherently contains limited sources of water and carbon for microorganisms and can also swell to seal off potential introduction of new carbon and energy sources from the surrounding environment. For microorganisms to survive the relatively low water activity environment of bentonite clay, there is heavy reliance on cycling of energy sources such as H_2 from other microorganisms with differing metabolic capabilities. As an abundant electron donor, H_2 is considered a primary driver in clay microbial networks [22, 25, 27, 34]. In clay, H_2 can be generated biotically either through microorganisms like anaerobic methane oxidizers or by heterotrophs through

fermentation [22, 26, 27]. Generation of H_2 in clay can also occur through abiotic processes like radiolysis of water and serpentinization (the change of rock with addition of water) [22, 25, 27]. Subsequently, carbon dioxide and H_2 act as carbon and electron sources for sulfate reduction in autotrophic SRB [22, 25, 35, 36]. The H_2 also acts as an electron donor for autotrophic denitrifying bacteria [27]. Therefore, through generation by fermenting microorganisms or methane oxidizers and consumption by autotrophic microorganisms, H_2 may be cycled in DGR-associated subsurface environments.

Low biomass microbial communities, such as those found in clay formations of the deep biosphere, have metabolic capabilities that require cycling of carbon. Hydrogenotrophic bacteria, such as some SRB, can use energy from the oxidation of H_2 to fix carbon from carbon dioxide [22, 27, 36]. Heterotrophic microorganisms may then assimilate the organic carbon that was fixed by hydrogenotrophic bacteria [22]. As an example, fermenting microorganisms present in the microbial community could produce acetate through oxidation of the assimilated organic carbon [22, 26]. With the by-products of fermentation, heterotrophic SRB could oxidize acetate and methane could be oxidized by methanotrophs thus completing the carbon cycle through a process that would result in production of carbon dioxide [26, 33, 37]. In clay metabolic networks, organic matter from clay, dead microbial biomass, or chemolithoautotrophs can all fuel the metabolism of heterotrophic organisms.

Microbial activity in clay requires available terminal electron acceptors. For microorganisms like SRB, that are capable of creating corrosive by-products, sulfate (SO_4^{2-}) acts as the terminal electron acceptor. Sulfate is naturally present in clay in the form of celestite ($SrSO_4$) [38] and sulfur is present in the form of pyrite (FeS_2) [25, 39]. In clay, nitrate reduction and sulfur oxidation of elemental sulfur or the clay mineral pyrite (FeS_2), can produce sulfate that in turn can fuel sulfate reduction by SRB or methane oxidation by anaerobic methanotrophs [18, 32, 33]. When iron is present in clay, corrosive hydrogen sulfide produced through sulfate reduction or methane oxidation can be removed from clay by spontaneously reacting with iron (Fe^{2+}) to form a non-corrosive ferrous sulfide precipitate (FeS) [25]. Because bentonite clay contains Fe^{2+} that is capable of spontaneously reacting with corrosive sulfide, it can potentially increase the stability of the DGR [25].

1.1.3 Suppression of microbial activity and growth

The clay barrier in a DGR is designed to prevent movement of radionuclides, water, and microorganisms between the engineered and the natural components in Canada's nuclear waste storage system. The bentonite buffer functions to slow the diffusion of ground water to, and corrosion

products away from, the container surface thus limiting the kinetics of possible corrosion reactions. Importantly, the bentonite also serves to ensure that SRB are not able to produce sulfide and cause container corrosion. Although it is not anticipated, should there be a breach of a used fuel container and release of radionuclides, the bentonite buffer acts as a strong sorbent which will immobilize or greatly hinder the movement of any radioactive elements. Corrosion, alterations to the clay, and nutrient cycling can only occur if microorganisms are viable and able to survive inhibitory DGR conditions. For microorganisms to be active, electron donors, electron acceptors, carbon sources, and water must be available. Under engineered anoxic conditions, the community of microorganisms present in highly compacted bentonite clay will likely die or enter a dormant state due to restricted access to all the nutrients required for metabolism and/or physical space to grow [10].

There is a set of physical parameters including specific dry density, swelling pressure, pore size, and water activity that are required to prevent growth of microorganisms in highly compacted bentonite clay [10]. Dry density is a value that represents the degree of compaction of clay prior to introduction of a saturating fluid. As clay at a higher dry density saturates and swells, it results in a higher swelling pressure, lower water activity and smaller pore sizes. Reduced pore sizes and water limitation prevent the growth and movement of microorganisms [10, 40]. Pressure vessels containing saturated clay under conditions analogous to those in a proposed DGR have allowed for predictions regarding the influence of microorganisms on a temporal scale in a DGR. A study conducted on samples of highly compacted bentonite with dry densities ranging from 0.8 to 2.0 g/cm^3 demonstrated physical parameters required to maintain or reduce the number of culturable microorganisms in clay [10]. Ultimately, dry density $\geq$1.6 g/cm^3, water activity <0.96, and swelling pressure >2 MPa successfully suppressed an increase in culturable microorganisms (Figure 1.3). As well, a salinity >100 g/L resulted in lower water activity and fewer culturable aerobic heterotrophs. Dry densities <1.6 g/cm^3, therefore, may allow for microbial growth and activity [10, 41]. This suppression of microbial growth and activity has additionally been verified for a period of time of up to eight years [42].

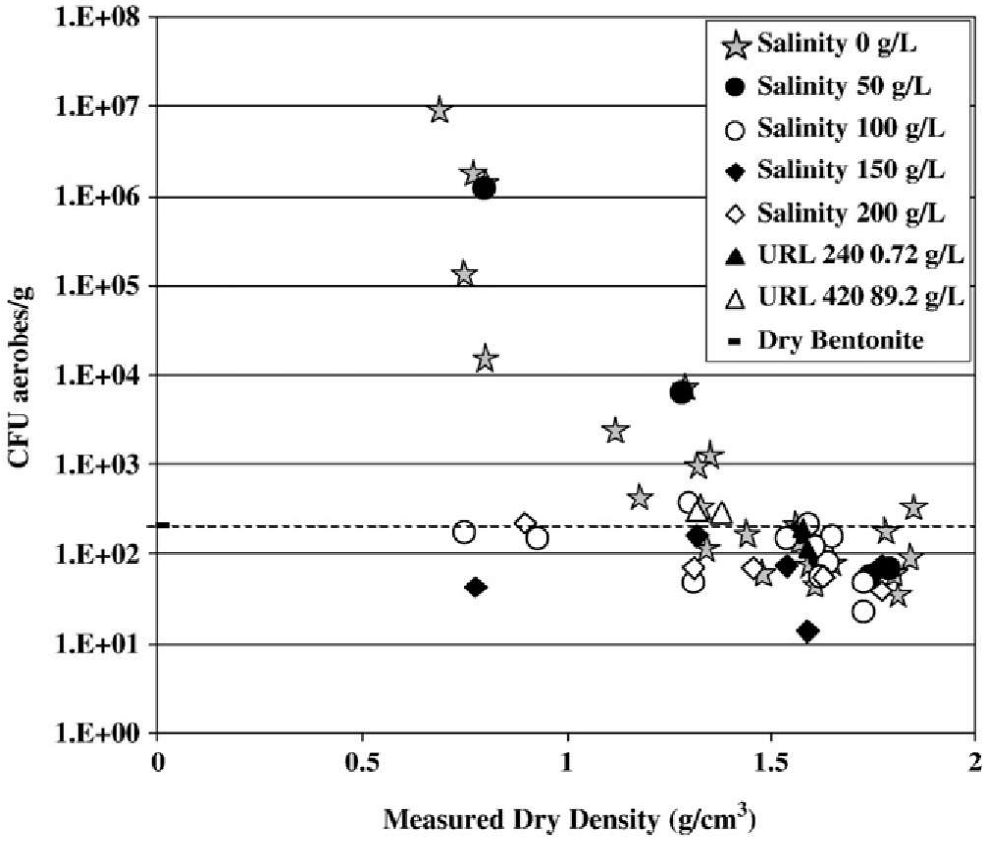

Figure 0.3 Culturable aerobic heterotrophs as a function of measured dry density in compacted Wyoming MX-80 bentonite. Data points denoted "URL" represent *in-situ* clay samples from an underground research laboratory. Along with various dry densities, different salinities of saturating water are reported in this figure. The dashed line represents the CFU aerobes/g in dry bentonite clay before incubation. This figure was reproduced from a previous publication [10].

Physical parameters can only effectively inhibit microbial growth if they are established evenly throughout the entire bentonite barrier. The outer layers of bentonite, at interfaces with other materials, tend to have higher water activity and therefore larger numbers of culturable microorganisms compared to enclosed/subsurface bentonite [10, 41–43]. These interfaces can be located between bentonite and the pressure vessel used in experiments. Likewise, bentonite interfaces in a DGR occur between bentonite and the host rock or bentonite and the used fuel container. These interfaces are identified as more favourable for microbial growth [42] and, therefore, are important to study regarding the clay component of a DGR.

After exposure to high pressure, high temperature, or gamma radiation microbial communities in bentonite clay samples changed [21]. Specifically in Wyoming bentonite, after each treatment there was a reduction in the most probable numbers of SRB and iron-reducing bacteria, but microbial activity was not completely inhibited [21]. Notably, pressure, heat and gamma radiation treatments of clay samples each impacted different phyla that made up the microbial community [21]. Aside from this, many experiments conducted with highly compacted bentonite introduce high pressure but do not account for the high temperature and gamma radiation that will be in a DGR. Experiments involving only high pressure are potentially the most important when predicting the state of a DGR after a long period of time because the high temperature and radiation of the radioactive waste will decrease over time.

1.1.4 Detection of microorganisms in clay

Assessment of clay microorganisms is required for predictions of their long-term impact on a DGR. To do so, multiple strategies have been employed to detect microorganisms present in clays before or after incubation under comparable conditions to DGRs. Responses of clay microbial communities to high compaction and swelling pressure, are often compared. Detection is required for interpretation of abundance, taxonomy, and metabolism of microorganisms inhabiting clay materials. Analyses of clay microorganisms are often based on cultivation, measurement of metabolites, and phospholipid fatty acids (PLFA). Because of adhesion and high sorption properties within clay matrices, separation of bacterial cells, DNA and RNA from clay is difficult, so analysis of nucleic acids is less frequently achieved [44, 45].

1.1.4.1 Cultivation

Microorganisms in powdered, as-received bentonite clays have been detected through traditional culturing mechanisms in multiple studies. Aerobic heterotrophs are typically isolated on R2A agar plates [46] and can range from 10^2 to 10^5 colony forming units (CFU) per gram of bentonite clay [10, 24, 39, 42, 47]. Anaerobic heterotrophs, also cultivated on R2A plates, are seldomly reported over 10^3 CFU/g [24, 42, 47]. The enumeration of sulfate- or iron-reducing bacteria is commonly based on most probable number (MPN) tubes [48] and reported abundances from bentonite clay samples range from below detection limit to ~10^3 MPN/g [21, 42, 47]. Cultivation enumerations are additionally employed to assess microbial communities before and after carrying out high pressure tests on clay, simulating DGR conditions [10, 21, 23, 42]. This has allowed researchers to determine temporal

changes in culturable microorganisms and draw conclusions about conditions that should be present to suppress microbial growth in a DGR.

There are strengths and weaknesses associated with microbial cultivation techniques. Because microbial cultivation confirms viability in the sample, such approaches do not overestimate abundances in the same way that quantification of microbial DNA can also target biomarkers from dead microorganisms or "relic" DNA [49, 50]. Although extracellular or "relic" DNA can have minimal impact on taxonomic and phylogenetic estimates, in some ecosystems bias can occur when nucleic acids are protected from extracellular enzymes within aggregates, biofilms, or other complexes [51]. Additionally, copy number variation of 16S rRNA genes within microbial genomes is also of concern because it can lead to incorrect estimations of microbial abundances [52]. However, traditional cultivation is selective for a subset of a microbial community depending on laboratory conditions applied and inherent cultivability of a given microorganism. As well, extracellular polymerase substances of microorganisms can adhere to clay, leading to ineffective removal separation from the clay matrix [44]. Sorption and adhesion of microorganisms to clay surfaces can prevent growth and therefore detection, resulting in underestimations of bacterial abundance [45]. Overall, traditional culturing allows for confirmation of living microorganisms in clay but is limited by cultivability and sorption.

1.1.4.2 Detection of microbial activity

Microbial activity in clay can be detected through measurement of microbial metabolites. Some common strategies used for metabolite measurement include redox indicator systems, detection of changes in pH, and fluorometry [53]. These studies utilize microcosms of clay consisting of a defined medium and periodic measurement of compounds in the airspace or liquid component to assess microbial activities [24, 25, 34]. Compounds measured have included terminal electron acceptors like sulfate and nitrate, as well as energy sources and electron donors such as H_2, lactic acid and acetic acid. Through the introduction of sulfate as a terminal electron acceptor, microcosms containing bentonite clay have demonstrated that the active microbial community can convert sulfate to hydrogen sulfide [24, 34]. Through measurement of sulfate, sulfide, iron, and pH in an Opalinus clay microcosm, it was demonstrated that the corrosive hydrogen sulfide from SRB can react with iron components of clay minerals, forming non-corrosive ferrous sulfides [25]. As well, measurement of acetate, sulfide, H_2, and pH from other clay microcosms have indicated that levels of H_2 gas influence the rate of sulfide production by SRB [54]. Clay microcosms are advantageous for assessment of the

potential microbial activity in a DGR. Analysis of microbial metabolism allows for an understanding of what drives changes in microbial activity. Microcosms also promote the growth of clay microorganisms, increasing the potential yield of DNA and allowing for microbial community analysis [21, 24, 25]. Although detection of microbial activity in clay demonstrates dominant metabolites produced by viable microorganisms under specified laboratory conditions, so the tests are limited to the conditions applied in each specific experiment.

1.1.4.3 Analysis of lipids

Based on phospholipids extracted from cell membranes, PLFA analysis assesses microbial abundances and composition without bias towards culturable microorganisms. Previous laboratory experiments conducted with bentonite and Opalinus clays have identified that the microbial cell abundance estimates based on PLFA exceed those based on cultivation by ~1000-fold [10, 43, 55]. Analysis of PLFA is also the only strategy that has detected eukaryotic biomarkers in clay [55]. Such PLFA analyses demonstrate that, whereas culturable microorganisms in saturated and highly compacted clay decrease over time, PLFA abundances remain constant [10, 56]. However, detection of PLFA does not distinguish between microorganisms that are alive and background "noise" present in clay samples. As well, conclusions regarding changes in detected PLFA abundances should be made with caution because rate of PLFA turn-over can be dependent upon environmental conditions [57]. It has been suggested that high PLFA abundances detected in soil samples can be correlated to higher pH [58] or that some PLFA detected in clay can reflect organic matter preserved in the clay matrix [43, 55]. Overall, the quantity of background "noise" that may be detected relative to the abundance of living microorganisms remains unknown.

1.1.4.4 Analysis of clay-associated nucleic acids

Nucleic acids extracted from clay materials are commonly below detection limits [21, 43, 53, 55, 59]. Low nucleic acid extraction efficiency is often due to properties of clay that cause sorption and adherence of charged nucleic acids to clay surfaces [44]. To overcome low nucleic acid extraction yields, microorganisms from clay may be cultivated first to increase the quantity of cells and thus DNA [24, 25, 34]. Microorganisms such as *Firmicutes* [24, 25], *Proteobacteria* [24, 34], and *Bacteroidetes* [24] have been detected from such clay microcosms. Common SRB-associated taxa detected in bentonite and Opalinus clay samples are affiliated with *Desulfosporosinus* and *Desulfobulbus* [24, 25, 34].

Another approach to overcome low DNA quantity in clay involves extraction from borehole water that flows around clay rock [17, 22, 37, 60]. Metagenomic sequencing of microorganisms filtered from borehole water surrounded by Opalinus clay identified a community of both autotrophic and heterotrophic microorganisms that cycle carbon dioxide, H_2, and sulfate [22, 61]. The microorganisms detected in the borehole water included *Proteobacteria, Firmicutes,* and *Actinobacteria* [17, 60]. One study of filtered borehole water detected archaea comprising up to ~0.3% of the relative proportion of the 16S rRNA gene sequences [60]. Although these borehole water studies account for non-culturable microorganisms, the microbial communities of clay rock, specifically, are poorly understood.

Even with the high sorption and the low quantity of DNA in clay, nucleic acids have been successfully extracted directly from the natural clay formations [62, 63], highly compacted bentonite clay [64], and as-received bentonite clay [65]. Recently, of the DNA extracted from as-received Wyoming MX-80 bentonite clay samples it was estimated that ~70% of high-throughput-sequencing reads are associated with "background" DNA that might arise from laboratory contaminants [65]. Whether the sequences obtained from clay are associated with viable cells, remains unknown. In natural bentonite clay formations in Spain, sequencing of the 16S rRNA genes from clay microorganisms generated a profile with 14 dominant phyla [63]. Most operational taxonomic units (OTUs) detected were associated with strict or facultative aerobes and there was high variation in microbial diversity between different sites [63]. Another recent study compared microorganisms in borehole water and compacted bentonite clay, revealing different dominant taxa in the borehole water and compacted clay [64]. *Desulfosporosinus*, *Rikenellaceae*, and *Smithella* genera were detected in borehole fluid and *Streptomyces, Paracoccus, Micrococcus,* and *Xanthomonas* were some of the dominant bentonite-associated microorganisms [64].

A common limitation of previous studies of bentonite clay microbial communities involves assessment of quantity and taxonomic diversity using culture-dependent strategies. Cultivation methodologies exclude non-culturable microorganisms and possibly viable but non-culturable (VBNC) microorganisms. The exclusion of non-culturable microorganisms from cultivation studies also biases the taxonomic distributions observed by 16S rRNA gene sequencing to readily cultured species. Of the studies that assess nucleic acids derived directly from clay, few have compared different types of clay. As well, few studies have compared microbial communities in clay with regards to factors such as different manufacturer, batch date, or lot number.

Despite recent advances, methods for analysis of nucleic acids from clay microorganisms still require further development. Studies of other low biomass environments explain the benefits to applying contaminant removal software, such as Decontam, because low biomass samples are known to contain a higher proportion of contaminant DNA [66–69]. Research involving other low biomass environments reinforces the importance of controls [67, 70, 71], unbiased cell lysis and PCR amplification methods [70], and identifying common sources of contamination [67, 72]. Additionally, confirmation that collection of larger sample prevents bias due to sample heterogeneity [73], and the impact of different sequencing platforms [74] have been assessed with regards to low biomass environments. As analysis of DNA from low biomass environments becomes more common, the repeatable and reliable protocols based on studies discussed in the preceding paragraph [62-74] will likely become standardized.

1.1.4.5 Viability of microorganisms

The bacterial state known as VBNC has been considered in studies of clay microbial communities [10, 56]. In a VBNC state, a bacterial cell is undetectable through traditional cultivation techniques, but can continue to take up nutrients and maintain metabolism, respiration, gene transcription, and membrane integrity [49]. The number and type of bacteria that enter the VBNC state depends on environmental conditions. In the laboratory, different methods for preservation of methane-oxidizing bacteria, for example, resulted in varying abundances of cells that entered the VBNC state [75]. Often a VBNC state is induced through stressful conditions, such as starvation, improper temperature, low oxygen availability, desiccation, and high saline concentrations [49]. This phenomenon is different from sporulation, persistence, and dormancy survival states because VBNC cells can maintain metabolic processes. There is skepticism about the VBNC state regarding whether it is a part of the life-cycle rather than an “end-of-life process” and whether activation from a VBNC state or “resuscitation” is even possible [76]. Furthermore, whether bacteria in powdered clay or highly compacted clay are in a VBNC state, remains unknown. Because long-term survival of microorganisms can be accomplished by maintenance of low cellular activity [49, 77], it has been suggested that this is a survival mechanism utilized by microorganisms in clay [56]. Assessment of changing microbial abundances at different dry densities of bentonite clay revealed that culturable microorganisms from the as-received clay increased after incubation at lower dry densities [10, 56]. However, the measured PLFA abundances detected were approximately the same no matter the dry density of the clay (Figure 1.4). This finding led to the hypothesis that though the viable cells detected

through PLFA analysis are similar in clay at all tested dry densities, the cultivability increased at lower dry densities as a result of activation from a VBNC state.

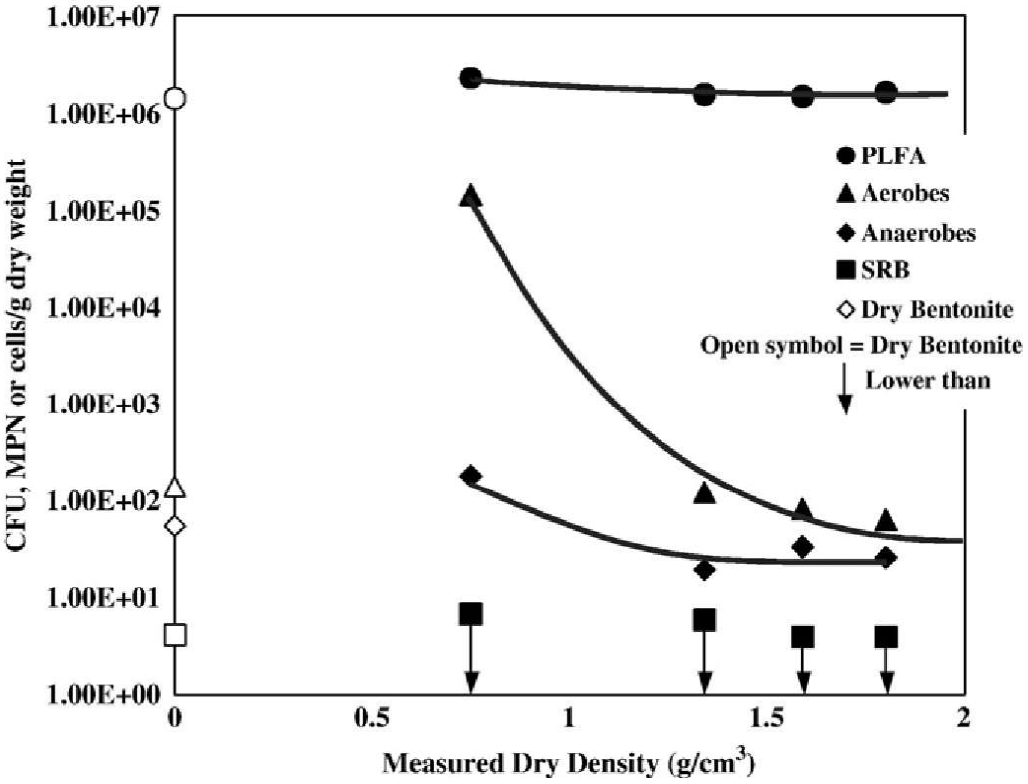

Figure 0.4 Comparison of culturable and PLFA-indicated biomass in Wyoming MX-80 bentonite. Compared to cultivation and PLFA analysis from the dry bentonite (data positioned on the y-axis), at the lowest tested dry density of bentonite the culturable bacteria increased in abundance, whereas PLFA abundances remained relatively consistent. At higher dry densities $\geq$1.6 g/cm^3, measured microbial abundances remained at or below those detected in the dry bentonite. This figure was reproduced from a previous publication [10].

The hypothesis that many clay bacteria are in a VBNC state is only based on PLFA and cultivation enumerations. To further test this hypothesis, a different culture-independent analyses, other than PLFA analysis, must validate the initial abundances of bacteria in bentonite clay. For example, assessing quantity of 16S rRNA gene copies through qPCR amplification would determine whether the relative quantity of DNA remains constant, similar to the PLFA abundance estimates. Previously qPCR quantification of DNA from Wyoming MX-80 bentonite has not been attempted for this purpose because of the inherent difficulty of extraction from the charged clay matrix.

1.2 Research overview

The overarching aim of this project was to combine nucleic acid profiling techniques and cultivation-based approaches to explore microbial community profile heterogeneity within diverse bentonite clay samples and assess how those profiles change when clay is saturated under DGR-relevant conditions. To better characterize the microbiology of bentonite clay, the research described in this thesis addressed two main objectives. The first objective was to explore microbial community profiles associated with representative bentonite clay samples using both cultivation-dependent and cultivation-independent techniques. Understanding the microbiology of bentonite clay is important to ensure long-term stability of associated barrier components in a DGR. Although characterization of culturable bentonite microorganisms has been achieved previously, the microbial profiles from nucleic acids are often left unexplored. Here, nucleic acid extraction from multiple diverse bentonite clay samples permitted comparison of microbial community profiles in manufactured bentonite clays. Heterogeneity of microbial communities within the same batch and among different batches were explored through assessment of biomarker (DNA/PLFA) profiles. Additionally, aerobic heterotrophs, denitrifying bacteria, and SRB from bentonite samples were enriched and quantified using common cultivation approaches and corresponding 16S rRNA gene profiles from all cultures were generated by high-throughput sequencing. Comparison of microbial profiles detected through cultivation approaches and from dry clay DNA extracts indicated a common core microbial community for clay representing viable/culturable and relatively abundant taxa within bentonite.

To ensure stability of a DGR over time, it is important that microorganisms present in clay materials are unable to grow and metabolise, potentially altering components of engineered barriers in the DGR. The second objective of this research was to assess microbiological changes associated with bentonite clay after containment at different dry densities. Combining strategies of cultivation and biomarker analysis provided data that are comparable to the assessments of as-received clay samples. Quantification of PLFAs, 16S rRNA genes, and aerobic heterotrophs was used to assess microorganisms from compacted and saturated bentonite clay. Comparison of these approaches tested the hypothesis that clay-associated microorganisms exist within a viable but nonculturable state and resuscitation depends on dry density conditions following saturation [10].

Chapter 2
Comparison of bentonite clay microbial communities

2.1 Introduction

Many countries are developing plans involving deep geological repositories for long-term storage of used nuclear fuel. Canada's used nuclear fuel bundles will be placed within copper-coated carbon steel containers. The carbon steel will provide strength and the copper will provide corrosion resistance [7, 78]. Used fuel containers will then be placed within highly compacted bentonite clay boxes and emplaced in the underground. As the clay slowly saturates with groundwater from the host rock it will swell, lowering water activity, preventing microbial growth, and retarding radionuclide transport [33]. Bentonite clay thus serves as an important component of the engineered barrier system within the natural host rock barrier. Together these barriers will provide protection against human activity and natural events such as glaciation cycles. The deep geological repository (DGR) barrier components are intended to ensure safe storage of spent nuclear fuel as it decays to safe levels of radiation over more than one million years.

Because microorganisms are present in mined materials, such as bentonite clay, several studies have evaluated their potential impacts on engineered barrier system components due to gas production, biofilm formation, radionuclide transport, transformation of clay minerals, and copper corrosion [33]. One of the primary microbiological considerations relevant to long-term containment of nuclear waste is microbiologically influenced corrosion (MIC). In a poorly designed DGR environment, MIC might occur when active microorganisms release metabolites that directly or indirectly accelerate metal corrosion [79, 80]. For example, sulfate-reducing bacteria (SRB) may contribute to MIC through anaerobic respiration and hydrogen sulfide (H_2S) production [21, 22, 25, 27, 79]. In turn, hydrogen sulfide can be corrosive to metals such as copper and steel. Heterotrophic bacteria are also relevant for DGR safety assessments because fermentation produces hydrogen gas (H_2) and acetate that can fuel sulfate-reduction by SRB [25, 61]. Denitrification can also lead to oxidation of the clay mineral pyrite (FeS_2) to sulfate, changing the structure of the clay and potentially providing a terminal electron acceptor for SRB activity [18, 31, 32]. Denitrifiers also produce gases that may cause formation of fissures in compacted clay, potentially allowing microorganism and radionuclide transport in the unlikely event of escape from the container [14, 33, 81]. Together, the potential for microbial activity to compromise DGR stability underlines the

importance of characterizing microbial activities in DGR design components and identifying conditions that minimize or prevent this activity over geological timeframes.

A predicate to modeling the potential impacts of microorganisms on DGR stability is developing an understanding of the microbial communities naturally present in mined bentonite clays. Several studies have enumerated SRB from bulk clay samples or clay from experimental treatments under conditions mimicking a DGR [10, 17, 21, 24, 41, 42, 56, 65, 82, 83]. In these studies, most probable number (MPN) tubes with sulfate-containing medium are often used. Sulfate-reduction is identified by the formation of a black precipitate that is produced as a result of hydrogen sulfide reacting with reduced iron within the medium to form ferrous sulfide (FeS). Heterotrophic clay bacteria are frequently grown on agar plates and R2A medium is commonly chosen because it contains minimal nutrients, mimicking the nutrient availability in clay environments [10, 17, 24, 42, 45, 47, 53, 56, 65, 84]. Detection of both aerobic and anaerobic heterotrophs is relevant because conditions in a DGR will change from oxic to anoxic after a short period of time [10, 24, 41, 42, 56]. Microorganisms capable of utilizing nitrate, such as denitrifiers, have also been studied in the context of a DGR [17, 18, 27]. The presence of denitrifying microorganisms is assessed with MPN tubes containing inverted Durham tubes. If nitrogenous gases are generated by denitrifiers, they collect in the Durham tube as a bubble.

Despite progress in quantifying and characterizing cultured bacteria from bentonite clays, the extent to which cultivation faithfully captures all viable clay microbes is unknown. Enumeration through traditional cultivation limits detection to only those microorganisms that can grow well under specific laboratory conditions. Furthermore, traditional cultivation excludes viable but non-culturable bacteria and slow-growing clay-associated microorganisms. Although community profiling methods based on extracted nucleic acids could help assess cultivation bias, sequencing of amplified 16S rRNA genes from bentonite clays has seldom been reported, presumably due to low biomass and sorption of DNA onto charged montmorillonite clay layers [44]. However, a protocol for successful extraction of DNA from bentonite clay samples has recently been validated, allowing for DNA-based quantification and identification of clay-associated microorganisms [65].

Building on benchmarking progress made by exploring bentonite clay-associated nucleic acid detection limits, this present study performed a comparison of cultivation and nucleic acid approaches for measuring microbial communities in bentonite clay. Although a primary goal of this study was to assess the heterogeneity in as-received bentonite clay samples from different sources and lots,

microbial community profiles were also used to test the hypothesis that cultivation-based approaches would reveal only a subset of the detectable microbial community profiled by direct 16S rRNA gene amplification and sequencing. In addition to microbial community profiling methods described above, clay microorganisms were assessed through quantification of 16S rRNA gene copies and characterization of the phospholipid fatty acid (PLFA) signatures within the selected clay samples. Establishing a microbial baseline for diverse clay samples and lots, and directly comparing results obtained by cultivation and nucleic acid analyses, informs future pressure cell experiments mimicking *in situ* conditions and ultimately informs specifications for a stable DGR system design.

2.2 Materials and Methods

2.2.1 Receiving and processing samples

Representative bentonite clay samples were collected for comparison, including those containing sodium and calcium exchangeable ions, from four different countries and prepared by five different manufacturers (Table 2.1). Coarse clay samples were ground to fine grain sizes using a glass DNA-free mortar and pestle. Replicate samples CC1 and CC2, and MX6 and MX7, differed only in starting granularity. Wyoming MX-80 samples with known production dates and lot numbers were used for assessment of time and batch characteristics on the microbial communities of clay.

Table 2.1 Diverse samples of as-received bentonite clays included in this study. Samples AB1, CC2, IR1, and MX7 received as small stones were ground prior to analysis. All available lot numbers and production dates are included.

Sample	Clay type	Exchangeable cation	Origin	Manufacturer	Production date[a]	Lot number[a]	Accession sample ID
MX1	MX-80 bentonite	Na^+	Wyoming, USA	American Colloid Company	Jun 2015	65275772	01
MX2	MX-80 bentonite	Na^+	Wyoming, USA	American Colloid Company	Nov 2016	116315319	02
MX3	MX-80 bentonite	Na^+	Wyoming, USA	American Colloid Company	Mar 2017	37324182	03
MX4	MX-80 bentonite	Na^+	Wyoming, USA	American Colloid Company	Mar 2017	37324184	04
MX5	MX-80 bentonite	Na^+	Wyoming, USA	American Colloid Company	Mar 2017	37324190	05
MX6	MX-80 bentonite	Na^+	Wyoming, USA	American Colloid Company	NA	NA	06
MX7	MX-80 bentonite	Na^+	Wyoming, USA	American Colloid Company	NA	NA	07
MX8	MX-80 bentonite	Na^+	Wyoming, USA	American Colloid Company	Jun 2015	65275768	08
MX9	MX-80 bentonite	Na^+	Wyoming, USA	American Colloid Company	NA	NA	14
AB1	Asha bentonite	Na^+	Kutch, India	Ashapura Minechem	NA	NA	09, 10
CC1	Sodium bentonite	Na^+	Saskatchewan, Canada	Canadian Clay Products	NA	NA	13
CC2	Sodium bentonite	Na^+	Saskatchewan, Canada	Canadian Clay Products	NA	NA	17
DC1	Deponit Ca-N	Ca^{2+}	Milos, Greece	S&B Industrial Minerals S.A.	NA	NA	15
IR1	IBECO-RWC	Ca^{2+}	Milos, Greece	S&B Industrial Minerals S.A.	NA	NA	11, 12
NB1	National standard	Na^+	Wyoming, USA	Opta Minerals	NA	NA	16

[a]NA, data not available

2.2.2 Moisture content and water activity

Approximately 2-5 g of clay was evenly placed into a steel cup and assessed using a water potentiometer (WP4 Dew Point Potentiometer, Meter Group). Water potential was measured following the manufacturer instructions at 25°C on fast mode. Water activity was calculated according to manufacturer instructions, using the potentiometer output of pressure (kPa) and temperature (°C). Moisture content was determined by measuring the loss of water after heating clay in an oven at 110°C for 22-28 hours. Samples were weighed on an analytical scale before (g_{wet}) and after drying (g_{dry}) to determine moisture content (mc) of the clay (Equation 2.1). Average moisture content and water activity were calculated from values obtained from triplicate samples of clay. Average moisture content for each clay sample was used to normalize all abundance estimates to gram dry weight (gdw), adjusting for variance caused by water mass within the clay samples.

$$mc = (g_{wet} - g_{dry}) / g_{wet} \quad \text{(Equation 2.1)}$$

2.2.3 Cultivation of bentonite clay bacterial communities

A dilution series was prepared in sterile phosphate-buffered saline (PBS; 0.01 M NaCl buffered to pH 7.6 with 9 mM Na_2HPO_4 and 1 mM $NaH_2PO_4 \cdot H_2O$) with clay dilutions of 10^{-1}-10^{-3}. The 10^{-1} dilution was prepared in a 50 mL tube by slowly adding 2 g of clay to 18 mL of PBS while vortexing continuously, followed immediately by gentle agitation for 30 minutes at room temperature. The agitation time was necessary to allow the clay to suspend and swell evenly, resulting in greater homogeneity of the clay-PBS solution and a higher efficiency of cell removal from clay interfaces [45]. Remaining dilutions were prepared by transferring 1 mL of the previous dilution into 9 mL PBS. Aliquots from all dilutions were dispensed into most-probable number (MPN) test tubes or onto R2A agar spread plates as described previously [10].

For enrichment and enumeration of SRB and denitrifying bacteria, MPN test tubes consisted of 9 mL of sterile sulfate-reducing medium (HiMedia Laboratories, M803) or liquid R2A medium (HiMedia Laboratories, M1687), amended with 12 mM sodium nitrate. Each sample was analyzed using a five tube MPN method. Test tubes were placed into a stainless-steel anaerobic culture chamber (Best Value Vacs), containing a GasPak EZ Anaerobe Container System Sachet (BD) and an anaerobic indicator strip (BD). Culture chambers were evacuated and flushed with N_2 gas up to 5 times before incubation for 28 days at 30°C. Every 7 days during incubation of denitrifying bacteria the chamber holding MPN tubes was gently shaken to encourage passage of gas bubbles from the

swollen clay and prevent clogging of the inverted Durham tubes. After incubation, positive MPN tubes were identified by a black precipitate for SRB and by a gas bubble within inverted Durham tubes for denitrifying bacteria activity. The MPN was determined using an MPN table (retrieved from: https://www.fda.gov/food/laboratory-methods-food/bam-appendix-2-most-probable-number-serial-dilutions) and the MPN per gram dry weight (gdw) was calculated using moisture content data for each sample. Mean MPN/gdw and standard deviation were calculated based on averaging the MPN/gdw obtained from each of three replicate sets of MPN tubes.

Aerobic and anaerobic heterotrophs were cultivated on triplicate plates of R2A medium with 1.5% agar [46]. Plates were incubated at 30°C under oxic conditions for 5-7 days or under anoxic conditions for 28 days. After the incubation period, colonies were counted and colony forming units (CFU)/gdw were calculated by adjusting for moisture content of each sample. In tandem with the MPN tubes, average CFU/gdw and standard deviation were calculated based on averages determined on three sets of triplicate plates, each inoculated with replicates of the clay sample. The lower limit of quantification for heterotrophic plate counts was determined to be 2500 CFU/g because plates with fewer than 25 colonies do not generate statistically valid data [85]. Additionally, the limit of detection was 100 CFU/g because CFU estimations could not be made if no colonies were present.

2.2.4 Genomic DNA extraction from clays and enrichment cultures

Genomic DNA was extracted from 2 g clay aliquots using the PowerMax Soil DNA Isolation kit (Qiagen) following manufacturer's instructions, with modifications as described previously [65]. After adding clay to PowerBead buffer, and before addition of lysis buffer, bead beating tubes were gently vortexed for 20 minutes to fully suspend and swell clay. Kit controls were included for each batch of DNA extractions. Quantification of DNA was performed using qPCR because all extracts were below fluorometric detection.

For extraction of enrichment culture genomic DNA from each sample, colonies on replicate agar plates for each dilution were slurried by adding 1 mL of sterile, DNA-free water and gently sweeping over plate surfaces with a sterile disposable spreader. The resulting colony slurry for each dilution was transferred to a microfuge tube. Contents of positive MPN tubes were mixed by vortexing, then 2 mL of suspended culture was transferred to a microfuge tube. All enrichment culture slurries were pelleted by centrifugation at 10,000 rpm for 2 minutes. Genomic DNA was recovered from the pellets following the protocol for DNeasy Ultraclean Microbial Kit (Qiagen),

using a bead beater (FastPrep-24 Instrument MP Biomedicals, OH, USA) at 5.5 m/s for 45 seconds. Genomic DNA from replicate MPN tubes of the same dilutions were pooled before amplification.

2.2.5 Quantitative polymerase chain reaction (qPCR)

The qPCR standard curve template was generated from the V3-V5 16S rRNA gene fragment of *Thermus thermophilus* that was cloned into vector pUC57-Kan. The template was amplified through PCR with primers M13F (5'- TGTAAAACGACGGCCAGT -3') and M13R (5'- CAGGAAACAGCTATGAC -3') that flank the 719 base-pair insert. The PCR product was separated on a 1% agarose gel and purified using the Wizard SV Gel and PCR Clean-Up System (Promega, WI, USA). The concentration of purified product was determined using a Qubit 4.0 Fluorometer (Thermo Fisher Scientific). A dilution series was prepared with qPCR standard DNA template.

Genomic DNA extracts from clay samples were quantified with qPCR using universal 16S rRNA gene primers 341F and 518R [86]. All qPCR amplifications were performed in duplicate and each contained 15 µL total volume with 1 × SsoAdvanced Universal SYBR Green Supermix (Bio-Rad), 0.3 µM of each primer, 7.5 µg bovine serum albumin (BSA), and 4 µL of undiluted template DNA (concentrations were below detection limit of Qubit 4.0 Fluorometer). The qPCR amplification was performed on a CFX96 Real-Time PCR detection system (Bio-Rad) beginning with 98℃ for 3 minutes followed by 40 cycles of 98℃ and 55℃ at 15 s and 30 s intervals, respectively. Starting 16S rRNA gene copy numbers were calculated from the linear regression equation produced from the standard curve with a 0.98 coefficient of determination (R^2).

2.2.6 Amplification of 16S rRNA genes and high-throughput sequencing

All PCR tube preparations were conducted in a PCR hood with ISO 5 HEPA filtered air and surfaces that were cleaned with 70% ethanol and treated with UV light for 15 minutes (AirClean Systems, ON, Canada). Each 25-µL PCR mix contained: 1× ThermoPol Buffer, 0.2 µM forward primer, 0.2 µM reverse primer, 200 µM dNTPs, 15 µg BSA, 0.625 units of hot start *Taq* DNA polymerase (New England Biolabs, MA, USA), and 1-10 ng of template DNA. The V4-V5 region of the 16S rRNA gene was amplified in triplicate using primers 515F-Y [87] and 926R [88]. The primers for each sample were modified to contain a unique 6 base index in addition to Illumina flow cell binding and sequencing sites [89]. Thermocycle conditions were: 95°C initial denaturation for 3 min, followed by 40 cycles of 95°C denaturation for 30 sec, 55°C annealing for 30 sec, and 68°C extension for 1 min, followed by a final extension of 68°C for 7 min. Negative controls containing no template (NTCs)

were included in the 96-well PCR plates to test for well-to-well contamination, and outside of plate controls tested for reagent contamination. Positive controls contained a 1:1 ratio of V3-V5 hypervariable regions of the 16S rRNA genes of *Thermophilus* and *Vibrio fischeri*, each separately cloned into pUC57-Kan plasmids.

Based on agarose gel quantification, amplicon DNA from all samples was normalized and equal nanogram quantities were pooled into a single DNA-free microfuge tube. The pooled amplicons were gel purified using the Wizard SV Gel and PCR Clean-Up System (Promega, WI, USA) and the pooled library was denatured and diluted following Illumina guidelines (Document no. 15039740 v01). The 8 pM library containing 15% PhiX control v3 (Illumina Canada Inc, NB, Canada) was sequenced on a MiSeq (Illumina, CA, USA) using a 2 × 250 cycle MiSeq Reagent Kit v2 (Illumina Canada). Samples were sequenced in two runs and sequences were merged in the post sequence analysis. All sequences were deposited into the European Nucleotide Archive (https://www.ebi.ac.uk/ena) with study accession number PRJEB39383.

2.2.7 Post sequencing analysis

Samples were analyzed using Quantitative Insights Into Microbial Ecology 2 (QIIME2, release 2019.10, [90]) that was managed by automated exploration of microbial diversity version 3 (AXIOME3;[91]; github.com/neufeld/AXIOME3). The DADA2 (version 2019.10;[92]) pipeline was used to remove primer sequences and trim low-quality sequences after 250 bases for forward and reverse reads. Taxonomy was assigned to ASVs using a naive Bayes classifier (feature-classifier classify-sklearn version 2019.10) pre-trained with SILVA database release 138 [93]. Decontam (Release 3.11) assigned prevalence-based *p* values to each ASV, and those with *p* values higher than the recommended cut-off value of 0.5 were identified as potential contaminants [66]. Controls assessed through Decontam included a swab of a sterile agar plate, kit controls for each DNA extraction batch, reagent controls for sequencing, and positive controls for sequencing. The ASVs identified as contaminants were manually removed from the sample ASV table. Ordinations and collapsed ASV tables were generated using QIIME2 plugins. The PCoA coordinates were based on rarefication to 2530 reads.

To assess members of the core microbial communities detected in clay and enrichment cultures (Section 2.3.3), read counts for all ASVs detected in all enrichment cultures for each sample were pooled together. Read counts for ASVs detected in replicate clay samples were also pooled. All read counts for clay and enrichment culture pools were normalized to 10 000 reads for each

sample. From the normalized reads, ASVs detected at ≥0.1% relative abundance in at least one of the cultured samples and one of the dry clay samples were labelled as core ASVs. To compare ASVs detected in dry clay and/or enrichment cultures, read counts from all samples were summed for each dry clay ASV. Read counts detected in enrichment cultures across all samples were also summed for each ASV.

2.2.8 Phospholipid fatty acid analysis

A 50 g aliquot of each clay sample was aseptically weighed and phospholipid fatty acid (PLFA) analysis was carried out by Microbial Insights (Knoxville, TN). Lipids were recovered with a modified Bligh and Dyer method [94]. Lipids were fractionated on disposable silicic acid columns into neutral-, glycol-, and polar-lipid fractions after being extracted, using a one-phase chloroform-methanol-buffer extractant, and dissolved in chloroform. To recover polar lipid fractions as methyl esters in hexane, PLFA were transesterified with mild alkali. The PLFA were analyzed with gas chromatography and peak confirmation was performed with electron impact mass spectrometry (GC/MS). The detection limit of this method was 150 pmole and the limit of quantification for PLFA was 500 pmole. A Level III raw data report was generated containing estimates of total biomass based on the assumption that 1 pmole of PLFA represented 20,000 cells. Relative proportions of PLFA were separated into structural categories that were assigned according to PLFA chemical structure.

2.3 Results and Discussion

2.3.1 Heterogeneity in representative clay samples

In this present study, bentonite clay from India, Canada, Greece, and the United States of America, with various exchangeable cations, production dates, and lot numbers (Table 2.1 and Figure 2.1), were assessed to establish a baseline for variance among bentonite clay microbial communities. Visually similar grain sizes were identified in each clay sample prior to performing analyses. The selected clays were dry, with moisture contents ranging from 5-15% (Figure 2.2). In addition, water activities for as-received clays ranged from 0.26-0.70 and were therefore below the minimum water activity (0.96) required for suppression of microbial growth [10].

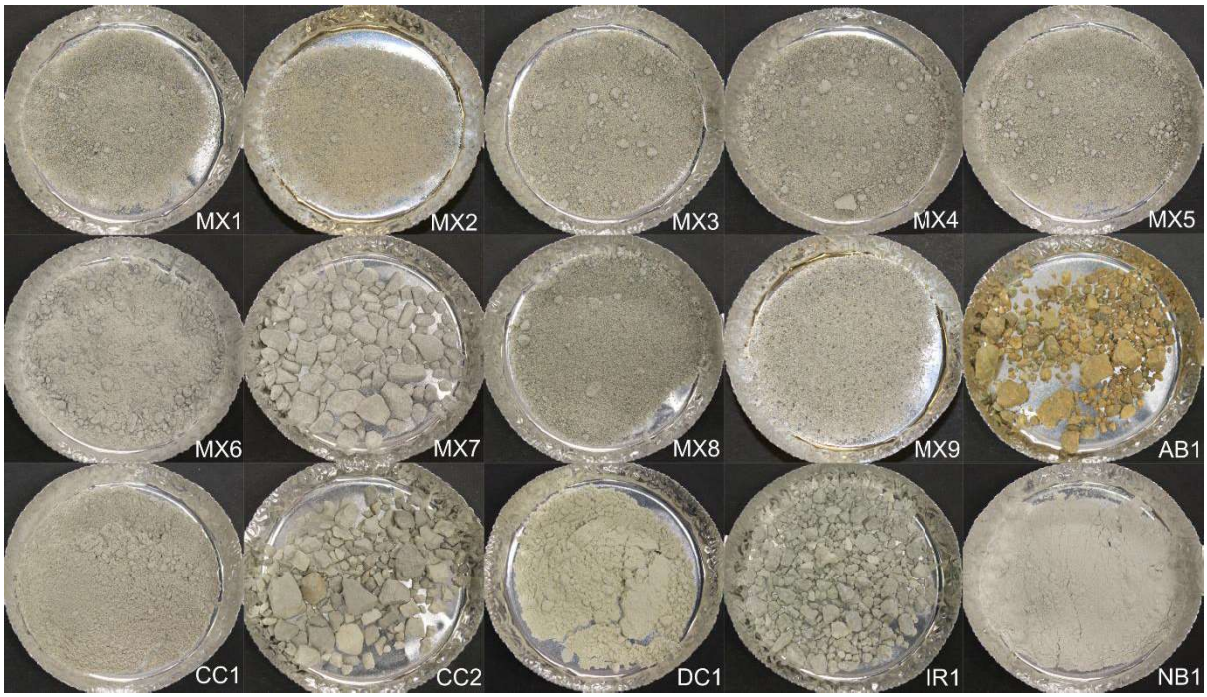

Figure 2.1 Photographs of the as-received bentonite clay samples used to perform this study. Coarse samples (AB1, CC2, IR1, and MX7) were ground to smaller grain sizes before analysis.

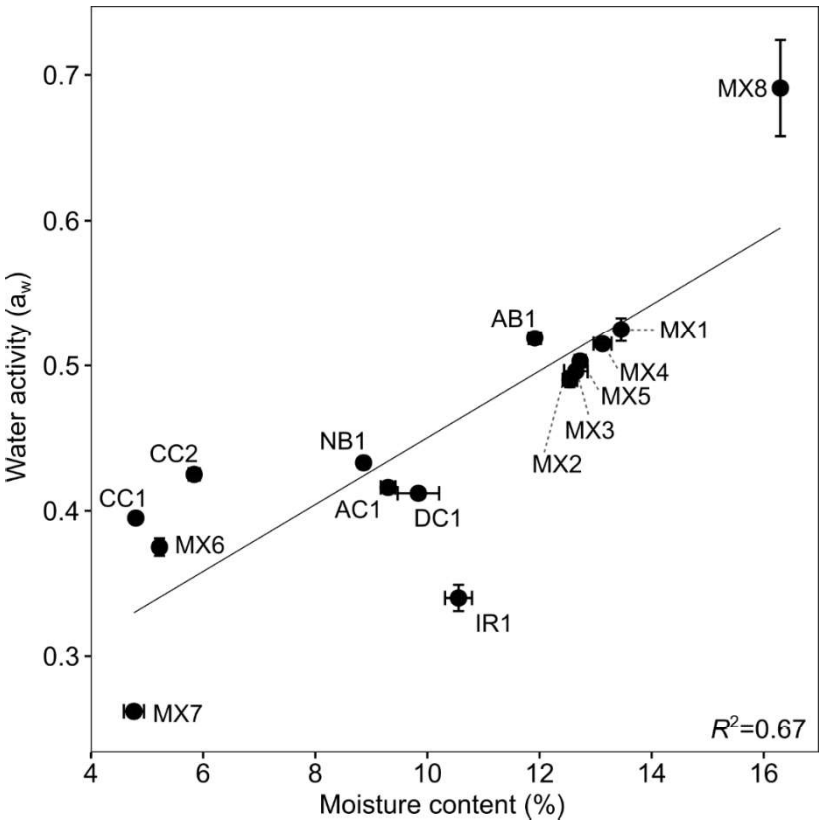

Figure 2.2 Moisture contents and water activities for all as-received clay samples. Error bars represent standard deviation of triplicate analyses and the solid line is the linear regression. Water activity was calculated using a water potentiometer and moisture content values were calculated after oven drying.

Quantities of culturable aerobic and anaerobic heterotrophs, SRB, and denitrifying bacteria in clay samples were compared. As well, biomarker analysis involving 16S rRNA gene profiling and PLFA analysis provided cultivation-independent microbial community profiles. Cultivation of clay samples resulted in lower microbial abundance estimates than methods using DNA or PLFA quantification (Figure 2.3), in most cases by orders of magnitude. Aerobic heterotroph abundances ranged from 10^2 to 10^4 CFU/gdw, which is within the range of previously reported aerobic heterotroph abundances for as-received bentonite clays (10^2 to 10^5 CFU/g; 16, 18, 25, 27). Eleven samples contained average abundances of aerobic heterotrophs that were below the limit of plate count quantification (2500 CFU/gdw), and one sample (NB1) additionally contained aerobic heterotroph abundances that were below the limit of detection (100 CFU/gdw). Anaerobic heterotrophs were less abundant than aerobic heterotrophs in all samples. All anaerobic heterotroph enumerations contained averaged microbial abundances below the lower limit of quantification, and

nine of the fifteen samples were additionally below the detection limit (Figure 2.3). Average MPN estimates for SRB from the bentonite samples were 63 ± 87 MPN/gdw, comparable to previously studied as-received bentonites with SRB abundances of up to 42 MPN/g [10, 24, 47]. Denitrifying bacteria enumerations detected an average abundance of 57 ± 36 MPN/gdw. Overall, Asha bentonite (AB1) was associated with the highest abundances for all enumeration methods, except for PLFA analysis, for which it had the second highest abundance estimate (Figure 2.3).

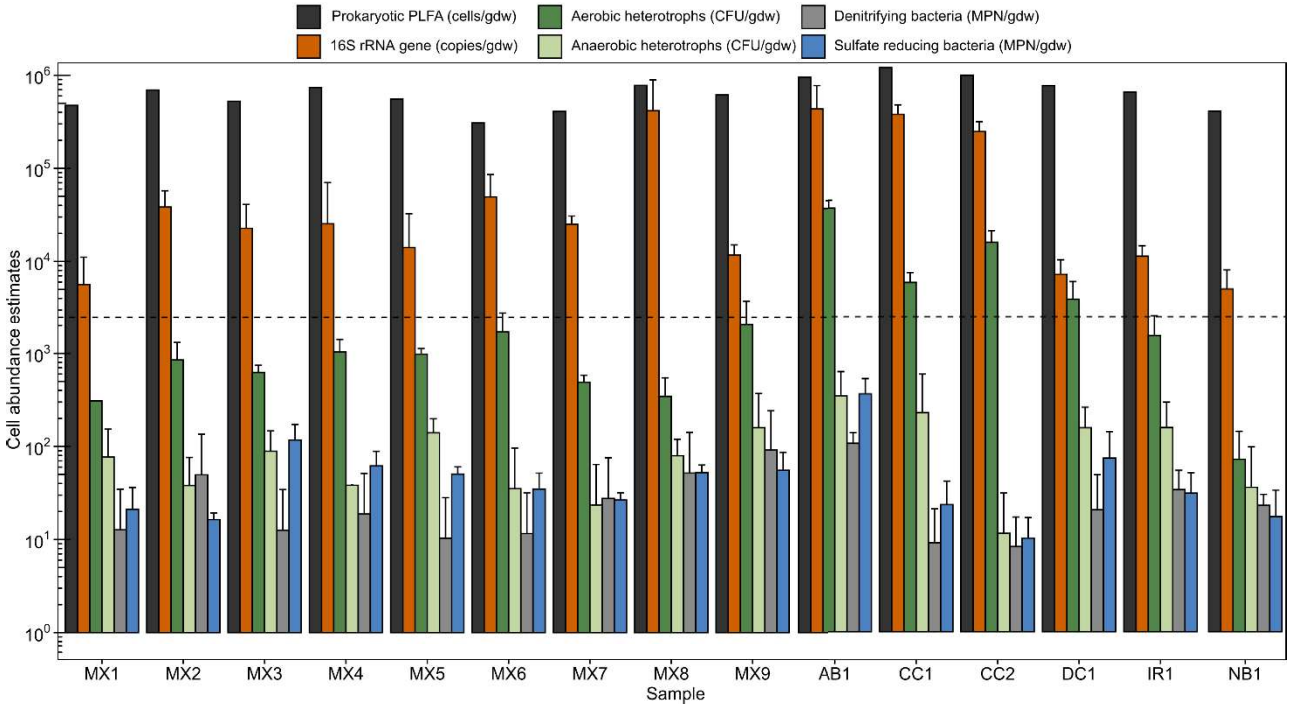

Figure 2.3 Microbial abundances estimated through culture-dependent and culture-independent assessments of bentonite clays. Abundance estimates were all normalized to gram dry weight (gdw) using average moisture content values for each clay sample. The dashed line represents the lower limit of plate count quantification (2500 CFU/g). Error bars show the standard deviation of triplicate culture-based enumerations and the standard deviation of quadruplicate qPCR amplifications. The PLFA analyses were performed without replication and only the predicted prokaryotic abundances are reported here for comparison.

The highest microbial cell abundance estimates were associated with PLFA data (Figure 2.3). Detected PLFA ranged from 16-61 pmol/g, with an average of 32 ± 12 pmol /g. Previous reports of PLFA quantities in bentonite and Opalinus clays presented 10^6 cells/g [10, 43, 55] that is comparable to the total PLFA abundances of prokaryotic and eukaryotic biomarkers detected in this study, ranging from 10^5 to 10^6 cells/gdw. Between all tested bentonite clay samples, PLFA community structures contained some variation (Figure 2.4). For two bentonite samples, PLFA was performed in duplicate (AB1 and AB2, and IR1 and IR2), resulting in similar relative abundances of each community structure profile (Figure 2.4). The SRB/Actinomycetes community structure group accounted for an average of $4.0 \pm 2.5 \times 10^4$ cells/gdw and eukaryotic biomass ranged from 8.0×10^3 to 9.6×10^4 cells/gdw, accounting for 1.6% to 11.0% of the PLFA-associated predicted cell abundances (Figure 2.4).

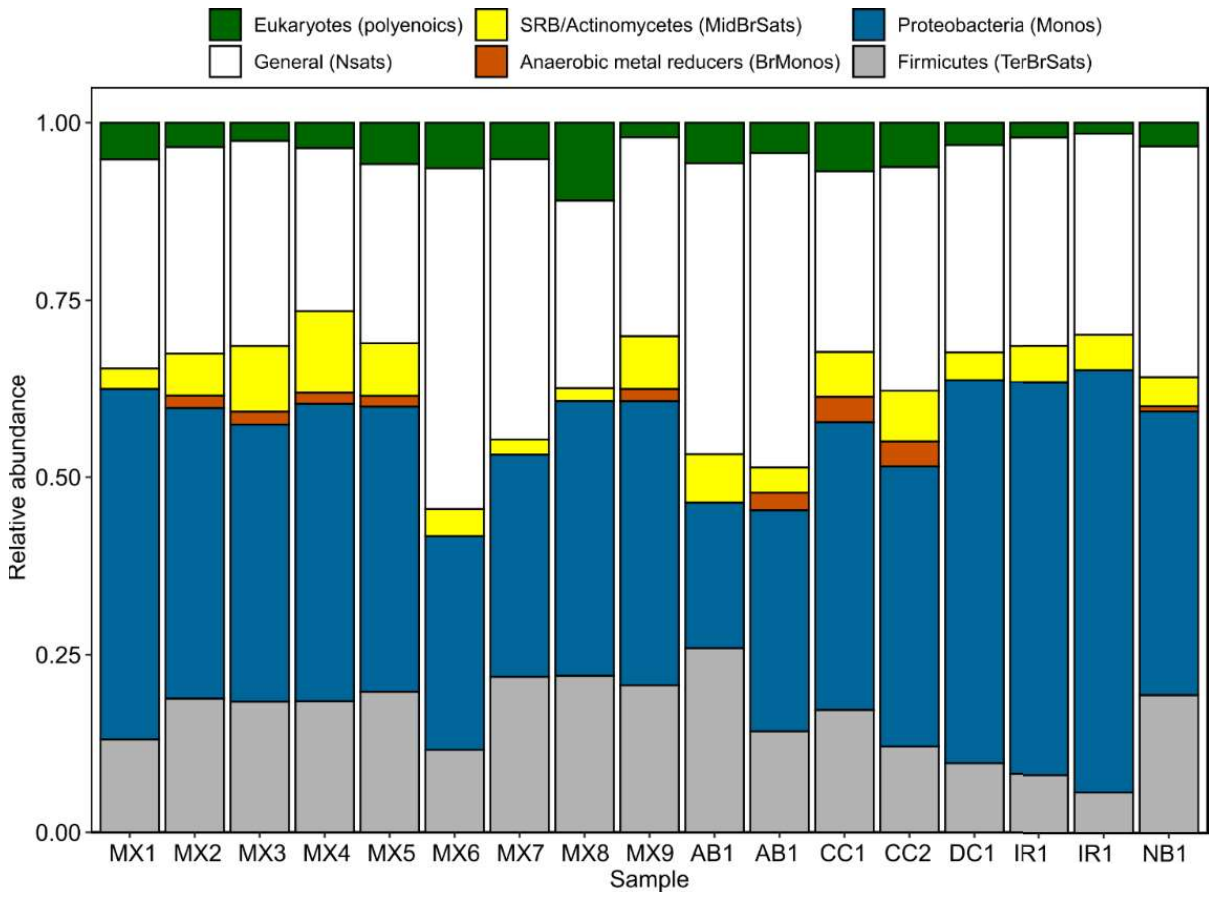

Figure 2.4 Phospholipid fatty acid (PLFA) community structure relative abundances across diverse clay samples. Samples AB1 and IR1 were assessed in duplicate. Structural groups were separated into different categories based on monoenoic (Monos), terminally branched saturated (TerBrSats), branched monoenoic (BrMonos), mid-chain branched saturated (MidBrStats), normal saturated (Nsats), and polyenoic phospholipid fatty acid profiles detected in each sample. Each category of PLFA is generally associated with specific groups of microorganisms, except for Nsats fatty acids because they can be found in all organisms.

Microbial community profiles were generated for all clay samples using high-throughput sequencing of 16S rRNA genes and classification to the ASV-level. After assessment of sequences from 21 positive and negative controls, Decontam identified potential reagent contaminant ASVs that were associated with DNA-extraction kit controls and PCR no-template controls (NTCs; Figure 2.5). Three contaminant ASVs detected in enrichment culture samples were affiliated with *Afipia*, *Escherichia-Shigella*, and *Pelosinus*, and seven unique contaminant ASVs were detected in as-received clay samples (Figure 2.5). The presented ASVs were removed from the ASV table before

further analyses (Figure 2.5). After removal of contaminant ASVs, the 16S rRNA gene read counts were compared across different DNA sources (Figure 2.6). Average quantities of read counts from control samples were lower than average read count quantities in samples (Figure 2.6). Sample read counts were normalized to 2530 before downstream principal coordinate analysis of clay microbial community profiles ensued.

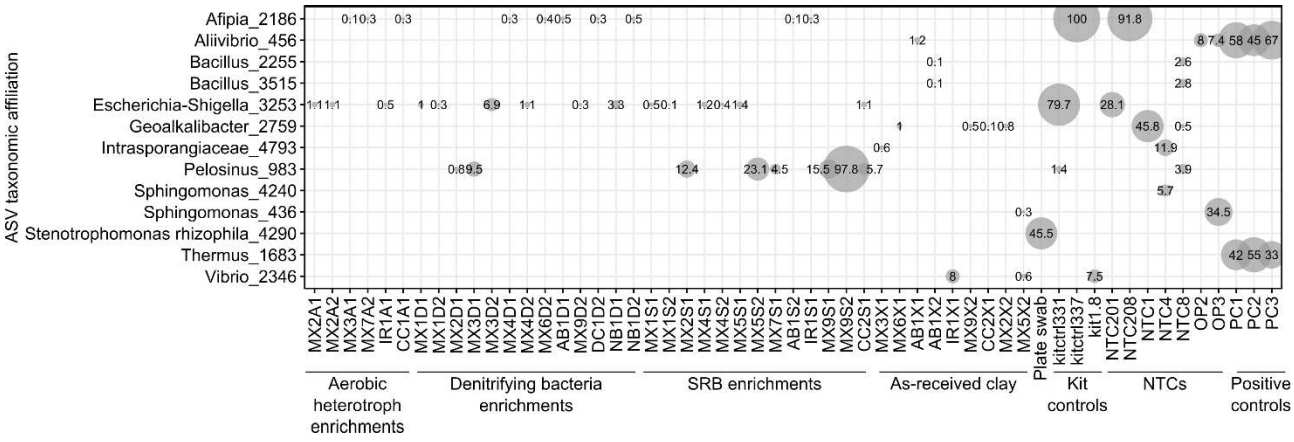

Figure 2.5 Contaminant ASVs identified in enrichment culture and as-received clay samples. Samples and controls containing contaminants with ≥0.1% relative abundance are shown. Sample names include the sample ID, the source of DNA (A, aerobic heterotroph enrichments; D, denitrifying bacteria enrichments; S, SRB enrichments; X, dry clay) and the replicate number (1 or 2). Negative controls include a "plate swab" from the surface of uninoculated R2A agar, kit controls used for each batch of DNA extractions, and no-template controls (NTCs).

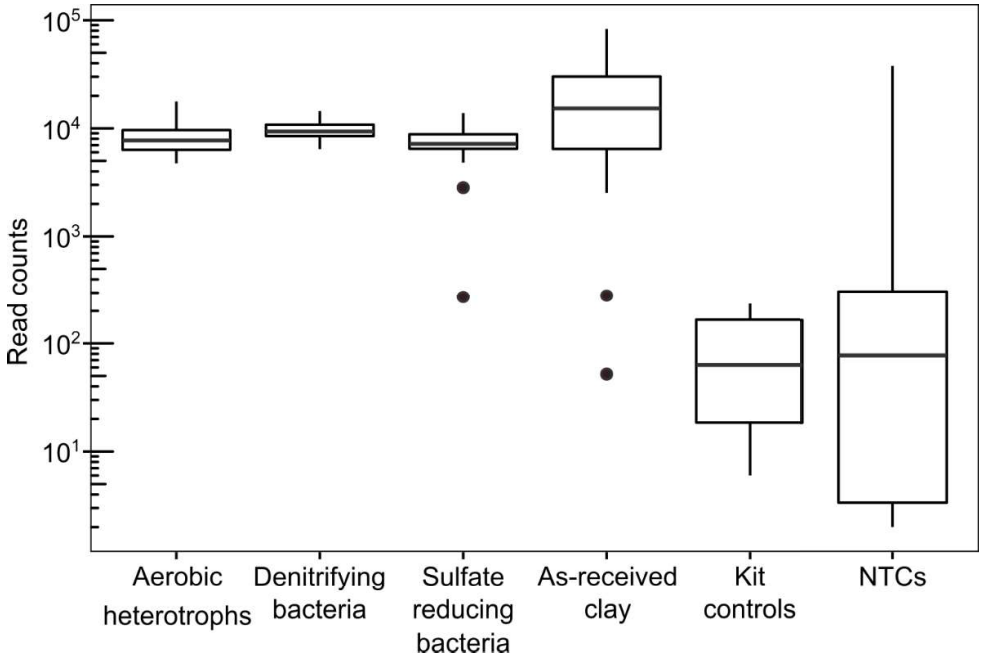

Figure 2.6 Clay, enrichment culture, and control 16S rRNA gene high-throughput sequencing read counts. The no-template controls (NTCs) were used to assess possible reagent contamination from the laboratory. Total read counts exclude reads from contaminant ASVs identified by Decontam.

Distinct microbial community profiles were detected in each sample of bentonite. Among the clay samples, duplicates often grouped together within ordination space (Figure 2.7). The bentonite samples from Milos (Greece; IR1), MX-80 bentonite from Wyoming (USA; MX1 and MX5) and National bentonite from Wyoming (USA; NB1) exhibited higher dissimilarity compared to other sets of duplicates. Dissimilarity among duplicates may have been due to a combination of clay sample heterogeneity or extraction efficiency from respective bentonites. As well, of the microbial community profiles detected in the clay samples, the Wyoming MX-80 clays with the same production dates contained similar profiles, despite different lot numbers (Figure 2.7). Clay samples from March 2017 (MX3, MX4, and MX5) grouped closer together than samples from June 2015 (MX1 and MX8). Previous analysis of bentonite clays also revealed similarities in the microbial community profiles detected from clays with similar production dates [65]. Considering the countries of origin, bentonite clay samples from Saskatchewan, Kutch, and Milos grouped among the bentonites from Wyoming. The unique profile of ASVs detected in each clay sample lead to these patterns or groupings (Figure 2.8). Many ASVs were unique to a single clay sample, but some, such as those affiliated with *Streptomyces*, *Sphingomonos*, *Thiobacillus*, and *Xanthomonas*, were more

broadly detected in clay samples. Overall, ASVs detected in bentonite clay samples were commonly related to members of the *Actinobacteria* and *Proteobacteria* phyla. Assessment of the heterogeneity in the clay-associated microbial profiles is important for planning pressure-cell experiments that will help inform predictions of microbial activity in the context of a DGR.

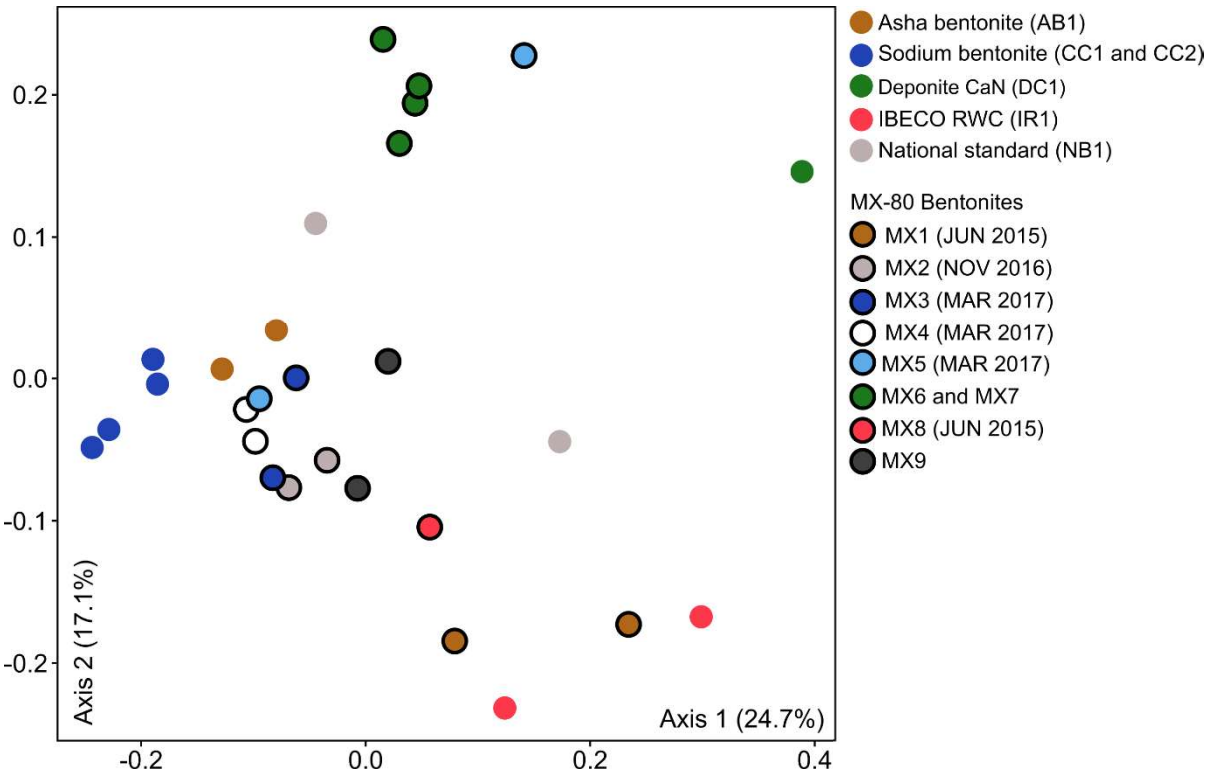

Figure 2.7 Principle-coordinate analysis (PCoA) ordination plot based on the weighted UniFrac distance metric of 16S rRNA gene sequences generated for clays. Replicates of DC1 and MX8 were removed during normalization to 2530 bases.

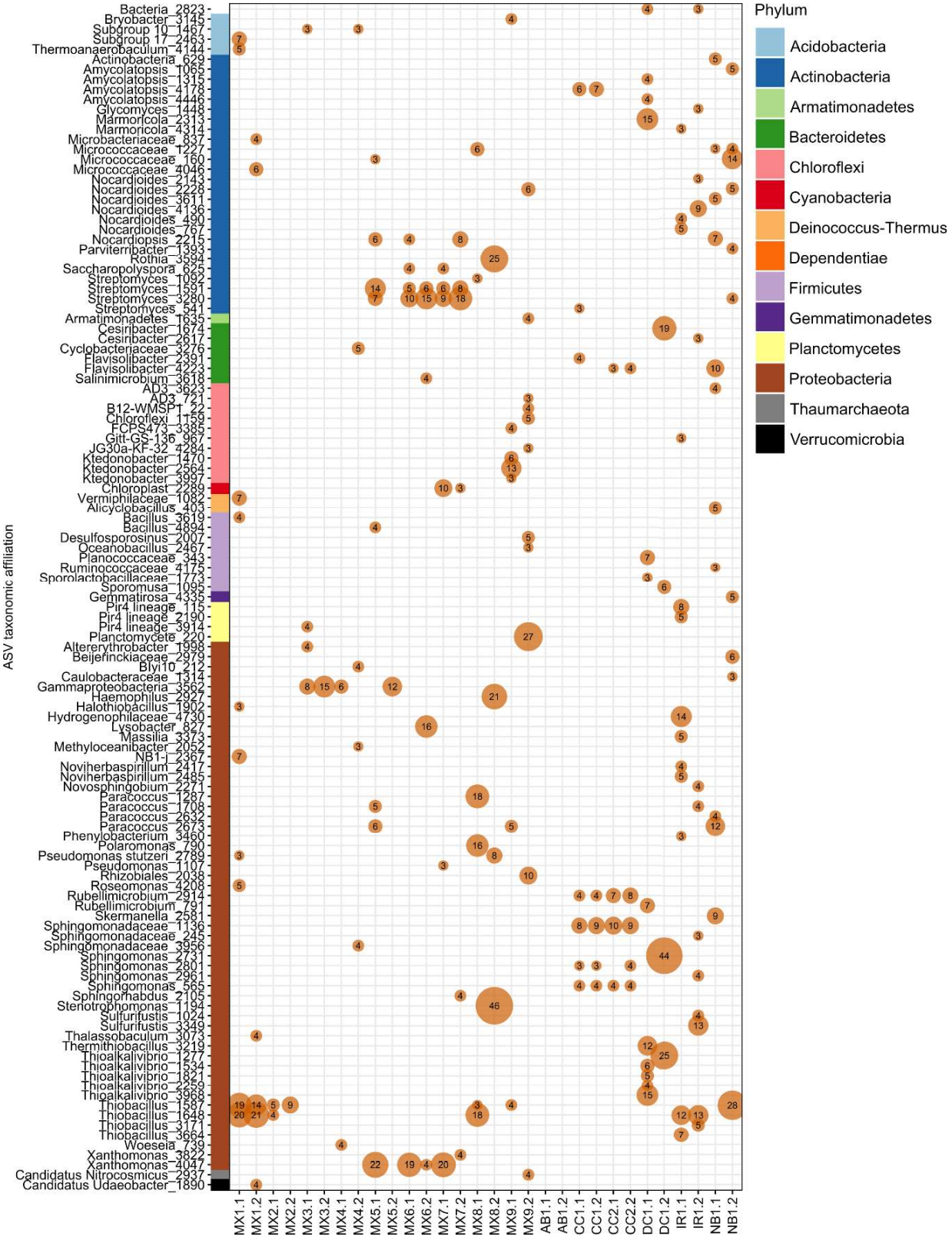
ASV taxonomic affiliation
Bacteria_2823
Bryobacter_3145
Subgroup_10_1467
Subgroup_17_2463
Thermoanaerobaculum_4144
Actinobacteria_629
Amycolatopsis_1065
Amycolatopsis_1315
Amycolatopsis_4178
Amycolatopsis_4446
Glycomyces_1448
Marmoricola_2313
Marmoricola_4314
Microbacteriaceae_837
Micrococcaceae_1227
Micrococcaceae_160
Micrococcaceae_4046
Nocardioides_2143
Nocardioides_2228
Nocardioides_3611
Nocardioides_4136
Nocardioides_490
Nocardioides_767
Nocardiopsis_2215
Parviterribacter_1393
Rothia_3594
Saccharopolyspora_625
Streptomyces_1092
Streptomyces_1591
Streptomyces_3280
Streptomyces_541
Armatimonadetes_1635
Cesiribacter_1674
Cesiribacter_2617
Cyclobacteriaceae_3276
Flavisolibacter_2391
Flavisolibacter_4223
Salinimicrobium_3618
AD3_3623
AD3_721
B12-WMSP1_22
Chloroflexi_1759
FCPS473_3385
Gitt-GS-136_967
JG30a-KF-32_4284
Ktedonobacter_1470
Ktedonobacter_2564
Ktedonobacter_3997
Chloroplast_2289
Vermiphilaceae_1082
Alicyclobacillus_403
Bacillus_3619
Bacillus_4894
Desulfosporosinus_2007
Oceanobacillus_2467
Planococcaceae_343
Ruminococcaceae_4175
Sporolactobacillaceae_1773
Sporomusa_1095
Gemmatirosa_4335
Pir4 lineage_115
Pir4 lineage_2190
Pir4 lineage_3914
Planctomycete_220
Altererythrobacter_1998
Beijerinckiaceae_2979
Blyi10_212
Caulobacteraceae_1314
Gammaproteobacteria_3562
Haemophilus_2927
Halothiobacillus_1902
Hydrogenophilaceae_4730
Lysobacter_827
Massilia_3373
Methyloceanibacter_2052
NB1-j_2367
Noviherbaspirillum_2417
Noviherbaspirillum_2485
Novosphingobium_2271
Paracoccus_1287
Paracoccus_1708
Paracoccus_2632
Paracoccus_2673
Phenylobacterium_3460
Polaromonas_790
Pseudomonas stutzeri_2789
Pseudomonas_1107
Rhizobiales_2038
Roseomonas_4208
Rubellimicrobium_2914
Rubellimicrobium_791
Skermanella_2581
Sphingomonadaceae_1136
Sphingomonadaceae_245
Sphingomonadaceae_3956
Sphingomonas_2731
Sphingomonas_2801
Sphingomonas_2961
Sphingomonas_565
Sphingorhabdus_2105
Stenotrophomonas_1194
Sulfurifustis_1024
Sulfurifustis_3349
Thalassobaculum_3073
Thermithiobacillus_3219
Thioalkalivibrio_1277
Thioalkalivibrio_1534
Thioalkalivibrio_1821
Thioalkalivibrio_2259
Thioalkalivibrio_3968
Thiobacillus_1587
Thiobacillus_1648
Thiobacillus_3171
Thiobacillus_3664
Woeseia_739
Xanthomonas_3822
Xanthomonas_4047
Candidatus Nitrocosmicus_2937
Candidatus Udaeobacter_1890
MX1.1
MX1.2
MX2.1
MX2.2
MX3.1
MX3.2
MX4.1
MX4.2
MX5.1
MX5.2
MX6.1
MX6.2
MX7.1
MX7.2
MX8.1
MX8.2
MX9.1
MX9.2
AB1.1
AB1.2
CC1.1
CC1.2
CC2.1
CC2.2
DC1.1
DC1.2
IR1.1
IR1.2
NB1.1
NB1.2
Phylum
Acidobacteria
Actinobacteria
Armatimonadetes
Bacteroidetes
Chloroflexi
Cyanobacteria
Deinococcus-Thermus
Dependentiae
Firmicutes
Gemmatimonadetes
Planctomycetes
Proteobacteria
Thaumarchaeota
Verrucomicrobia

Figure 2.8 Relative abundances of ASVs affiliated with as-received clay samples. Bubbles represent the ASVs, grouped by phyla, detected with relative abundances ≥3% from each clay sample. Sample replicate numbers (i.e., 1 or 2) are indicated after the decimal place at the end of each sample code. Sample AB1 contained no ASVs with ≥3% relative abundance.

2.3.2 Comparison of community profiling approaches

Along with assessment of heterogeneity among the DNA from various bentonite clays, the microbial profiles detected through direct sequencing of DNA isolated from enrichment cultures were compared. Cultivated community profiles grouped separately from the corresponding clay after cultivation, (Figure 2.9), because enrichment cultures promoted the growth of a subset of clay microorganisms (Figure 2.10). Sulfate-reducing bacteria cultured from all clay samples were frequently from the order *Clostridiales* (Figure 2.10). In general, aerobic R2A plates isolated bacteria almost exclusively affiliated with the order *Bacillales* (Figure 2.10). Orders of bacteria detected in enrichment cultures for denitrifying bacteria were similar to those detected in SRB and aerobic heterotroph enrichments (Figure 2.10). Similarities were likely due to the culture conditions that were supportive of anaerobic heterotrophy. At the order level of taxonomic classification, cultivation of clay microorganisms resulted in detection of microbial community profiles that were similar across diverse clay samples and different from those detected in clay via DNA profiling (Figure 2.10). Alternatively, at an ASV-level resolution it was apparent that the microbial community profiles were unique for every sample (Figure 2.11, 2.12, and 2.13).

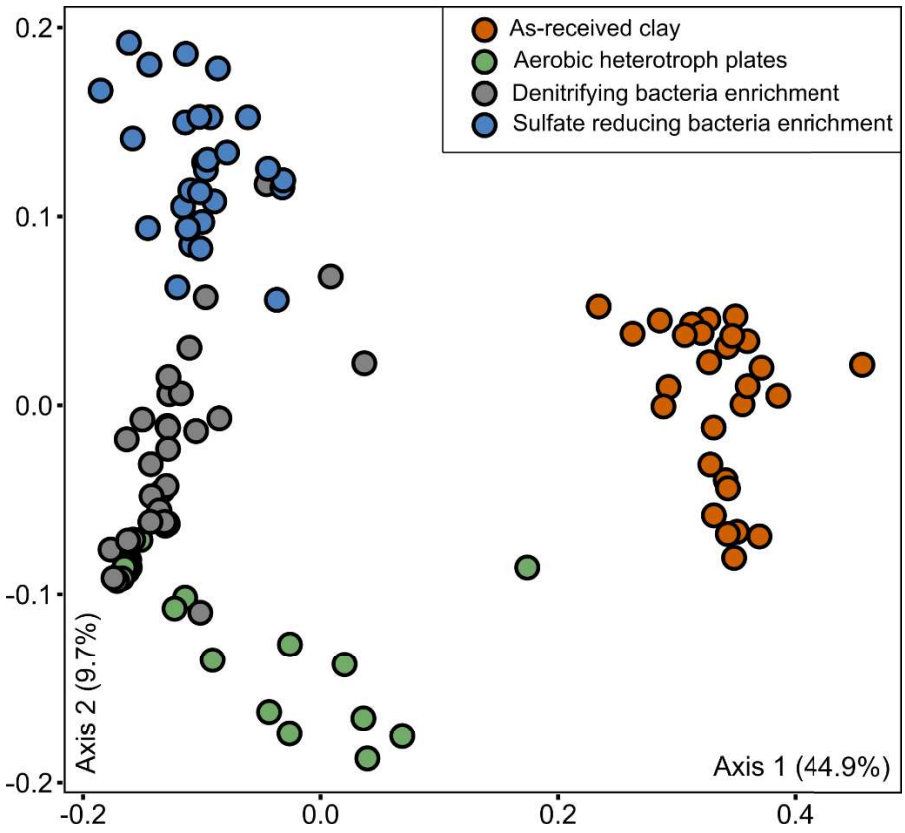

Figure 2.9 Comparison of clay microbial profiles based on nucleic acid and cultivation-based analyses. This weighted UniFrac PCoA plot of 16S rRNA gene sequences was generated from as-received clay and enrichment culture DNA extract profiles. Sequences were normalized to read counts of 2530 reads.

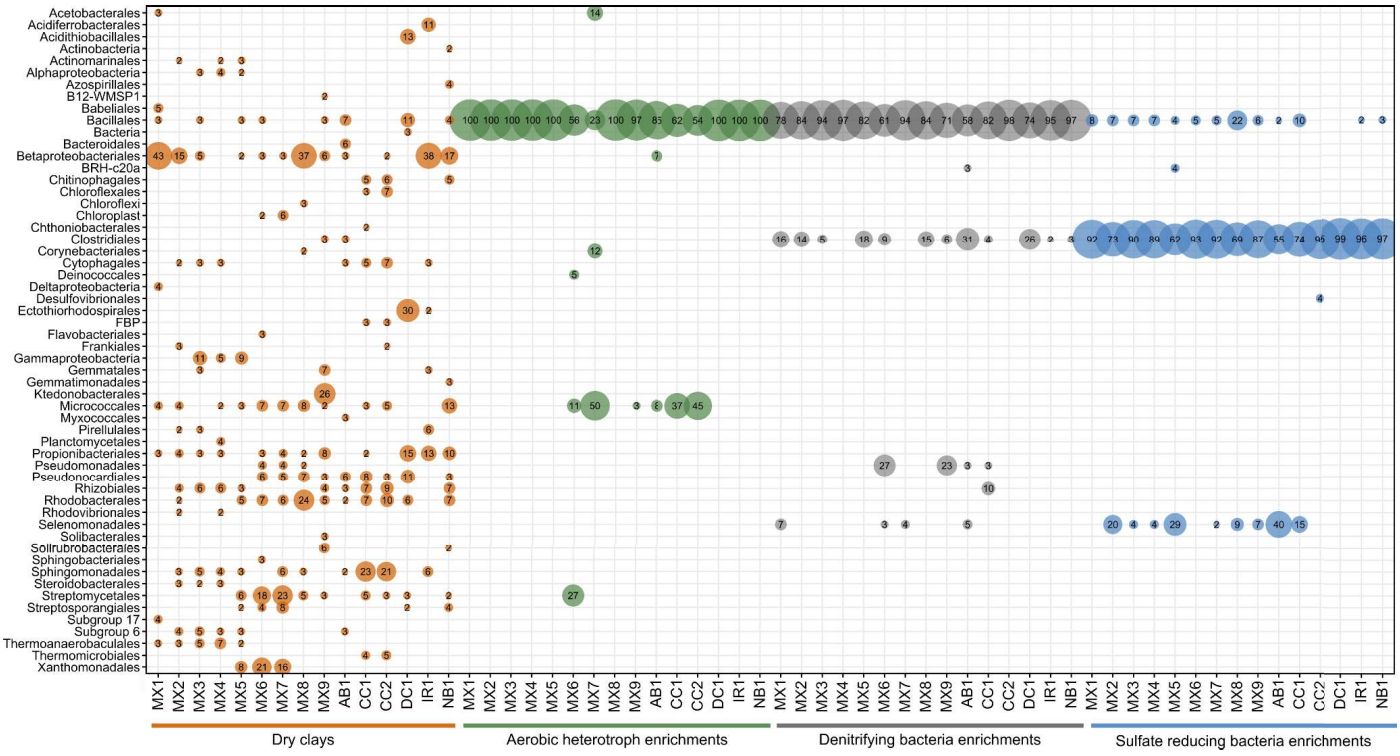

Figure 2.10 Order-level taxonomic relative abundances of enrichment culture and clay sample ASVs. All affiliated taxa displayed were ≥2% relative abundance within samples.

Different ASV profiles were detected in enrichment cultures compared to dry clays. Although ASVs associated with *Actinobacteria* and *Proteobacteria* were primarily detected in dry clays (Figure 2.8), the three enrichment culture conditions tested primarily encouraged growth, and detection of *Firmicutes* bacteria resulted (Figures 2.11, 2.12, and 2.13). Cultivation of aerobic heterotrophs from clay resulted in detection of many ASVs associated with *Bacillus* (Figure 2.11) and anoxic cultivation from clay resulted in detection ASVs associated with *Clostridium*, *Bacillus*, and *Desulfosporosinus* genera (Figures 2.12 and 2.13). *Desulfosporosinus,* a common sulfate-reducing bacterium [26], was cultivated from many bentonite samples tested (Figure 2.13) but was rarely detected at relative abundances >3% in the initial clay samples (Figure 2.8). This genus has been detected in previous studies assessing microcosms and MPN tubes of bentonite clay [21, 24]. Representatives of *Paracoccus* and *Thiobacillus*, that have previously been identified as denitrifiers and sulfur oxidizers [26, 27, 79], were detected in clays (Figure 2.8) but not in the corresponding enrichment cultures at ≥2% relative abundance (Figures 2.11, 2.12, and 2.13), although they have previously been detected in bentonite clay enrichment cultures [21, 47]. *Pseudomonas* is frequently reported from aerobic heterotroph and SRB enrichment cultures from natural clay deposits or saturated, highly compacted clays [39, 42, 47, 64, 95], and sometimes in uncompacted as-received clays [24]. Here, ASVs associated with *Pseudomonas* were also detected in enrichment cultures, but in relatively low abundances (Figure 2.12). Although enrichment culture samples contained ASVs associated with similar genera, every sample of clay enriched a different microbial set of microorganisms, resulting in unique microbial profiles (Figures 2.11, 2.12, and 2.13).

Figure 2.11 Relative abundances of ASVs affiliated with enrichment cultures for aerobic heterotrophs from clay samples. Duplicate sample community profiles represented at the ASV level with a relative abundance ≥2%. Sample replicate number is indicated at the end of each sample name.

Figure 2.12 Relative abundances of ASVs affiliated with enrichment cultures for denitrifying bacteria from clay samples. Duplicate sample community profiles represented at the ASV level with a relative abundance ≥2%. Sample replicate number is indicated at the end of each sample name.

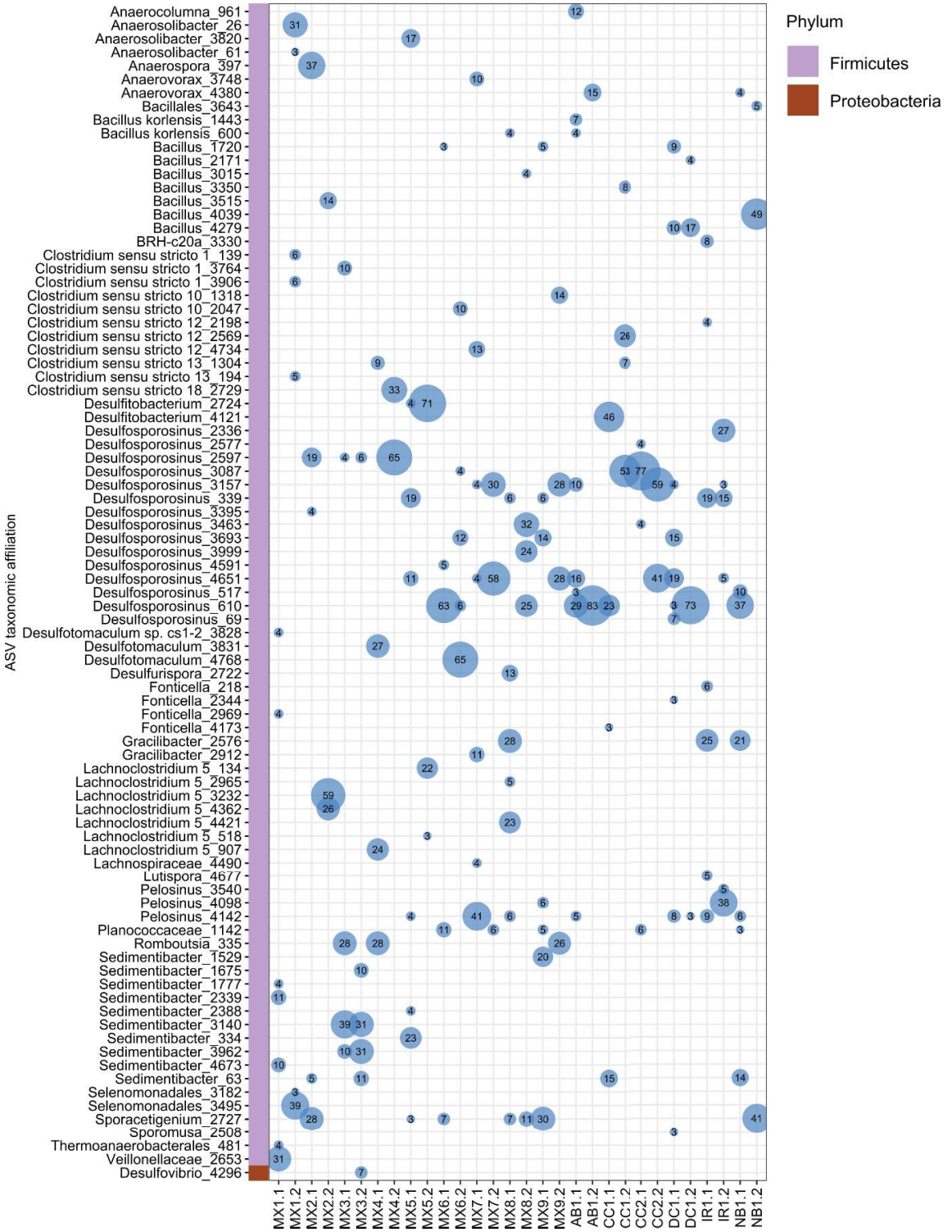

Figure 2.13 Relative abundances of ASVs affiliated with enrichment cultures for sulfate-reducing bacteria from clay samples. Duplicate sample community profiles represented at the ASV level with a relative abundance ≥3%. Sample replicate number is indicated at the end of each sample name.

2.3.3 Core microbial community

Assessments of microbial profiles from cultivation and clay DNA extracts can provide different results regarding actual abundance or viability of microorganisms in the clay. Sequencing of enrichment culture microorganisms is beneficial because it distinguishes clay microorganisms that were viable and readily cultured; however, such an approach does not measure the relative abundances of non-culturable microorganisms within clay samples. For comparison of all clay microorganisms detected in enrichment cultures and clay DNA extracts, each ASV detected was categorized as present in enrichment cultures only, clay only, or both. From these categories, 81.9% (1604 ASVs) were only detected in clays, 16.8% (330 ASVs) were only detected in enrichment cultures, and 1.3% (25 ASVs) were detected in both, highlighting that few taxa were both culturable and relatively abundant with 16S rRNA gene profiles. Through cultivation, relative abundances of microorganisms from the original clay samples changed because of conditions like temperature, available nutrients, and atmosphere that selected for specific bacteria to proliferate to higher abundances. Enrichment cultures therefore contained many ASVs with larger read count quantities from high-throughput sequencing (Figure 2.14A). Most ASVs detected in clay 16S rRNA gene profiles were not detected in high-throughput sequencing of enrichment culture 16S rRNA genes (Figure 2.14A), indicating that dominant sequences from clays correspond to taxa that resist cultivation or were not associated with viable cells. Of the 600 most abundant ASVs on the rank abundance curve for as-received clays (Figure 2.14B), 582 were not detected in enrichment cultures, again suggesting that they represent free DNA or bacteria that were not readily cultivated. If 16S rRNA gene profiles from clay represented viable cells, then bentonite clay enrichment cultures would respond similarly to samples from soils and the human gut, where low abundance microorganisms from the rare biosphere were commonly recovered with cultivation [96, 97].

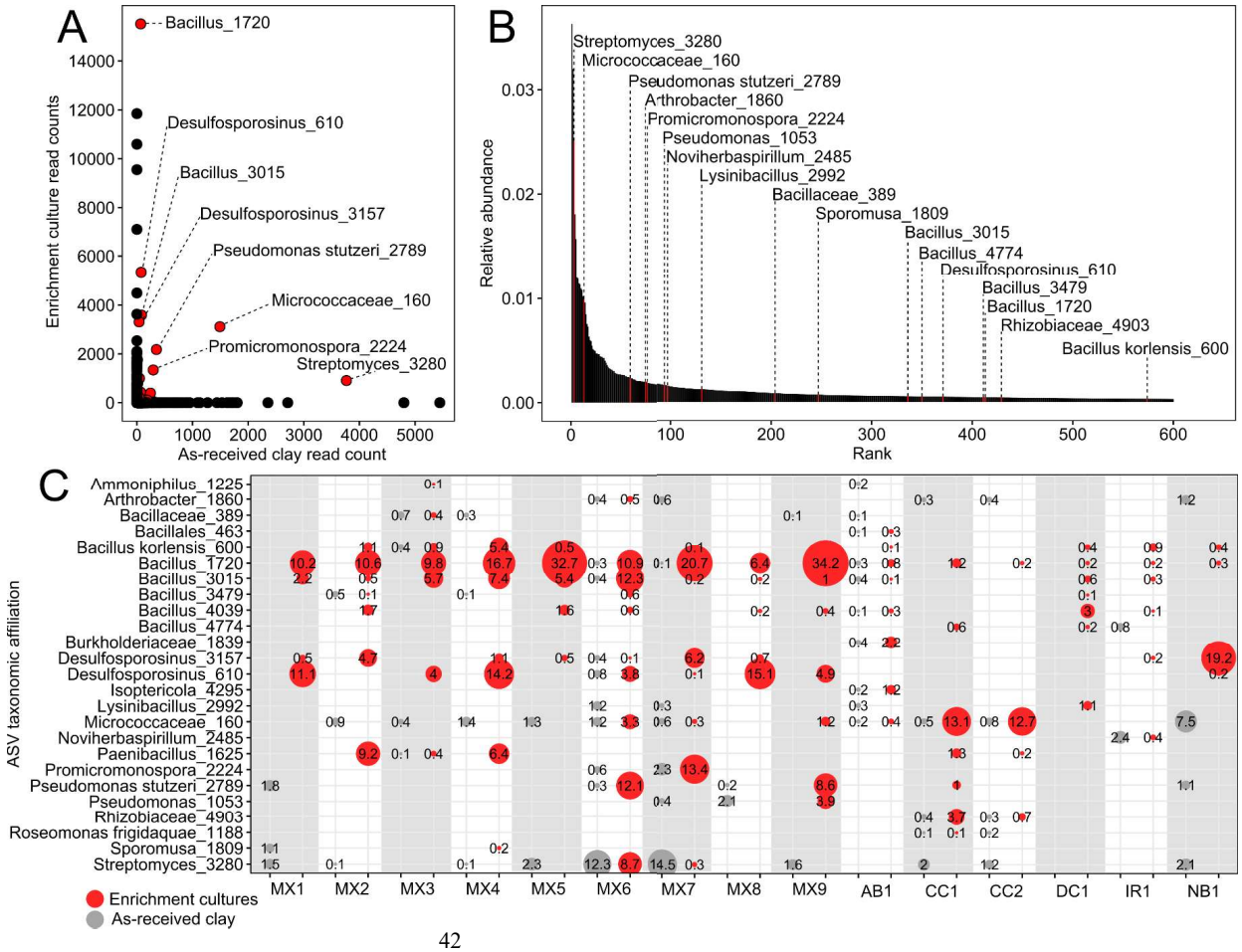
A
Bacillus_1720
Desulfosporosinus_610
Bacillus_3015
Desulfosporosinus_3157
Pseudomonas stutzeri_2789
Micrococcaceae_160
Promicromonospora_2224
Streptomyces_3280
Enrichment culture read counts
As-received clay read count
B
Streptomyces_3280
Micrococcaceae_160
Pseudomonas stutzeri_2789
Arthrobacter_1860
Promicromonospora_2224
Pseudomonas_1053
Noviherbaspirillum_2485
Lysinibacillus_2992
Bacillaceae_389
Sporomusa_1809
Bacillus_3015
Bacillus_4774
Desulfosporosinus_610
Bacillus_3479
Bacillus_1720
Rhizobiaceae_4903
Bacillus korlensis_600
Relative abundance
Rank
C
ASV taxonomic affiliation
Ammoniphilus_1225
Arthrobacter_1860
Bacillaceae_389
Bacillales_463
Bacillus korlensis_600
Bacillus_1720
Bacillus_3015
Bacillus_3479
Bacillus_4039
Bacillus_4774
Burkholderiaceae_1839
Desulfosporosinus_3157
Desulfosporosinus_610
Isoptericola_4295
Lysinibacillus_2992
Micrococcaceae_160
Noviherbaspirillum_2485
Paenibacillus_1625
Promicromonospora_2224
Pseudomonas stutzeri_2789
Pseudomonas_1053
Rhizobiaceae_4903
Roseomonas frigidaquae_1188
Sporomusa_1809
Streptomyces_3280
MX1
MX2
MX3
MX4
MX5
MX6
MX7
MX8
MX9
AB1
CC1
CC2
DC1
IR1
NB1
Enrichment cultures
As-received clay

Figure 2.14 Commonly detected members of the core microbial community in bentonite clays. (A) Scatter plot of 16S rRNA gene sequence read counts of ASVs from clays and enrichment cultures. Core ASVs, detected in both clays and enrichment cultures, are indicated with a red point (B) Rank abundance curve of ASVs detected in clays highlighting ASVs additionally from enrichment cultures in red. (C) Bubble plot of core clay ASVs present in clays and enrichment cultures. The bubbles represent ASVs present at or above 0.1% relative abundance in samples. For each sample, sequences were normalized to 10,000 reads and those obtained from all three enrichment cultures or from duplicate clay samples were merged into enrichment culture or as-received clay categories, respectively.

From the most abundant dry clay ASVs, 25 were detected in both clay and enrichment cultures, confirming the viability and relatively high abundance of the associated microorganisms. These 25 ASVs were affiliated with *Streptomyces*, *Micrococcaceae*, *Promicromonospora*, *Bacillus, Rhizobiaceae, Pseudomonas, Burkholderiaceae, Desulfosporosinus, Noviherbaspirillum,* and *Isoptericola* (Figure 2.14C). Detected in ten of the fifteen clay samples, *Streptomyces* was the most abundant ASV detected from all clay DNA extracts combined (Figure 2.14B), although, five samples (AB1, IR1, DC1, MX3, and MX8) did not contain detectable *Streptomyces* with relative abundances greater than 0.1% (Figure 2.14C). Members of *Streptomyces* were also detected through enrichment cultivation of two samples (MX6 and MX7; Figure 2.14C) confirming the viability of these taxa in clay. Previous studies of microorganisms within compacted bentonite corroborate the presence [64, 95] and viability [95] of *Streptomyces* members. Representatives of *Micrococcaceae* and *Bacillus* that have previously been detected in compacted bentonite [64], natural bentonite formations [63], and clay enrichment cultivations [24], were also detected in the bentonite clays and enrichment cultures used in this study (Figure 2.14C). This suggests that *Micrococcaceae* and *Bacillus* were also viable components in the core microbial community of bentonite clay. Furthermore, considering all samples together, the endospore-forming genus *Bacillus* was the most abundantly detected genus of bacteria from enrichment cultivations (Figure 2.14A), was also among those with the highest relative abundance in corresponding clay DNA extracts (Figure 2.14C). Even though each clay sample contained unique microbial profiles, *Bacillus* ASVs were detected in every sample either through enrichment cultivation, clay extracts, or both (Figure 2.14C). Overall, detection of ASVs in both enrichment culture and clay implies that the associated bacteria were relatively abundant and viable in clay, perhaps as spores, and therefore are core microbial taxa that can be cultivated readily. Furthermore, members of the core microbial community may potentially serve as targets for

monitoring microorganisms in pressure vessel experiments that simulate saturated DGR barrier conditions (Chapter 3).

Chapter 3
Microbiology of compacted bentonite clay

3.1 Introduction

Highly compacted bentonite clay represents an essential barrier in the underground repository that will contain Canada's used nuclear fuel. The proposed deep geological repository (DGR) will consist of an engineered barrier system that is designed to withstand relatively high temperatures and radiation from used nuclear fuel as it decays for the duration of one million years. Current plans include encasing used fuel bundles in copper-coated used fuel containers surrounded by buffer boxes of highly compacted Wyoming MX-80 bentonite clay. Bentonite clay has been chosen as the barrier between the used fuel container and host rock because of its ability to swell when saturated, preventing transport of water, microorganisms, and radionuclides throughout the storage system [33].

An understanding of microbial communities within bentonite clay is important for predicting whether they will contribute to the corrosion of barrier components. One of the main considerations regarding clay microorganisms is microbiologically influenced corrosion (MIC). Corrosion due to microorganisms occurs as a result of corrosive by-products generated by anaerobic microorganisms, such as sulfate-reducing bacteria (SRB). Other reasons for the investigation of clay microorganisms under DGR-relevant conditions include determination of whether microbial activity can result in transformation of clay minerals, gas production, and formation of biofilms [22, 28, 81].

The activity of microorganisms within saturated bentonite clays can be controlled through alteration of physical characteristics of clay such as pore size, water activity, and swelling pressure. To physically restrict microorganisms, bentonite must be compacted to a dry density ≥ 1.6 g/cm^3 [10]. This compaction, along with swelling pressure exceeding 2 MPa, achieves pore sizes within clay that are smaller than the average size of a microbial cell [10]. Additional measures to restrict microbial activity include relatively low water activity. For this purpose, the clay barrier must maintain water activity below 0.96 [10]. Research has demonstrated that when physical restrictions are applied, culturable microorganisms in clay remain at, or below, the original clay abundances [10, 42, 56].

Previous research has established profiles of culturable microorganisms that persist in bentonite clay after incubation under laboratory conditions or in natural bentonite clay formations. Methods used to cultivate clay-associated microorganisms commonly involve R2A agar [46] for the enumeration of heterotrophs and sulfate-containing medium within most probable number (MPN)

tubes for the enumeration of SRB [10, 21, 41, 42, 56, 82]. Members of *Desulfosporosinus, Pseudomonas,* and *Streptomyces* genera have been detected previously in enrichments of SRB and heterotrophic bacteria from pressure-treated bentonite clay [21, 95]. After incubation with a dry density of 1.6 g/cm^3 for ~8 years, saturated bentonite samples plated on R2A agar primarily *Bacillus* and *Paenibacillus* spp. [42]. Natural bentonite clay formations harbour culturable members of phyla such as *Proteobacteria* (including *Pantoea*, *Sphingomonas*, and *Paracoccus*) and *Actinobacteria* (including *Arthrobacter*, *Micrococcus*, *Kocuria*, *Dermacoccus*, *Janibater*, and *Modestobacter*) [84]. The same cultivation methods are often employed to pre-enrich clay microorganisms prior to genomic DNA extraction and sequencing of 16S rRNA genes [21, 24, 84, 95]. A strategy of pre-enrichment is used because extraction and detection of nucleic acids directly from clay is often unsuccessful, primarily because of low biomass and the adsorption of nucleic acids onto the clay matrix [44, 65]. Sequencing of DNA from cultured clay microorganisms comes with limitations. For example, non-culturable or slow-growing microorganisms typically remain undetected. However, a recent validation of a DNA extraction protocol for bentonite clays allows for future studies of microbial community profiles of bentonite clays [65].

Combining cultivation and molecular (e.g., DNA/PLFA) approaches for bentonite clay has demonstrated repeatable results [10, 56], serving as validation for future experiments involving approaches that will further identify whether increases in culturable microorganisms are because of growth or possibly a return from viable but non-culturable (VBNC) states. When saturated and compacted clay was incubated under pressure, clay with lower dry densities exhibited an increase in the number of culturable microorganisms even though post-incubation PLFA-based abundances were similar [10]. Such increases in culturable microorganisms resulting from low dry density and high water activity were attributed to activation from a VBNC state.

This study examined the microbiology of saturated pressure vessels containing compacted clay with different dry densities that bracket the DGR-relevant dry density of 1.6 g/cm^3. Powdered and gapfill forms of bentonite clay were used to fill pressure vessels. Gapfill clay is different from powdered because it has been processed by physical compaction and subsequent redistribution into fine particle sizes. This study combined cultivation, PLFA, and 16S rRNA gene analyses to characterize changes in microbial community profiles associated with saturated bentonite clay under DGR-relevant conditions. In addition, temporal changes were assessed by analyzing multiple pressure vessel time points.

3.2 Materials and Methods

3.2.1 Pressure vessel assembly and disassembly

Pressure vessels composed of steel and a Teflon lining, were used for incubation of saturated bentonite clay under high pressure. Given the volume of the pressure vessels and the targeted dry density, calculated quantities of powdered or gapfill clay were collected and compressed into pressure vessels. Target dry density was achieved using a mechanical press to compact the clay into the pressure vessels. Gapfill bentonite clay was compacted into pressure vessel experiments when sufficient quantities of powdered bentonite material were not available. Throughout saturation, swelling pressure was monitored with pressure gauges secured to the top of the pressure vessel. Initiating at the start of saturation, pressure vessels at dry densities of 1.1, 1.4, or 1.6 g/cm^3 were incubated for 1, 3, or 6 months, with deviations of up to 14 days (Table 3.1).

After removal from the Teflon lining of the pressure vessels, compacted clay was divided into six different samples, consisting of top, middle, and bottom for each of the inner and outer regions of clay. Aerobic heterotrophs, anaerobic heterotrophs, and SRB were enumerated from each sample of clay. New scalpel blades, sterilized tweezers, and clean aluminum foil were used to slice each sample. For the "outer" region of the pressure vessels, approximately 5 mm of clay from the outer edge of the clay was removed with a scalpel and weighed. Samples from the innermost component of the pressure vessel clay were used for analysis of "inner" regions of the pressure vessel. Weighed sample aliquots for culturing were stored at 4°C for up to seven days and samples of clay before DNA extraction and the subsequent DNA extracts were all stored at -20°C.

Table 3.1 Pressure vessel samples of bentonite clay. Variation in treatments was achieved through three different targeted dry densities and three lengths of incubation. Because unforeseen lab access limitations prevented analysis with all methods for all pressure vessels, X designations indicate analyses that were performed for corresponding pressure vessel clay samples.

Pressure vessel	Dry density (g/cm^3)	Incubation (months)	Rep	Type of clay[a]	ID[b]	Cultivation	Sequencing	qPCR	PLFA
1.1_1moA	1.1	1	A	powdered	15	X			
1.1_1moB	1.1	1	B	powdered	16	X			
1.1_1moA	1.1	1	A	gapfill	9		X		
1.1_1moB	1.1	1	B	gapfill	6		X		
1.1_3moA	1.1	3	A	powdered	1	X	X		
1.1_3moB	1.1	3	B	powdered	2	X	X	X	X
1.1_6moA	1.1	6	A	powdered	5	X	X		
1.1_6moB	1.1	6	B	powdered	10	X	X		
1.4_1moA	1.4	1	A	powdered	13	X			
1.4_1moB	1.4	1	B	powdered	17	X			
1.4_3moA	1.4	3	A	powdered	4	X	X	X	X
1.4_3moB	1.4	3	B	powdered	14	X			
1.4_3moA	1.4	3	A	gapfill	1.6		X		
1.4_6moA	1.4	6	A	gapfill	7	X			
1.4_6moA	1.4	6	A	powdered	8	X			
1.6_3moA	1.6	3	A	gapfill	12	X	X	X	X

[a]Powdered clay was lot number 116315324, gapfill clay lot number was not available.

[b]ID, experiment identification assigned by collaborators.

3.2.2 Moisture content and water activity

Measurement of moisture content and water activity were performed as described in Section 2.2.2.

3.2.3 Cultivation of bentonite clay bacterial communities

Dilution series were prepared using the methods described in Section 2.2.3. For enrichment and enumeration of SRB, MPN test tubes consisted of 9 mL of sterile sulfate-reducing medium (HiMedia Laboratories, M803). Each sample was analyzed using a five-tube MPN method. Inoculated test tubes were placed into a stainless-steel anaerobic culture chamber (Best Value Vacs), containing a GasPak EZ Anaerobe Container System Sachet (BD) and an anoxic indicator strip (BD). Culture chambers were evacuated and flushed with N_2 gas up to five times before incubation for 28 days at 30°C. After incubation, positive MPN tubes were identified by a black precipitate in the tube. The MPN per gram

dry weight (gdw) was calculated by accounting for moisture content for each sample. Mean MPN/gdw and standard deviation was calculated from the MPN/gdw quantified for the replicate top, middle, and bottom samples. Aerobic and anaerobic heterotrophs were cultivated using the same methods described in Section 2.2.3. Triplicate clay samples were collected from within the top, middle, and bottom of each pressure vessel.

3.2.4 Extraction of genomic DNA

Genomic DNA was extracted from 2 g clay powder samples using the PowerMax Soil DNA Isolation kit (Qiagen) following manufacturer instructions, with several modifications as described previously [65]. Furthermore, clay in bead beating tubes was gently vortexed for 20 minutes to allow swelling and dispersion prior to lysis buffer addition. Kit controls were included for each batch of DNA extractions. Quantification of DNA was performed using qPCR because all extracts were below the detection limit of the Qubit fluorometer.

3.2.5 Quantitative PCR

Quantification of 16S rRNA genes through qPCR was performed as described in Section 2.2.5. Averages were determined for inner and outer samples by combining values from top, middle, and bottom samples. Time constraints prevented performance of replicate qPCR on pressure vessel initial clay (Powdered Wyoming MX-80 clay), as an alternative, the dry clay 16S rRNA gene copies/gdw were estimated based on averaged 16S rRNA gene copies/gdw from three samples of Wyoming MX-80 clay (MX1, MX2, and MX3) from Chapter 2.

3.2.6 Statistical analysis

Statistical significance of changes across triplicate values from 16S rRNA gene copies/gdw, aerobic heterotroph CFU/gdw, and SRB MPN/gdw were assessed through calculation of p values using paired t-tests with 95% confidence intervals in RStudio (version 4.0.2). The p values <0.05 were considered to represent statistically significant changes in abundance.

3.2.7 Amplification of 16S rRNA genes and high-throughput sequencing

Section 2.2.6 outlines the method used for amplification and high-throughput sequencing of 16S rRNA genes from clay samples. All sequences were deposited into European Nucleotide Archive (https://www.ebi.ac.uk/ena) with study accession number PRJEB39478.

3.2.8 Post sequencing analysis

Samples were analyzed using Quantitative Insights Into Microbial Ecology 2 (QIIME2, release 2019.10, [90]) managed by automated exploration of microbial diversity version 3 (AXIOME3; [91]; github.com/neufeld/AXIOME3). DADA2 (version 2019.10; [92]) was used to remove primer sequences and trim low-quality sequences after 250 bases for forward and reverse reads. Taxonomy was assigned to ASVs using a naive Bayes classifier (feature-classifier classify-sklearn version 2019.10) pre-trained with SILVA database release 138 [93]. Controls assessed with Decontam [66] included kit negative controls for DNA extraction batches, reagent negative controls for sequencing, and positive controls for sequencing. The ASVs that Decontam identified as contaminants were removed from the sample ASV table.

3.2.9 Phospholipid fatty acid analysis

Phospholipid fatty acid analysis was performed as described in Section 2.2.8.

3.3 Results

3.3.1 Variation in compacted bentonite clay

Pressure vessels containing compacted Wyoming MX-80 bentonite clay at dry densities of 1.1, 1.4, and 1.6 g/cm^3 were each incubated for durations of either 1, 3, or 6 months (Table 3.1). The pressure vessels were filled with powdered or gapfill bentonite clay. Saturation of compacted clay within pressure vessels involved addition of pressurized water through the tops of pressure vessels. After incubation, the outer (interface) and inner layers of clay from each pressure vessel were separated into sections from the top, middle, and bottom of the pressure vessel. As well, each pressure vessel condition was tested in duplicate, except for the clay with 1.6 g/cm^3 dry density, that was tested only once due to COVID-19 related lab-access limitations.

In the initial bentonite clay used to prepare pressure vessels, the moisture content was 11.5 ± 0.9%, and water activity was 0.4 ± 0.05. After incubation of compacted bentonite in pressure vessels, higher dry density was associated with lower moisture content and water activity (Figure 3.1). In this way, moisture content and water activity levels corresponded to those reported in other research on compacted bentonite clay [10]. As expected, clay samples from pressure vessels with the lowest dry densities (1.1 g/cm^3) contained the highest moisture contents and water activities (Figure 3.1). Clay samples containing over 35% moisture content were considered to have been 100% saturated [13] and exhibited water activities of 0.99 or higher. Inconsistent moisture content and water activity values were apparent for clay in pressure vessels compacted to higher dry densities (1.4 and 1.6 g/cm^3; Figure 3.1). Uneven saturation of clay was likely due to the addition of water and swelling of clay at the top of the pressure vessel, thus slowing the passage of the subsequent water to the rest of the pressure vessel. Alternatively, some pressure vessels may have experienced pooling of the saturating water at the bottom of the pressure vessel. In such a case, the low compaction of clay at the outer interface of the pressure vessel likely permitted passage through to the bottom.

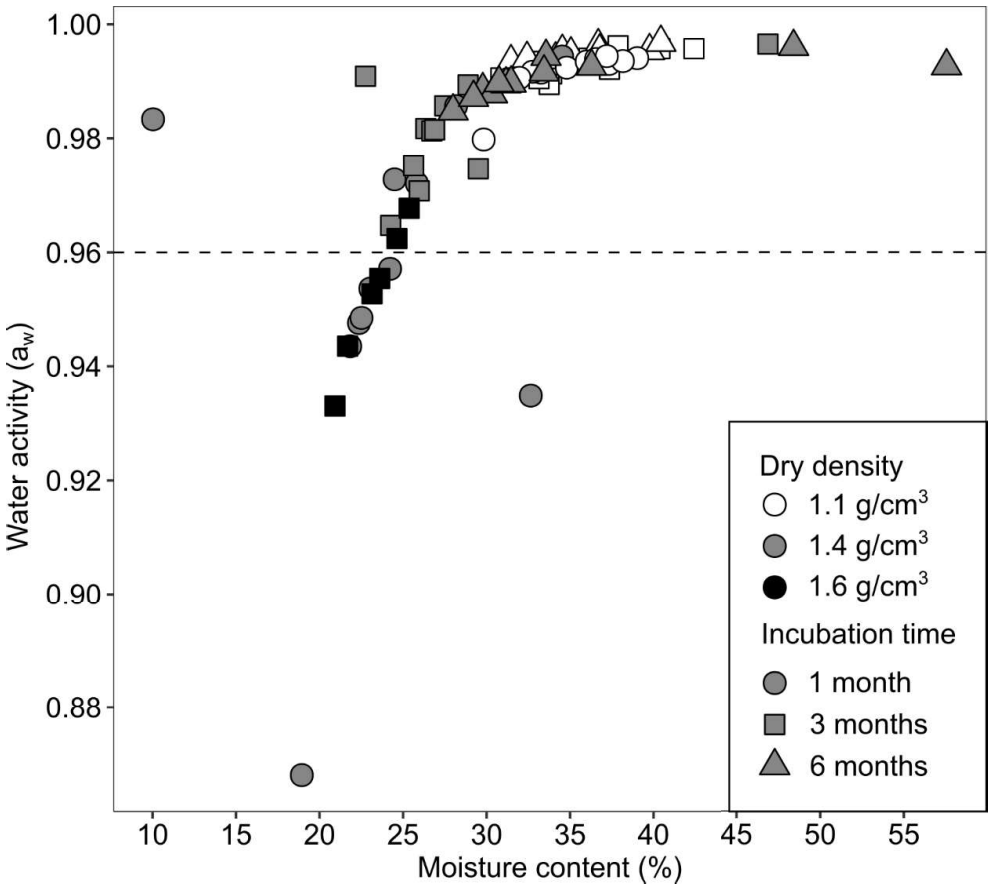

Figure 3.1 Moisture contents and water activities in compacted bentonite samples. Data points from each dry density represent single clay samples. The dashed line indicates water activity of 0.96, below which, is inhibitory to growth of culturable microorganisms [10]. Water activity was calculated using a water potentiometer and moisture content values were calculated after evaporation of water in a drying oven.

Before incubation, as-received pressure vessel clay was tested to determine the original abundances of culturable microorganisms. Detectable abundances of culturable bacteria from the initial clay samples were $3.0 \pm 1.4 \times 10^0$ CFU/gdw of aerobic heterotrophs (Figure 3.2), and anaerobic heterotrophs were below the detection limit of 100 CFU/g. Cultivation-based estimates of SRB abundances were $9.8 \pm 6.6 \times 10^0$ MPN/gdw (Figure 3.2). After incubation in pressure vessels, detectable aerobic heterotrophs from the inner and outer layers of clay in each pressure vessel were enumerated using the same plate-count and MPN methods. As a result, average detectable aerobic heterotroph and SRB abundances from inner layers were lower than the corresponding outer layers for several samples (Figure 3.2). Large differences between replicates resulted in few statistically significant differences between the outer and inner layers compared to initial clay or to each other (Figure 3.2). Only the outer layer from pressure vessel 1.1_1moB contained significantly more aerobic heterotrophs compared to initial clay (p value = 0.02) or inner layers (p value = 0.02). Despite the large variation in abundance between pressure vessel layers, increases in the average detectable abundances from the initial clay samples were observed (Figure 3.2). Anaerobic heterotrophs were additionally cultivated from all clay samples, but abundances were frequently below the detection limit of 100 CFU/g. Abundances of anaerobic heterotrophs were only above detection limit for the outer layer replicates for three pressure vessels (1.4_1moA, 1.4_1moB, and 1.1_1moB).

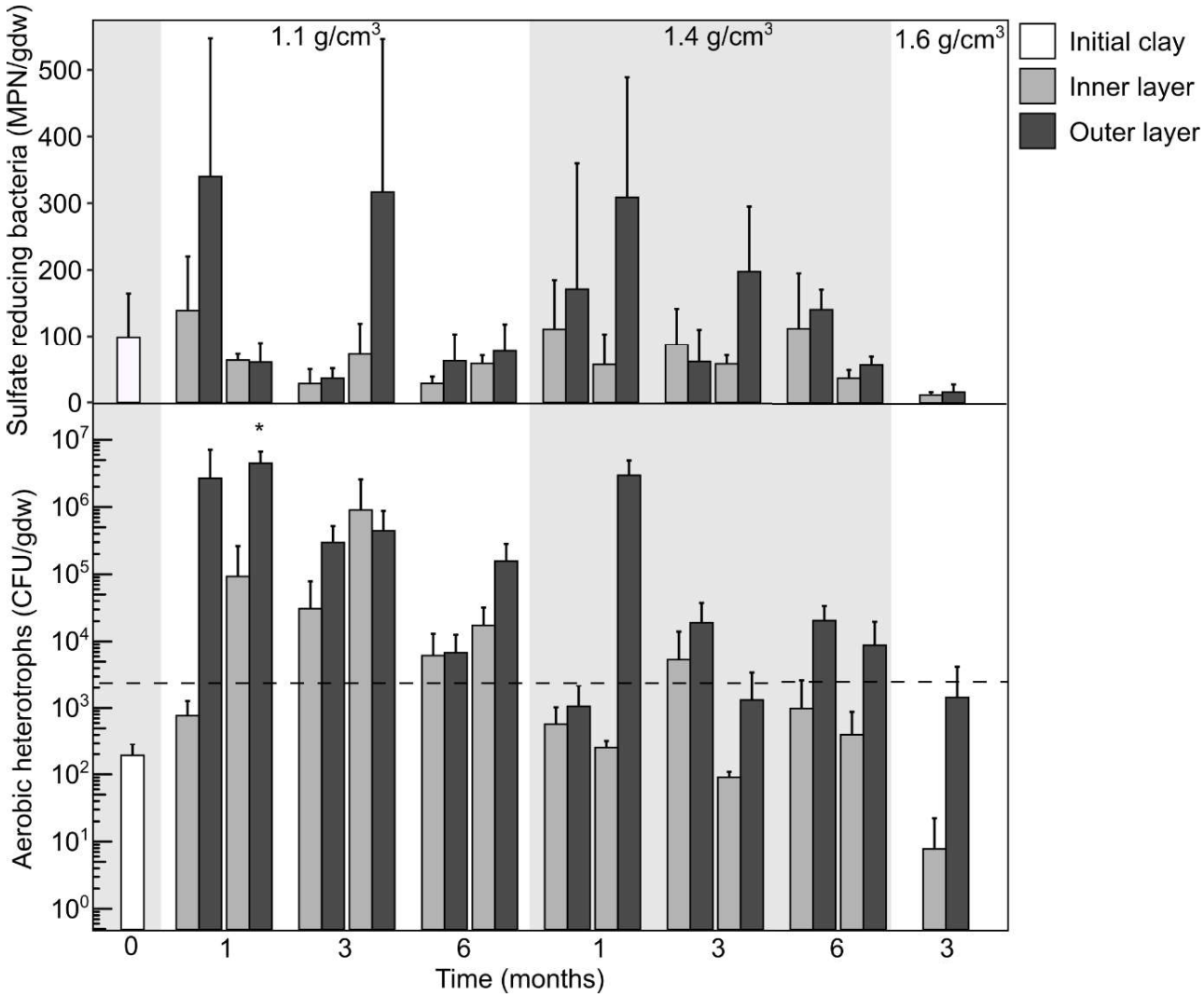

Figure 3.2 Abundances of cultivated microorganisms in compacted bentonite clay. Estimates of aerobic heterotrophs and sulfate-reducing bacteria are based on averaged abundances from triplicate samples of clay. Initial clay sample quantification was only performed on powdered initial clay. The asterisk (*) represents the pressure vessel with clay that contained significant differences (p value <0.05) compared to the corresponding inner layer and to the initial clay. The horizontal dashed line represents the lower limit of plate count quantification (2,500 CFU/g) and the lower limit of detection is 100 CFU/g.

3.3.2 Comparison of approaches

In addition to cultivation-based approaches, qPCR and PLFA analyses were used to further compare microbial profiles from three selected pressure vessels with 3-month incubations of clay compacted to 1.1, 1.4, and 1.6 g/cm^3 (Table 3.1). From these vessels, inner layer clay samples collected from the top, middle, and bottom were assessed as biological replicates. Although cultivation of initial clay was performed in triplicate, COVID-19 related time constraints prevented triplicate qPCR analysis, so for this comparison cultivation- and qPCR-based microbial abundances were estimated using the data collected from three production lots of Wyoming MX-80 clay (Chapter 2). Specifically, the lot numbers of clay used as the "mock" initial clay sample, were MX1, MX2, and MX3 (Table 2.1). These three samples were chosen because each had a different lot number and production date and are powdered Wyoming MX-80 bentonite, similar to the type of clay that was compressed into some pressure vessels. For PLFA analysis of the "true" initial powdered clay, the clay sample used in the pressure vessels was assessed.

Microorganisms from compacted and initial clay samples were quantified and compared. Among initial clay and pressure vessel samples, the PLFA detected increased from 21 pmols/g to up to 92 pmols/g. The clay with highest compaction (1.6 g/cm^3) experienced the largest increase in detectable PLFA (Figure 3.3A). Enumeration of microorganisms through quantification of the 16S rRNA genes resulted in copies ranging from 10^3 - 10^6 copies/gdw (Figure 3.3B). Compared to detected 16S rRNA gene abundances in the initial clay sample, clay from the pressure vessel with the lowest dry density (1.1 g/cm^3) experienced a 1,000-fold increase in 16S rRNA gene abundance. However, there was large variation from the top to the bottom of these pressure vessels. Within-vessel 16S rRNA gene abundances ranged from ~10^6 copies/gdw at the top, ~10^4 copies/gdw in the middle, and ~10^5 copies/gdw at the bottom of the pressure vessels. Although the average 16S rRNA gene quantities increased up to 1,000-fold from the 10^3 copies/gdw associated with the "mock" initial clay sample, the change was not significant (p value >0.05) because of the within-vessel differences. In comparison, at the highest tested dry density of 1.6 g/cm^3, there was a significant decrease (p value = 0.03) in the 16S rRNA gene copy abundance compared those from the initial powdered clay estimate (Figure 3.3B).

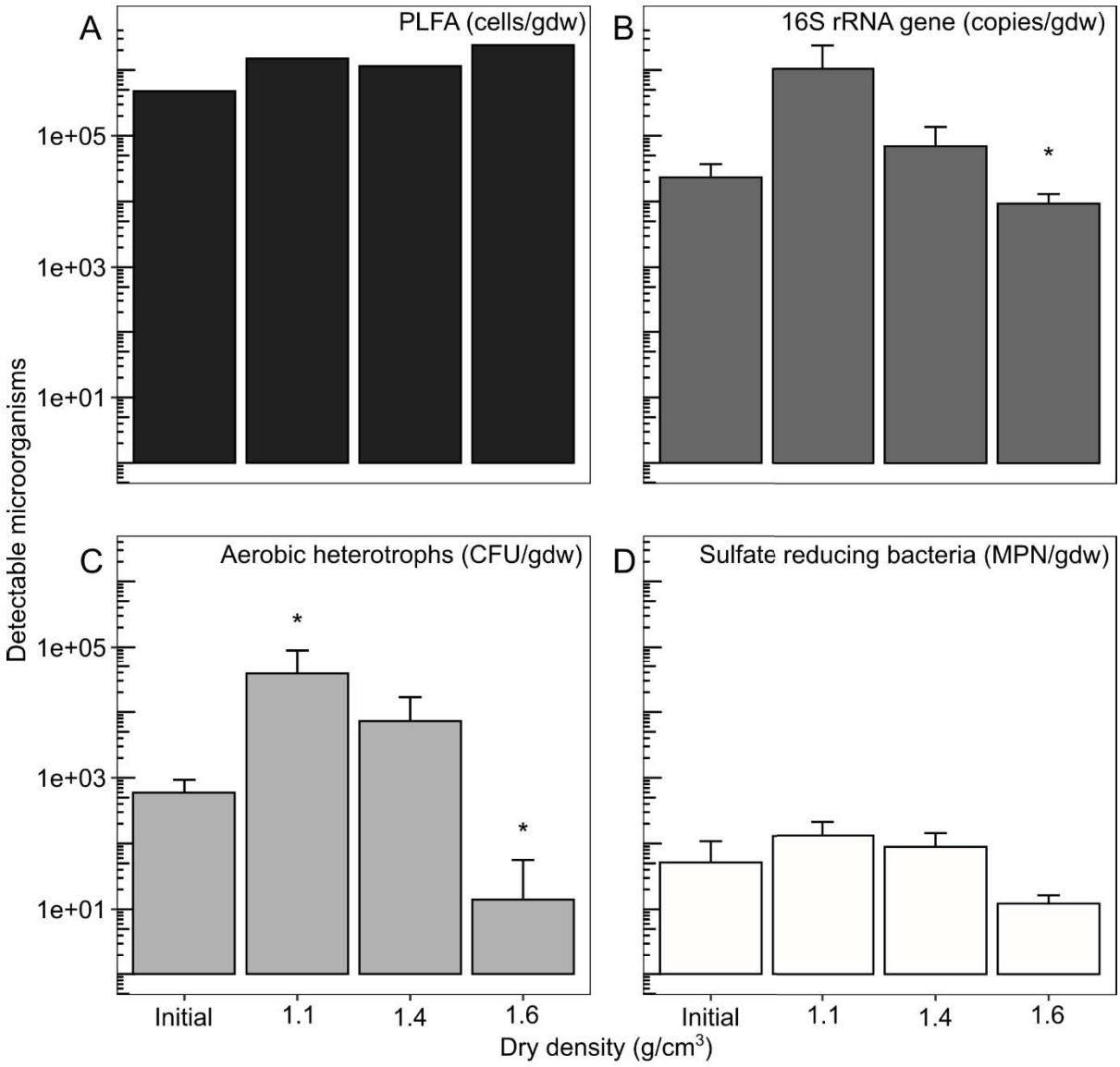

Figure 3.3 Quantified clay microorganisms based on cultivation, 16S rRNA genes, and PLFA abundances. All abundance estimates were based on values obtained from the inner layer clay of pressure vessels 1.1_3moB, 1.4_3moA, and 1.6_3moA. Standard deviation was calculated from triplicate pressure vessel clay samples. The 16S rRNA gene- and cultivation-based initial clay abundances are estimated based on averaged quantities from three bentonite clay samples with different lot numbers. These three initial clay samples were Wyoming MX-80 clay samples MX1, MX2, and MX3 from Chapter 2. Asterisks (*) indicate p values <0.05 compared to initial clay abundances.

In general, the 16S rRNA gene copy abundance estimates were similar to those observed through enumeration of culturable aerobes on R2A plates (Figure 3.3B and C). Compared to the initial clay sample, culturable aerobic heterotrophs increased significantly (p value = 0.04) after compaction at 1.1 g/cm^3 for 3 months and at the highest dry density the culturable aerobic heterotrophs decreased significantly (p value = 0.0001; Figure 3.3C). Changes in microbial abundances observed with PLFA-based abundance estimates in pressure vessel clay samples at 1.6 g/cm^3 were not corroborated by cultivated aerobic heterotroph abundances and 16S rRNA gene quantifications (Figure 3.3). Alternatively, the SRB abundance estimates determined from MPN tube enumerations did not change significantly compared to the initial clay abundances (p values >0.05; Figure 3.3D).

3.3.3 Microbial community profiles

High-throughput sequencing of 16S rRNA genes was used to generate microbial profiles for clay samples from the inner and outer layers of nine pressure vessels. Lab access restrictions (i.e., COVID19-related) prevented analysis of some samples (Table 3.1). The genomic DNA extracted from the powdered clay used to fill most pressure vessels was the source of the initial clay samples for this assessment. After post-sequencing processing of data, 30 contaminant ASVs associated with the 30 controls were removed (Figure 3.4). All contaminants ASVs except for Bacillus_29 were present in samples at relative abundances <3% (Figure 3.4). The average read counts generated from high-throughput sequencing of 16S rRNA genes were higher in clay samples than in controls (Figure 3.5).

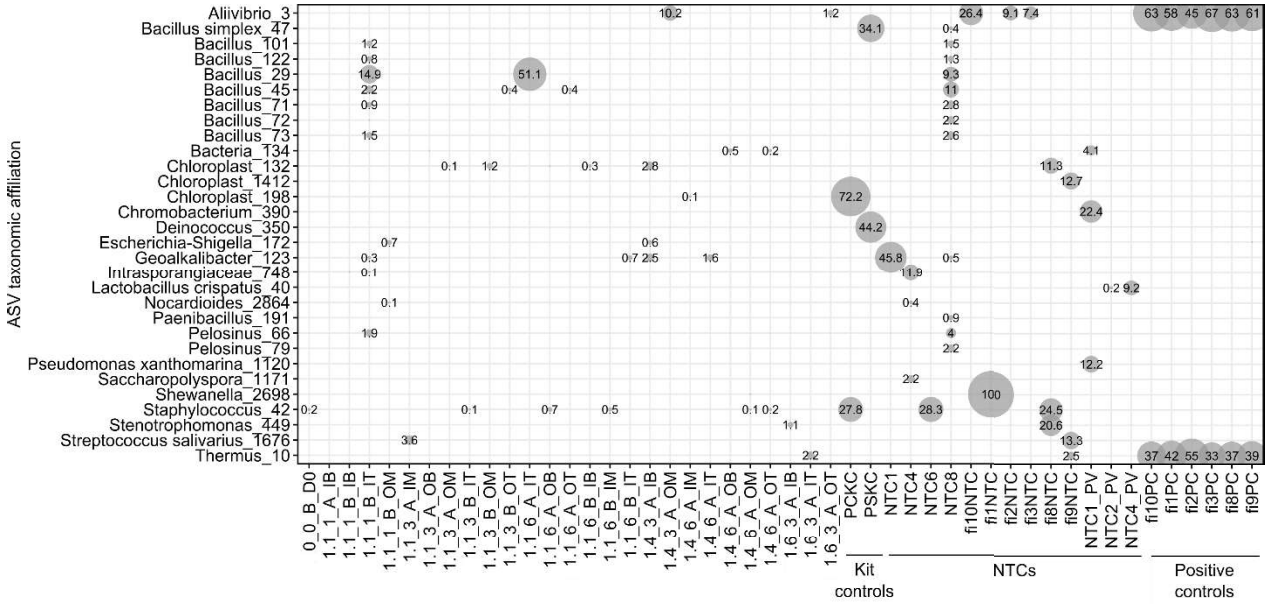

Figure 3.4 Contaminant ASVs from the compacted bentonite that were identified by Decontam. Only samples and controls containing contaminants with ≥0.1% relative abundance are pictured. Negative controls include kit controls used for each batch of genomic DNA extractions and no-template controls (NTCs) were used to assess reagent contamination. The presented ASVs were removed from the ASV table before further analysis was performed.

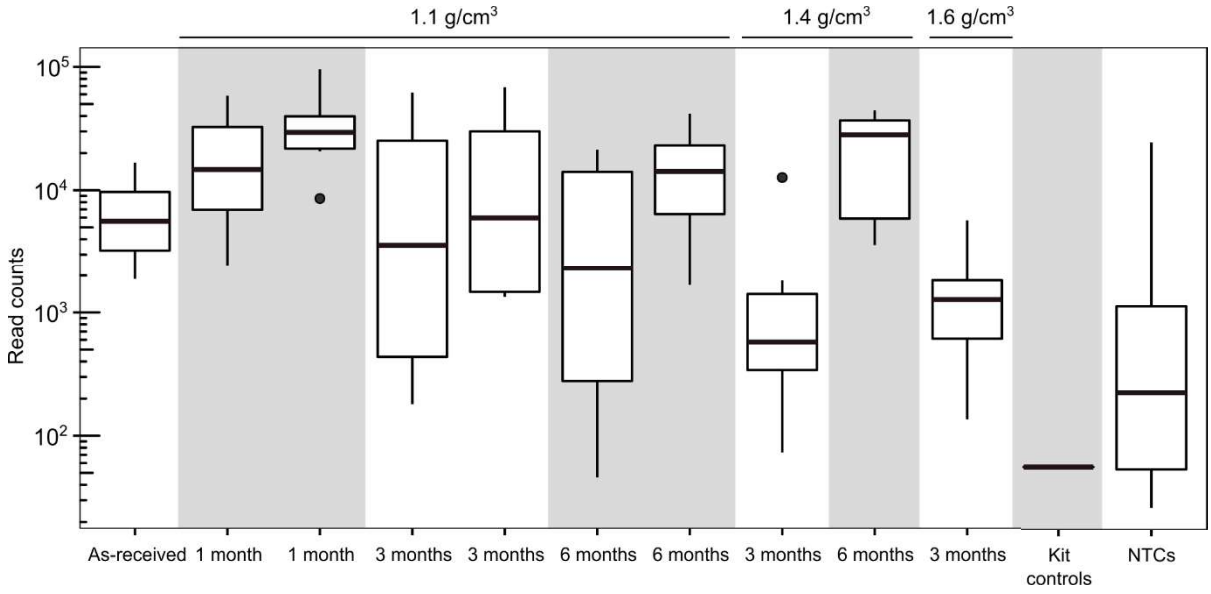

Figure 3.5 The 16S rRNA gene read counts from initial clay, compacted clay, and controls. Read counts are based on the quantity of high-throughput sequencing reads assigned to ASVs in the ASV table after removal of contaminant sequences. The kit controls and no-template controls (NTCs) were used to assess reagent contamination from the laboratory. Replicate pressure vessels A and B are plotted beside each other.

Of the sequences assessed from inner and outer layers of pressure vessel clays, ASVs affiliated with *Bacillus* and *Pseudomonas* genera dominated many samples (Figure 3.6). High relative abundances of *Pseudomonas* were only detected in microbial community profiles from pressure vessels with lowest tested dry density and correspondingly the highest measured water activities (Figure 3.6). The *Bacillus* genus was a dominant member of the microbial community profiles for clay after compaction to 1.1 and 1.4 g/cm^3 (Figure 3.6). Other genera that are members of *Firmicutes* and *Proteobacteria* phyla were also detected in several clay samples after incubation in pressure vessels (Figure 3.6). Notably, despite the heterogeneity among 16S rRNA gene profiles obtained from the initial clay samples, microbial profiles detected in most clay samples after incubation contained similar dominant genera (Figure 3.6). Compared to pressure vessel clay with low compaction, assessment of samples from highly compacted clay (1.6 g/cm^3) indicated a different change to the microbial community profile. Microbial community profiles from 1.6 g/cm^3 dry density and initial clays were similar because they both contained low relative abundances of ASVs affiliated with *Bacillus* and *Pseudomonas* (Figure 3.6). *Bacillus* and *Pseudomonas* were not detected in 16S rRNA gene profiles from initial clay samples above 4% relative abundance and following incubation, the microbial profiles generated from the inner layer of clay compacted at 1.6 g/cm^3 also lacked detection of ASVs affiliated with *Bacillus* and *Pseudomonas* at relative abundances >4% (Figure 3.6).

Generally similar microbial community profiles were generated by 16S rRNA genes and PLFA biomarker analyses. Clay compacted to lower dry densities (1.1 g/cm^3) exhibited increases in 16S rRNA gene sequences affiliated with orders of *Bacillus* and *Pseudomonas* after short-term incubations (Figure 3.6). The corresponding PLFA profiles showed increases in proportional abundances of *Firmicute*s and *Proteobacteria* (Figure 3.7). Compared to time-zero clay, each PLFA community structure observed in the 1.6 g/cm^3 pressure vessel clay underwent at least 3-fold increase in abundance (Figure 3.7). The largest change was PLFA associated with *Proteobacteria*, with a 6-fold increase after incubation at 1.6 g/cm^3.

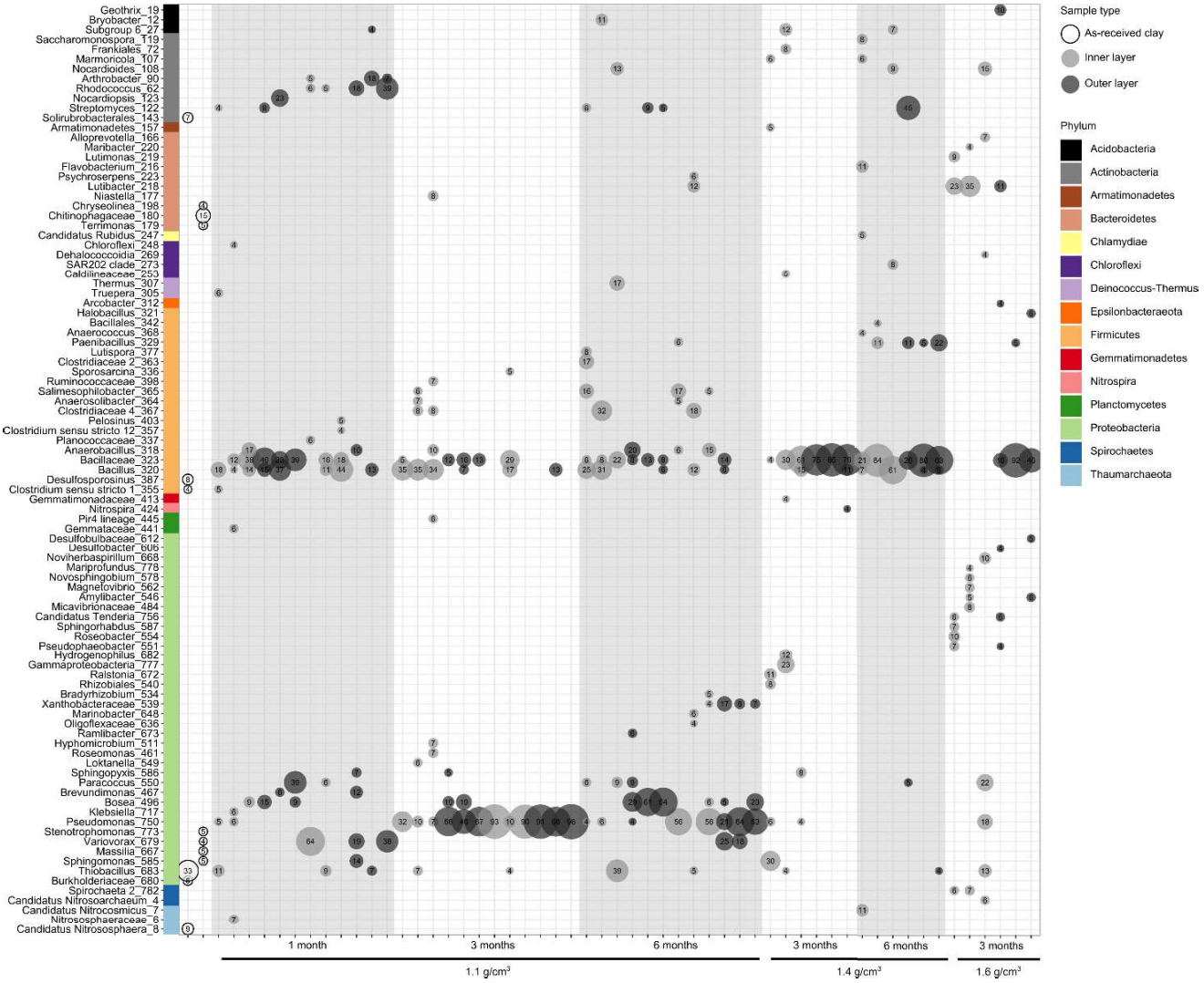

Figure 3.6 Relative abundances of taxa affiliated with 16S rRNA genes from compacted bentonite clays. Assigned phyla are collapsed to genus and make up ≥4% relative abundance of the detected microbial community profile. Replicate pressure vessels were only assessed at a target dry density of 1.1 g/cm^3. Sequencing was not performed on clay from some pressure vessels because of time constraints.

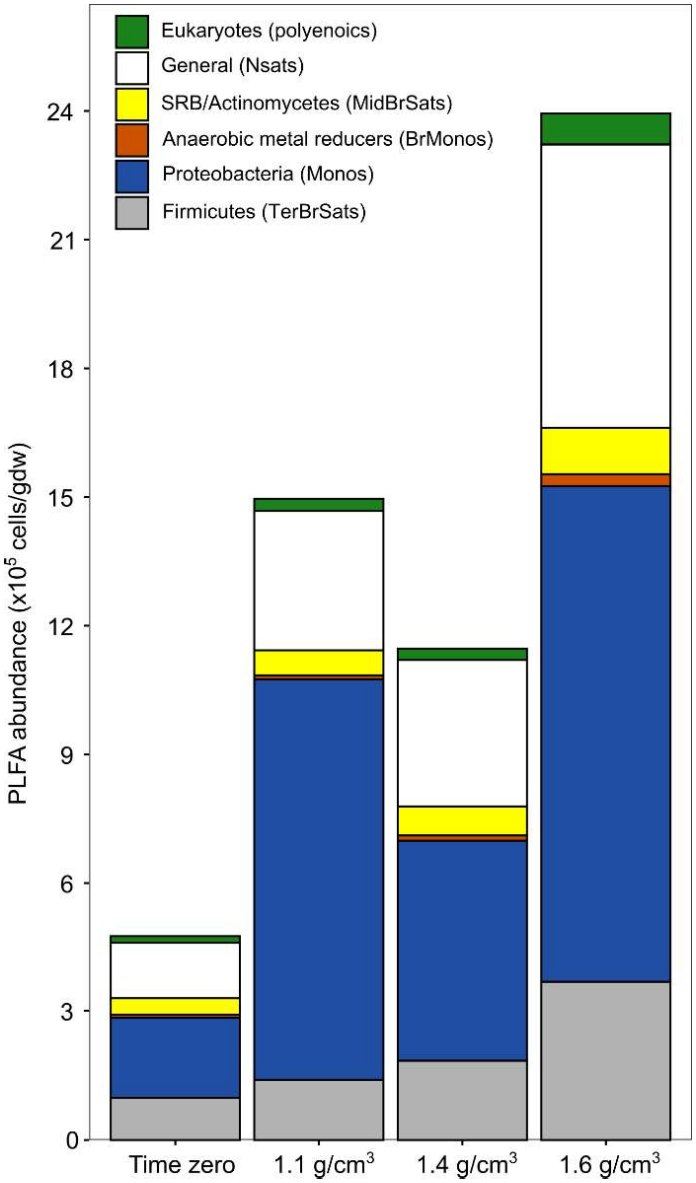

Figure 3.7 The PLFA community structure distribution in clay compacted to one of three dry densities. Proportional abundances are based on total estimated cells/gdw in initial clay powder and inner layers of pressure vessel clay at three different dry densities after three months of incubation. Structural groups are separated into different categories based on monoenoic (Monos), terminally branched saturated (TerBrSats), branched monoenoic (BrMonos), mid-chain branched saturated (MidBrStats), normal saturated (Nsats), and Polyenoic phospholipid fatty acid profiles detected in each sample. Each category of PLFA is generally associated with specific groups of microorganisms, except for Nsats because they are found in all organisms.

3.4 Discussion

Combining analysis of detectable abundances of culturable microorganisms, nucleic acids, and PLFAs permitted assessment of temporal changes to microbial community profiles of clay after incubation under conditions that mimic those of a DGR. High dry densities intended for a DGR (1.6 g/cm^3 or higher) typically restrict microbial growth, making assessment of the few culturable microorganisms and low nucleic acid abundances difficult [10]. Previous research showed that water activity less than 0.96 suppressed replication of culturable aerobic heterotrophs in compacted bentonite clay [10]. In this study, all saturated pressure vessels possessed clays with water activities greater than 0.96, and microbial growth was observed. Thus, assessments of clay with lower dry densities allowed for analysis of changes to the microbial community that can potentially occur in saturated bentonite clay.

Regarding the inner and outer layers of pressure vessel clay, there was only one pressure vessel where statistically significant differences in aerobic heterotroph quantities were detected between the inner and outer samples (Figure 3.2). Interfaces that are between bentonite clay and other materials are known to contain higher abundances of culturable microorganisms because the regions often contain localized decreases in dry density that provide favourable conditions for microbial growth [10, 42, 56]. In a DGR, important interface regions include between bentonite clay and the copper coating of the used fuel container, as well as between the bentonite clay and the concrete barrier [33].

Previous studies have demonstrated that higher aerobic heterotroph abundances corresponded to lower dry densities of clay [10, 41, 56]. In those studies, microorganisms were able to grow when the compaction of saturated bentonite clay was below a dry density of 1.6 g/cm^3 [10, 47, 83]. Although the large variation between replicates in this study yielded statistically insignificant changes, cultivation from saturated bentonite clay still revealed increases in the average detectable abundances of aerobic heterotrophs (Figure 3.2). However, statistically similar abundances of SRB were detected across all tested dry densities indicating that the selected pressure vessel conditions promoted neither growth nor death of SRB (Figure 3.2). In previous research, consistent abundances of SRB have been observed after incubation of bentonite or Opalinus clays at various dry densities [10, 41, 42, 56].

An understanding of potential microbial growth in the bentonite barrier of a DGR is required to estimate and prevent their impact on the long-term stability of the engineered barriers. Pressure

vessels containing clay compacted to 1.1 and 1.4 g/cm^3 commonly displayed high relative abundances of *Bacillus* and *Pseudomonas* ASVs (Figure 3.6), as well as increases in 16S rRNA gene copy numbers (Figure 3.3B). When combining these taxonomic profiles and the 16S rRNA gene abundance data, it is likely that growth of *Bacillus* and *Pseudomonas* members accounted for 16S rRNA gene abundance changes. Because *Bacillus* and *Pseudomonas* affiliated ASVs are associated with bacterial growth in clay, their absence in the inner layer of highly compacted bentonite (1.6 g/cm^3) suggests a lack of microbial growth. As well as lacking *Bacillus* and *Pseudomonas* affiliated ASVs, the quantity of 16S rRNA gene copies significantly decreased after incubation under high compaction, suggesting that degradation of nucleic acids and possibly death of cells occurred within this saturated clay environment.

Methods such as direct cell counting, fluorescence *in situ* hybridization, and nucleic acid quantification of clay microorganisms have been unsuccessful in past research because of low cell abundance or an inability to amplify nucleic acids [98]. Detection of clay microorganisms by PLFA analysis was commonly attempted to resolve this issue [10, 43, 53, 55, 99]. In a similar study, highly compacted bentonite clays with targeted dry densities ranging from 0.8 - 2.0 g/cm^3, were analysed using PLFA and cultivation techniques [10]. Similar PLFA abundances were reported across all samples despite increasing abundances of culturable aerobic heterotrophs at lower dry densities [10]. At the time, the hypothesized reason for the increase in culturable aerobic heterotrophs at lower dry densities was microorganism resuscitation from a VBNC state [10, 56]. Resuscitation would allow for cultivation of microorganisms that were present, but previously undetectable. However, our data indicates that increases in average abundances of 16S rRNA gene copies corresponded to bacterial proliferation (Figure 3.3B and C). Because the quantity of 16S rRNA genes of specific microorganisms changed after incubation in pressure vessels, it is unlikely that the microorganisms were already present and simply transitioned out of a VBNC state.

The PLFA-based estimates of microbial cell abundances from the most compacted clay (1.6 g/cm^3) did not reflect the changing quantities of 16S rRNA genes and aerobic heterotrophs in pressure vessels with various dry densities (Figure 3.3). The high background PLFA abundances may reflect organic matter preserved in the clay matrix [43, 55]. In this present study, the high background abundances of PLFA detected from clay may mask the comparatively small changes in culturable microorganisms, rendering them undetectable through PLFA quantification. In other words, the

change in the quantity of microorganisms observed through 16S rRNA genes was likely too small to overcome the large background abundances of PLFA from preserved organic material.

Microbial profiles from compacted bentonite clay after saturation and incubation revealed changes that provide valuable insight into the microbial taxa capable of growing within compacted clay. The genus *Bacillus*, which contains endospore-forming bacteria, has previously been cultivated from bentonite clay samples, as well as from natural or artificial formations of bentonite, Boom (Belgium), and Opalinus clay [39, 42, 84, 95, 100]. In Chapter 2, members of the *Bacillus* were also cultivated from various types of uncompacted bentonite clay. Although *Bacillus* spp. were core members of uncompacted clay microbial communities (Chapter 2), highly compacted bentonite at dry densities of 1.6 g/cm^3 provided unfavourable growing conditions for these microorganisms [64]. Species of *Pseudomonas* have also been detected previously in natural formations of Boom and bentonite clays [84, 100] as well as in highly compacted bentonite and Opalinus clays [39, 64, 95, 98]. In previous studies of highly compacted bentonite clay, microbial profiles detected using high-throughput sequencing were dominated by OTUs affiliated with *Xanthomonas*, *Streptomyces* and *Micrococcaceae* [64]. In that study, the lower density outer interface regions of the highly compacted clay contained *Pseudomonas*-associated OTUs that were consistently observed at relative abundances >6% [64]. Here, *Pseudomonas* associated ASVs also dominated some microbial community profiles of low-density saturated bentonite. Although absent in clay compacted to 1.6 g/cm^3 dry densities, *Pseudomonas* dominated microbial profiles of relatively low compaction (1.1 g/cm^3) bentonite clay (Figure 3.6).

Examination of saturated clay from pressure vessels containing dry densities both at and below the DGR-relevant dry density of 1.6 g/cm^3 was carried out through combined cultivation, PLFA, and 16S rRNA gene analyses. Changes in the microbial community profiles detected from clay that was compacted to low dry densities indicated proliferation of similar subsets of bacteria regardless of the microorganisms detected in the initial clay powder. In future pressure vessel experiments with better simulation of the saturated bentonite barrier conditions, the growth of these bacteria should be further assessed.

Chapter 4
Conclusions and future directions

4.1 Summary

For long-term storage of Canada's used nuclear fuel, a multiple-barrier storage system has been proposed. As proposed, a deep geological repository would combine engineered and natural barrier components to ensure safe storage of used nuclear fuel for over one million years. Engineered barriers consisting of stable used nuclear fuel and strong carbon steel containers with a corrosion-resistant copper coating will be encased in highly compacted bentonite clay. Approximately 500 m below ground, the layers of highly compacted and gapfill bentonite will be followed by thick concrete walls and then stable host rock. For long term stability of the bentonite clay barrier, microbial activity must be minimized. For that reason, the microbiology of bentonite clay has previously been assessed. Often, the microorganisms present in powdered and highly compacted bentonite clay are assessed using traditional cultivation methods [10, 24, 42]. To further account for naked DNA and non-culturable microorganisms that are not detected through cultivation, this thesis research adopted a recently validated protocol [65] for extraction and high-throughput sequencing of bentonite clay microbial DNA before and after treatments replicating conditions that will persist in a DGR. This thesis has brought together previous research of clay microorganisms through a combined analysis with cultivation and biomarker-based techniques.

A nucleic acid-based comparison of the microbial communities within diverse bentonite clay samples revealed distinct community profiles in each sample and some similarities among profiles from bentonite clays produced in the same time period (Figure 2.6). Nucleic acid analysis of as-received bentonite clay was also used to demonstrate the discrepancy regarding the most abundant nucleic acid sequences detected from clays and microorganisms that were captured through cultivation in the laboratory. At a broad taxonomic level, traditional cultivation primarily identified the presence of bacteria associated with *Bacillales* and *Clostridiales* (Figure 2.10). At the ASV-level of resolution, each clay sample contained a unique microbial community profile dominated by *Firmicutes* from enrichment cultures and *Actinobacteria* and *Proteobacteria* from clay (Figure 2.8). Combining cultivation data with nucleic acid sequences allowed for identification of ASVs affiliated with *Streptomyces*, *Micrococcaceae*, *Bacillus*, and *Desulfosporosinus* that were both viable and abundant members of the core clay microbial community (Figure 2.14). Without extraction of nucleic

acids from the bentonite clay samples, dominant ASVs affiliated with *Streptomyces* and the *Micrococcaceae* would have remained undetected. Whether the dominant nucleic acids detected in bentonite clay samples were associated with living cells, or were instead relic DNA from long deceased microorganisms, remains unknown and a topic for future research.

A thorough understanding of microbial communities within as-received initial clay samples allows for the detection of changes after performing experiments in compacted bentonite. Quantification and high-throughput sequencing of 16S rRNA genes from compacted bentonite clay indicated that putative microorganisms can grow and replicate under relatively high water activity and low dry density conditions. At the lowest dry density tested, several samples of clay showed a 1,000-fold increase in 16S rRNA gene abundances and a corresponding change in microbial community profiles (Figure 3.3B and 3.6). Despite the inherent heterogeneity among dry bentonite clay samples, after incubation with >0.96 water activity and low compaction (to a dry density <1.6 g/cm^3) similar microbial profiles were detected (Figure 3.6). In contrast, compared with initial clay samples, the 16S rRNA genes from the highly compacted bentonite clay (1.6 g/cm^3) decreased in abundance and similar microbial community profiles were maintained (Figure 3.3B and 3.6). The initial clay 16S rRNA gene profiles displayed heterogeneity between replicate samples (Figure 3.6). These preliminary results also reinforce that microbial growth is prevented, at least over relatively short incubation times, at a dry density of bentonite clay similar to that proposed for use in a deep geological repository.

Trends observed through quantification of 16S rRNA genes and culturable aerobic heterotrophs in compacted clays differed from those detected through PLFA analysis. The PLFA analysis showed that the number of microorganisms in compacted bentonite clay increased with higher dry densities (Figure 3.3A). It has previously been suggested that high PLFA abundances can be due to detection of viable but non-culturable bacteria [10, 56]. Because quantification of nucleic acids would permit detection of VBNC bacteria, tracking the quantities of 16S rRNA gene copies from clay samples was used to test the hypothesis that many existing VBNC bacteria should resuscitated during incubation at low dry densities. As a result, the changes observed in 16S rRNA gene abundances and the change in the microbial community profile do not support the resuscitation hypothesis. In clay at lower dry densities the average abundances of 16S rRNA genes increased compared to what was observed in the initial clay samples (Figure 3.3B). This is the same trend that was observed through abundances of culturable aerobic heterotrophs (Figure 3.3C). As well, at lower

dry densities, ASVs associated with bacteria such as *Bacillus* and *Pseudomonas* increased in relative abundance compared to the initial clay samples. The change in the microbial community profiles along with the increase in 16S rRNA gene abundances suggests that microbial growth occurred in the low-density pressure vessel clay not simply VBNC resuscitation. It is also suggested that the high PLFA abundances were a result of detecting background PLFA abundances or organic materials rather than changes in the microbial community.

The work in this thesis provides future researchers with a better understanding of microbial communities that inhabit diverse samples of bentonite clay and how those community profiles change after saturation. The data reinforce the importance of 1.6 g/cm^3 as a dry density for preventing microbial growth within saturated bentonite clay. As well as showing a decrease in microbial abundance, the dominant taxa that remain in highly compacted bentonite clay have been determined.

4.2 Future directions

In the future, new experiments testing additional DGR-relevant conditions should be performed, including longer incubation periods, higher dry densities and anoxic conditions. Because the DGR will only be oxic for a relatively short period of time during assembly, processing of pressure vessels and clay samples should therefore occur in an anaerobic chamber to mimic the long-term anoxic conditions in a DGR. For the assessment of compacted clay after anoxic incubation, the parallel methodological combination of cultivation-dependent approaches and nucleic acid assessments are recommended to provide complementary analysis in experiments. The quantity and phylogenetic classifications of SRB, like *Desulfosporosinus* that were outlined in this present study as members of the core microbial community in clay, should be assessed in clay from anoxic pressure vessels that are otherwise the same as the ones performed here (i.e., the same incubation times and dry densities). Future experiments involving the application of DNase or propidium monoazide to clay samples before extraction of DNA is recommended to assess microbial community profiles without the potential taxonomic and phylogenetic biases due to relic DNA or nucleic acids within dead microorganisms. Additionally, the same tests should be performed at higher dry densities and for longer lengths of incubation. Thorough assessment of the presence and viability of SRB is critical for prevention of microbiological impacts on a DGR.

Time restrictions caused by a global pandemic prevented a full analysis of multiple bentonite clay samples. In the future, qPCR and high-throughput sequencing should be performed on the remaining available bentonite samples to provide a more comprehensive dataset. In future pressure vessel experiments, the same type of clay should be used to pack pressure vessels to ensure no inconsistencies due to variation in processing method of clay. Otherwise, further analysis should also be performed to determine microbiological differences in gapfill and powdered clay materials. To reduce variation between biological replicates, it is suggested that a system that allows for even saturation throughout pressure vessel clay be established. Although, it is understood that the inherent swelling properties of clay can make even saturation very difficult in the laboratory.

As a result of eukaryote detection in the PLFA analysis, amplification and sequencing of microorganisms in powdered and compacted clay DNA extracts with primers that select for eukaryotes like fungi, should be performed. This will allow for a more comprehensive assessment of all microorganisms present in bentonite clay samples that mimic engineered barrier conditions to assess potential microbiology implications for a deep geological repository. To extend beyond what is

already known about bentonite clay microbial communities, future studies using metagenomic sequencing and transcriptomics are recommended to better characterize metabolic capabilities of the detected microbial communities. This assessment could lead to valuable risk assessments for clay-associated microorganisms and the long-term stability of a DGR.

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