

Microbiology of bentonite clay relevant to a deep geological repository for used nuclear fuel

by

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### **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 1*

As a co-op student, Cailyn Perry assisted with drawing the components of Figures 1.3 and 1.5. The deep subsurface portion of this chapter was previously published as part of a review paper:

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### *Chapter 2*

All pressure vessels were set up by Claire Tully as part of her PhD work at the University of Western Ontario under the supervision of Dr. James Noël. For the initial nine pressure vessels, sample processing and cultivation were completed by Melody A. Vachon as a part of her Master's thesis work at the University of Waterloo under the supervision of Dr. Josh Neufeld. Emilie Spasov assisted with the cultivation of bentonite from three pressure vessels. As undergraduate co-op students, Ana Fernández Galliano, Taylor Virgin, and Sarah Hammond assisted with cultivation of bentonite from pressure vessels and DNA extractions from culture biomass. In addition to the people mentioned above, Jeffrey Binns (NWMO), Peter Keech (NWMO), and Mehran Behazin (NWMO) had input into the design of these experiments. A version of this chapter has been published as:

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### *Chapter 3*

All pressure vessels were set up by Claire Tully as part of her PhD work and Jessie McDonald as part of her MSc work, both at the University of Western Ontario under the supervision of Dr. James Noël. As undergraduate co-op students, Cailyn Perry and Brianna Thomas assisted with cultivation, and Nathan Cheng with DNA extractions of culture biomass and swabs. In addition to the people mentioned above, Jeffrey Binns (NWMO), Peter Keech (NWMO), and Mehran Behazin (NWMO) had input into the design of these experiments.

#### *Chapter 4*

All microcosm setup, cultivation, and DNA extraction was completed in collaboration with Cailyn Perry, who was an undergraduate co-op student at the time. Peter Palermo, also as an undergraduate co-op student, assisted with making media for cultivation.

#### *Chapter 5*

Core sampling at the Tsukinuno mine was completed by Dr. Russell Alexander (Bedrock Geosciences) and Heini Reijonen (Geological Survey of Finland) in collaboration with Mr. Masa Ito (Kunimine Industries Company Ltd.). As undergraduate co-op students, Cailyn Perry, Shelby Papp, and Milena Rabu assisted with sample preparation, cultivation, and DNA extractions of culture biomass and swabs. In addition to the people mentioned above, Erik Kremer (NWMO) had input into the sampling plan.

#### *Chapter 6*

The microcosms used to test for VBNC and growth were prepared by Veronika Koziyeva, a postdoctoral fellow, and she additionally completed the cultivation and dPCR. The bentonite heterogeneity experiments were completed in collaboration with Cailyn Perry, who was an undergraduate co-op student. The cell counts for the SRB growth curve were completed by Stefan Grinchenko as an undergraduate co-op student.

## **Abstract**

Nuclear energy is an important source of energy globally, but results in the production of used nuclear fuel. When removed from a nuclear reactor after its useful lifetime, used nuclear fuel is still highly radioactive and must be stored safely for approximately one million years until it returns to the radioactivity level of naturally occurring uranium ore. Canada, along with other countries that have an inventory of used nuclear fuel, is in the process of designing a deep geological repository (DGR) for long-term storage of used nuclear fuel. In this system, used nuclear fuel, itself a stable solid, will be stored in copper-coated steel used fuel containers, and placed in bentonite clay buffer boxes made of highly compacted Wyoming MX-80 bentonite. Buffer boxes would then be stacked in placement rooms approximately 500 m below ground in a suitable host rock with spaces between buffer boxes and host rock filled in with a granulated form of bentonite referred to as gapfill material. To ensure the longevity of a DGR, it is important to consider the role that microorganisms could play, particularly through contributing to corrosion of used fuel containers through a process termed microbiologically influenced corrosion. Expected to dominate occurrences of microbiologically influenced corrosion under anoxic conditions, sulfate-reducing bacteria (SRB) are a primary target of research, but rarely live in isolation, thus necessitating the study of microbial communities that could live in the bentonite clay surrounding used fuel containers on a broader scale.

Due to the potentially detrimental role that some microorganisms could play in a DGR, a goal in designing a DGR is to compact bentonite, a type of swelling clay, to a sufficiently high dry density that microbial growth is suppressed upon saturation. The first goal of this thesis was to investigate the bentonite dry density necessary to suppress microbial growth in Wyoming MX-80 bentonite under oxic conditions and gapfill material under both oxic and anoxic conditions. A set of pressure vessel experiments demonstrated suppression of microbial growth under oxic conditions in bentonite compacted to a minimum dry density of  $1.4 \text{ g/cm}^3$ . Under anoxic conditions, growth of heterotrophs was suppressed in pressure vessels with a minimum dry density of  $1.45 \text{ g/cm}^3$ , but culturable SRB persisted in abundances higher than the as-received bentonite starting material in the outer layers of all pressure vessels throughout the full one-year experiment. Additional experiments were conducted to explain the increase in abundance of SRB, which had not previously been observed in other studies. These experiments

suggested that the increase in abundance of SRB was likely not inherent to the gapfill material, nor was there evidence for it being a result of differences in SRB medium or in amounts of trace oxygen between studies. In both oxic and anoxic pressure vessels, an initial increase in abundance of culturable heterotrophs was observed prior to complete saturation, presumably as water became available but swelling pressure remained sufficiently low to allow for their growth. Although previously proposed to potentially be associated with a recovery from the viable but not culturable state rather than growth, a follow-up experiment suggested that the initial increase in abundance of culturable heterotrophs was likely a reflection of growth (i.e., cell division).

Dry density is an important consideration in DGR design, but it is not the only physical property that could influence microbial growth within a DGR. Temperature is expected to fluctuate from natural subsurface temperatures of  $<20^{\circ}\text{C}$  to temperatures as high as  $94^{\circ}\text{C}$ , and little research has been conducted to explore the potential for microbial growth in bentonite at these elevated temperatures. This thesis includes experiments testing the abundance and community composition of microorganisms adapted to a variety of temperatures in as-received and hydrated bentonite. The results showed a low abundance of culturable microorganisms that survived incubation at  $60^{\circ}\text{C}$ , but 16S rRNA gene profiles dominated by presumably unculturable representatives of the thermophilic family *Thermoactinomycetaceae*. Hydrated bentonite was additionally incubated at temperatures of 75, 90, and  $105^{\circ}\text{C}$ , but DNA sequencing results did not show a shift in community composition from as-received bentonite, suggesting that the natural as-received bentonite microbial community may not include members adapted to these very high temperatures.

Lab-scale experiments allow for testing of very specific DGR-relevant conditions (e.g., dry density and temperature) and the effect these have on microbial community abundance and composition. However, a DGR is being designed to exist for upwards of one hundred thousand years, which is not a realistic timescale for any experiment. To circumvent this limitation, one approach is to couple lab-scale experiments to the study of natural analogues, which have naturally existed for DGR-relevant timeframes. This thesis presents a study of the Tsukinuno bentonite deposit in Japan, which can serve as a natural analogue to DGR bentonite. In this study, sequencing of DNA extracted from bentonite revealed microbial communities dominated by sequences associated with *Thiobacillus*, *Hydrogenophaga*, *Comamonadaceae*, and

*Pseudomonas*. Although differences in community composition were observed between samples, microbial communities were relatively similar for all four studied cores and at all depths into the clay bed. A series of geochemical parameters were measured to help identify factors that may influence microbial community composition. The abundance of culturable anaerobic heterotrophs was positively correlated with the concentration of nitrate, which could be used by anaerobes for denitrification, and the abundance of culturable aerobic and anaerobic heterotrophs was negatively correlated with the abundance of the clay mineral montmorillonite, increased concentrations of which would increase the swelling capacity of the bentonite. The results presented throughout this thesis will together be useful for incorporation into future models of microbial activity within a DGR and can ultimately be used to inform DGR design.

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## **List of Abbreviations**

|         |                                                             |
|---------|-------------------------------------------------------------|
| AECL    | Atomic Energy of Canada Limited                             |
| ANOVA   | Analysis of variance                                        |
| ASV     | Amplicon sequence variant                                   |
| ATP     | Adenosine triphosphate                                      |
| AXIOME  | Automation, Extension, and Integration of Microbial Ecology |
| BSA     | Bovine serum albumin                                        |
| CANDU   | Canada deuterium uranium                                    |
| CFDA-AM | 5-Carboxyfluorescein diacetate acetoxymethyl ester          |
| CFU     | Colony forming unit                                         |
| DADA2   | Divisive Amplicon Denoising Algorithm 2                     |
| DGR     | Deep geological repository                                  |
| DNA     | Deoxyribonucleic acid                                       |
| dNTP    | Deoxynucleotide triphosphate                                |
| dPCR    | Digital PCR                                                 |
| DPTA    | diethylenetriaminepentaacetic acid                          |
| dsDNA   | Doubles stranded deoxyribonucleic acid                      |
| gdw     | Gram dry weight                                             |
| GFM     | Gapfill material                                            |
| HCB     | Highly compacted bentonite                                  |
| HEPA    | High-efficiency particulate absorbing                       |
| HSD     | Honestly significant difference                             |
| MIC     | Microbiologically influenced corrosion                      |
| MPN     | Most probable number                                        |
| NTC     | No template control                                         |
| NWMO    | Nuclear Waste Management Organization                       |
| PBS     | Phosphate buffered saline                                   |
| PCR     | Polymerase chain reaction                                   |
| PLFA    | Phospholipid fatty acid                                     |
| PMA     | Propidium monoazide                                         |

|      |                                                   |
|------|---------------------------------------------------|
| PTFE | Polytetrafluoroethylene                           |
| qPCR | Quantitative polymerase chain reaction            |
| R2A  | Reasoner's 2A                                     |
| rRNA | Ribosomal ribonucleic acid                        |
| SKB  | Swedish Nuclear Fuel and Waste Management Company |
| SQ   | Starting quantity                                 |
| SRB  | Sulfate-reducing bacteria                         |
| SRS  | Scaling with ranked subsampling                   |
| UFC  | Used fuel container                               |
| UV   | Ultraviolet                                       |
| XRD  | X-ray diffraction                                 |

## Chapter 1: Introduction

### 1.1 Nuclear energy and the deep geological repository concept

Nuclear power is an important global source of energy and a key component of a fossil-fuel independent future. In Canada, and globally, nuclear reactors are powered by uranium. In Canada, a form of nuclear fuel known as Canada deuterium uranium (CANDU) is used. The CANDU fuel is made of  $\text{UO}_2$  powder pressed into solid pellets and then baked into ceramic fuel pellets (Figure 1.1A) [1]. Fuel pellets are stacked in fuel pencils made of zirconium-tin alloy, which are welded together to form fuel bundles (Figure 1.1A). When fuel bundles are removed from nuclear reactors at the end of their useful life using current technology, they are still highly radioactive and must be stored safely, isolated from people and the environment [1].

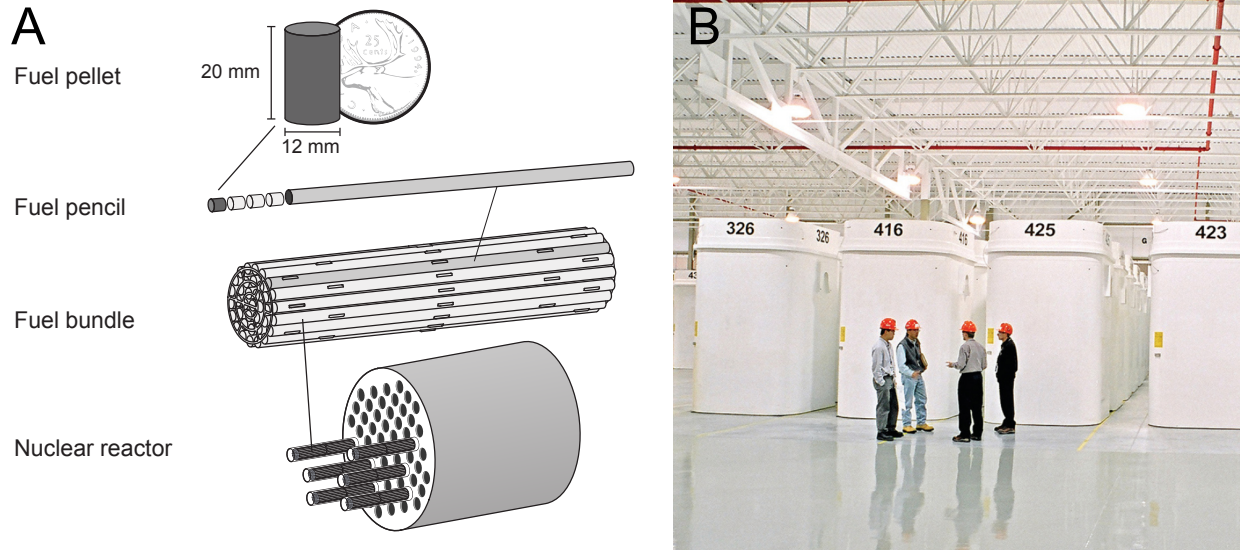


**Figure 1.1** Canadian nuclear fuel (A) within dry storage containers (B). Modified from a previous publication [1].

Used nuclear fuel must be stored for very long timeframes because it takes one million years for the radioactivity level of used nuclear fuel to return to that of naturally occurring uranium ore (Figure 1.2A) [1]. Current storage of used nuclear fuel bundles occurs at reactor sites (Figure 1.2B), where they are initially placed in water-filled pools for at least ten years while initial radioactivity and heat dissipate, then in dry storage containers indefinitely (Figure 1.1B). As of June 2023, Canada had 3.3 million used fuel bundles in interim storage at reactor sites, and this number is expected to increase to 5.6 million by the end of the planned operation of existing Canadian nuclear reactors [1].

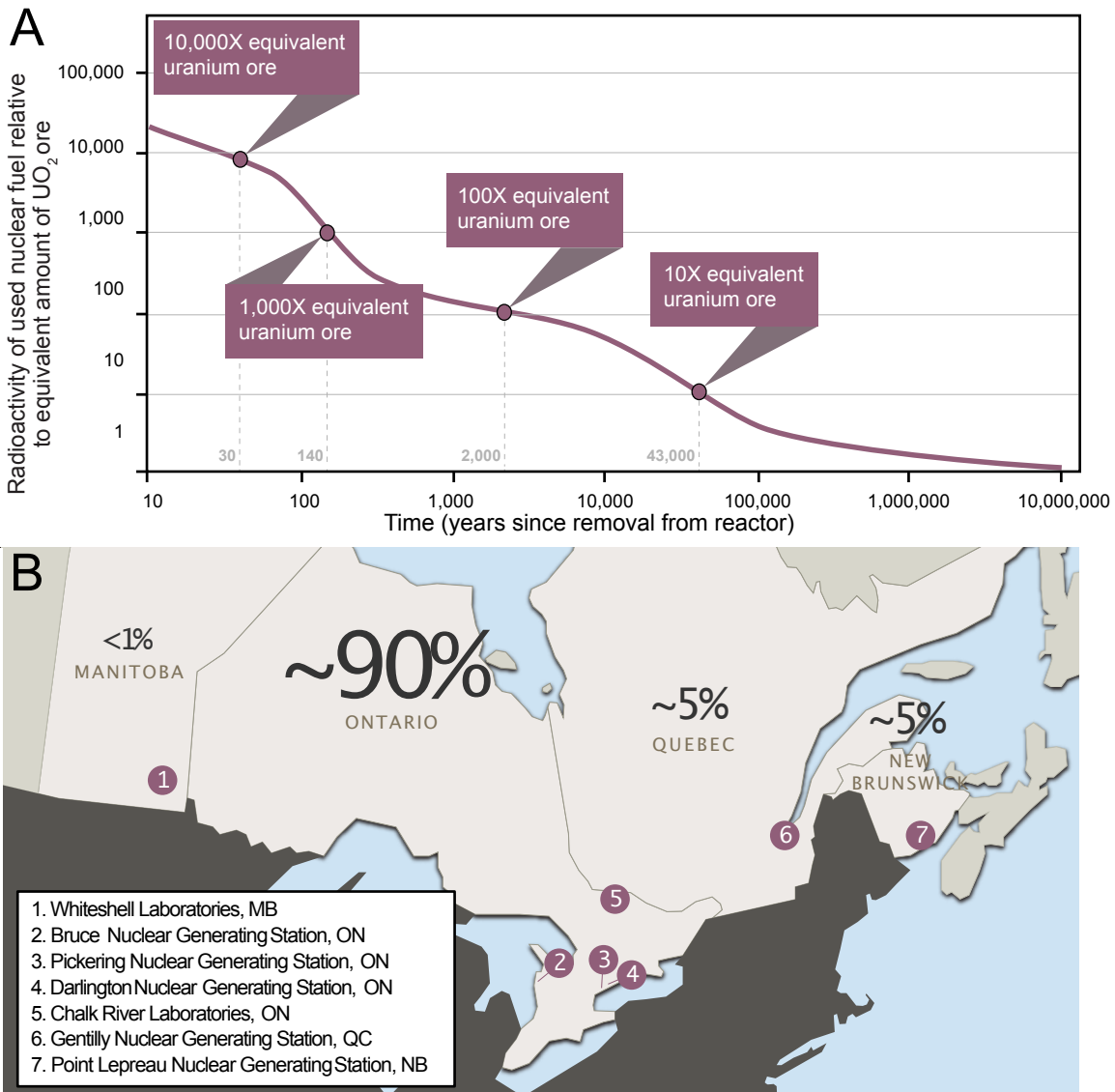


**Figure 1.2** Radioactivity of used nuclear fuel compared to uranium dioxide ore over time (A) and the current storage locations of Canadian used nuclear fuel at existing reactor sites (B). Modified from elsewhere [1].

Although storage at reactor sites is safe, it is only an interim solution because it is unrealistic to maintain above ground storage facilities for a million years, during which time dry storage containers would have to be replaced approximately every 50 years and storage facilities refurbished regularly, not to mention the ice ages expected to occur and unknown state of human civilizations that far into the future. In 2002, Canada's producers of nuclear energy (Ontario Power Generation, New Brunswick Power Corporation, and Hydro-Québec) established the Nuclear Waste Management Organization (NWMO), a not-for-profit organization tasked with

designing a safe long-term storage solution for Canada's used nuclear fuel. Along with other countries also in the process of designing and implementing long-term storage solutions for used nuclear fuel, the NWMO is designing a deep geological repository (DGR; Figure 1.3). In Canada, the DGR will be implemented through an approach called Adaptive Phased Management, which involves construction of a DGR, but also includes important steps like public engagement, especially with communities near potential repository host sites, developing a plan for transporting used nuclear fuel from reactor sites to the DGR site, and engaging with other countries to incorporate global best practices [1].

The Canadian DGR design is a multibarrier system that involves placing used fuel bundles in carbon steel used fuel containers (UFCs) that are coated in copper for corrosion resistance, which are then placed in bentonite buffer boxes made of high-compacted Wyoming MX-80 bentonite (Figure 1.3) [2]. Buffer boxes are then stacked in placement rooms approximately 500 m below ground in a suitable host rock, with highly compacted spacer blocks used to separate UFCs (Figure 1.3) [2]. Spaces between buffer boxes and the rock walls of the placement room would then be filled in with a granulated bentonite referred to as gapfill material (GFM; Figure 1.3).

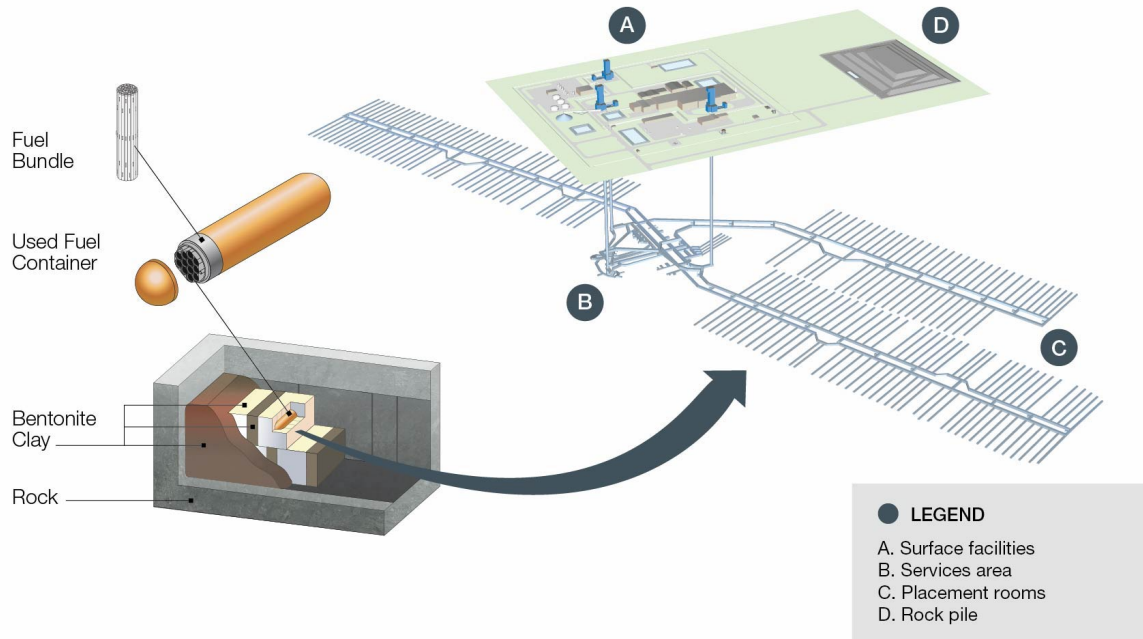


**Figure 1.3** Proposed design of the Canadian DGR. Modified from a previous publication [2].

## 1.2 Bentonite clay

Bentonite is a key component of DGR designs, where it is the material directly in contact with UFCs. Bentonite is a type of clay formed by the weathering of volcanic tuff and is defined by the presence of montmorillonite as the dominant clay mineral [3]. Montmorillonite is the most abundant clay mineral in the smectite group of clays and consists of two outer tetrahedral sheets of silica with a central octahedral sheet of alumina [4]. Due to isomorphic substitution of  $\text{Si}^{4+}$  for  $\text{Al}^{3+}$  in the tetrahedral sheets, and of  $\text{Al}^{3+}$  for  $\text{Mg}^{2+}$  in the octahedral sheets, montmorillonite has a net negative charge. This charge is balanced by exchangeable cations, either sodium or calcium, that exist between layers of montmorillonite [5]. Depending on the exchangeable cation, montmorillonite either displays swelling behaviour or not. In the case of sodium, a monovalent cation, attraction to negatively charged sheets of montmorillonite is weak, so when hydrated, water molecules between the montmorillonite sheets push the sheets apart and the clay swells. In contrast, divalent calcium cations are strongly attracted to the negatively charged sheets, and when hydrated, water molecules do not push the sheets apart and the clay does not swell [4].

The swelling nature of sodium bentonite makes it an ideal buffer material for use in a DGR. Montmorillonite has a very small average particle size of 0.5  $\mu\text{m}$ , resulting in small pore sizes that add to mechanical stability of the material and minimize space for microbial colonization [4, 6]. When hydrated in a confined space (e.g., a DGR placement room), swelling leads to the development of pressure (i.e., swelling pressure) that further limits microbial colonization of bentonite. Further, bentonite has a low hydraulic permeability, which minimizes flow of groundwater, potentially carrying with it corrosive compounds, from the host rock to the UFCs [6]. This low hydraulic permeability would additionally immobilize radionuclides in the event of UFC failure [6].

The type of bentonite proposed for use in a DGR is mined from Wyoming and processed by applying high isostatic pressure ( $\sim 100$  MPa) to achieve a maximum mesh size of 80 (i.e., Wyoming MX-80). This powdered bentonite is highly compressed to form highly compacted bentonite (HCB) buffer boxes and spacer blocks. It is also used to produce gapfill material, the second type of bentonite proposed for use in a DGR, through a roll compacting/milling process. The mineral composition of Wyoming MX-80 bentonite has a high montmorillonite content of  $\sim 81\%$ , and smaller abundances of other minerals like muscovite, plagioclase, pyrite, and quartz [7]. The bentonite received from a manufacturer prior to experimentation is commonly referred to as “as-received bentonite,” which is a relatively dry and finely ground material.

### **1.3 Microbiology in the context of a DGR**

In addition to including multiple natural and engineered barriers in the design of a DGR, there are several factors that must be considered to ensure that the integrity of a DGR and its components are stable over very long timeframes. One such factor is the role that microorganisms within or surrounding the DGR could play. In general, DGRs are predicted to be relatively inhospitable environments for microbial activity because of limited water availability, lack of space, increased swelling pressure due to the highly compacted and swelling nature of saturated bentonite clay, low nutrient availability, and high temperature [8]. Nonetheless, there are active microbial communities in deep subsurface environments, and microorganisms are additionally culturable from as-received bentonite [9, 10], thus necessitating the study of their potential viability and activity within a DGR.

In the context of a DGR, microorganisms may participate in microbiologically influenced corrosion (MIC), a process through which corrosion of a surface is initiated or propagated by corrosive compounds produced by microbial metabolism. Under anoxic conditions, sulfate-reducing bacteria (SRB) tend to dominate occurrences of MIC, likely due to the near-ubiquitous distribution of sulfate in the environment (Figure 1.4) [11–16]. Even in sulfate-limited systems, SRB have been shown to proliferate [17–19]. Additionally, SRB have been detected within commercially available bentonites proposed for use in a DGR [9, 10]. Apart from SRB, microorganisms with metabolisms that result in a net production of gas are also relevant. If produced in sufficient volume, gas could lead to the formation of permeability fissures in the clay or host rock, which would increase groundwater and ion transport to the surface of UFCs [13]. Although SRB, and to a lesser degree gas-producing microorganisms, are a major area of focus in DGR design, microorganisms rarely live in isolation and may have growth or metabolism influenced by various symbiotic relationships with other microorganisms living in the same community, such as those capable of sulfur oxidation or fermentation. As such, it is important to study the whole microbial community that may be expected within bentonite clay and the deep subsurface environment that will host the DGR.

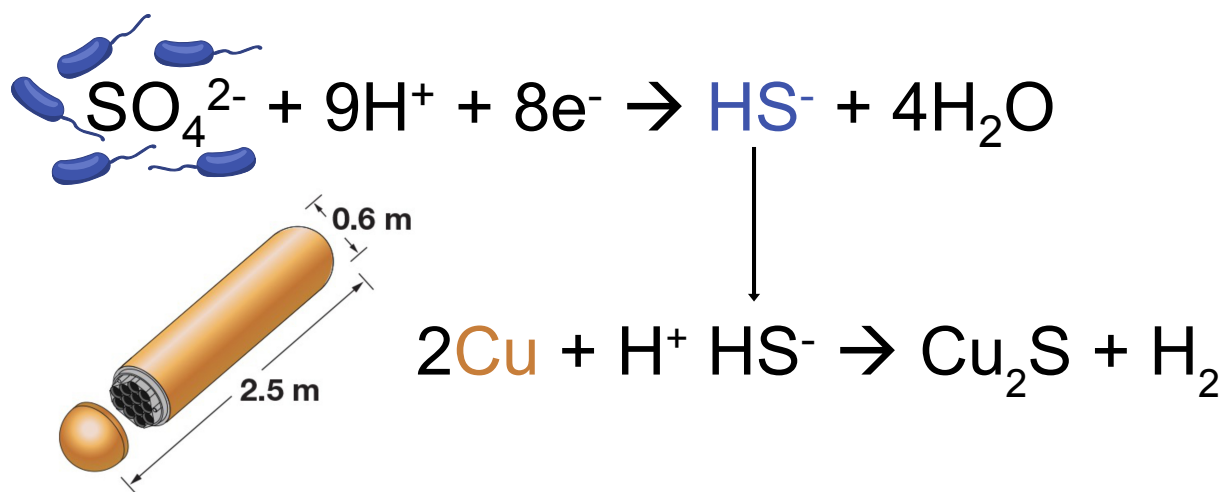


**Figure 1.4** Microbiologically influenced corrosion of the copper coating of a used fuel container via sulfide production by sulfate-reducing bacteria. UFC image taken from a previous publication [20].

## 1.4 Microbiology of subsurface environments

Because a DGR will be hosted in the deep subsurface, existing research on deep subsurface microbiology can provide important information about the types of microorganisms and microbial metabolisms that may be expected to occur in and around a future DGR. The terrestrial subsurface is one of Earth's largest environments and is predicted to host as many microbial cells as global surface soils, and more than all oceans combined [21–24]. Especially given the massive volume of this ecosystem, subsurface microorganisms play an important role in global biogeochemical cycling. The deep terrestrial subsurface is not only of interest to global nuclear waste management organizations, but also to industries interested in mining the valuable compounds, such as ores, minerals, oil, and natural gas, it contains, and for its potential in carbon capture and storage of hydrogen for use as an energy vector [25]. Further, certain deep subsurface environments on Earth can serve as analogues to saline subsurface environments on other planets like Mars [26–32]. Nonetheless, the deep terrestrial subsurface remains underexplored, particularly because of logistical challenges of sampling such inaccessible locations.

Microorganisms are diverse in their metabolic needs, but there are several common requirements for all known life on Earth: water, carbon, nutrients, physical space, and energy for growth and reproduction. In many environments, these requirements are met readily, but the deep subsurface is typically nutrient poor. Because availability of the necessities for life tend to decrease with depth, so do the average abundances of microbial cells (Figure 1.5) [33, 34]. In such nutrient-deprived conditions, life in the deep subsurface operates at a slower pace than it does in most surface environments. For example, the average generation time for microbial cells in terrestrial deep subsurface environments has been estimated to be centuries [35–40]. This, coupled with relatively small population sizes, may lead to evolution driven by stochastic processes, like genetic drift, rather than deterministic factors, such as selection [41].

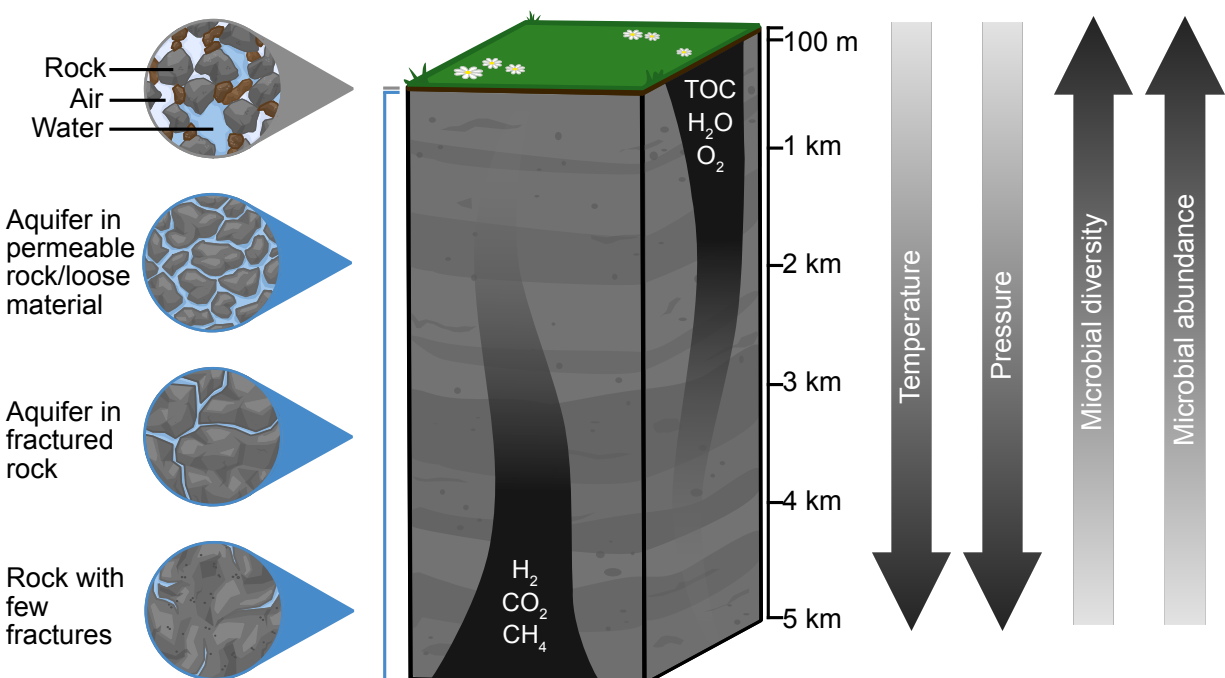


**Figure 1.5** Schematic of a terrestrial subsurface environment. The top layer of the subsurface is generally unsaturated, with saturated layers below. Most aquifers exist in the saturated zone, within the top 100 m from the surface. The saturated zone consists of aquifers in permeable rock, loose material, or fractured rock. Outside of aquifers, rock fractures within the saturated zone are often water-filled. As depth increases, so do the temperature and pressure, whereas microbial diversity and abundance are highest closer to the surface. Organic carbon, water, and oxygen become increasingly unavailable with depth, whereas  $H_2$ ,  $CO_2$ , and  $CH_4$  gases are abundant in the deep subsurface.

In the deep subsurface, water exists in the form of groundwater, which is a broad term used to describe fluid located below the surface in pore spaces of rocks and soil, in the fractures between rocks, and in aquifers (Figure 1.5). Aquifers typically occur in the first 100 m below the surface but can also extend to much greater depths [42]. They are commonly composed of unconsolidated porous rock/sediment (e.g., sand, gravel) or consolidated porous rock (e.g., sandstone). Other aquifers consist of water within interconnected fractures, cracks, or joints in solid rock (Figure 1.5). The salinity of groundwater increases with depth and can result in hypersaline environments at some of the greatest depths sampled [43]. Deep groundwater may also host high concentrations of heavy metals, which can be toxic to microorganisms [44, 45].

Given an absence of sunlight, and a lack of associated primary production from photosynthesis, access to organic carbon in the deep subsurface is more limited for

microorganisms than it is in surface environments. Some organic carbon in the deep subsurface was included with sediments at the time of their deposition and now through diagenesis exists primarily as oil and petroleum deposits. Subsurface organic carbon also exists in clay, shales, coal, and other deposits. Living near organic carbon deposits can be advantageous for microorganisms, especially heterotrophs, but it is not the only strategy. Organic carbon can also be produced *in situ* by chemolithoautotrophs that fix inorganic carbon, which allows for microbial life in the subsurface beyond carbon reservoirs [46]. In addition to a lack of organic carbon, deep subsurface environments are often anoxic, and with limited nutrients, thus most subsurface microorganisms rely on non-oxygen electron acceptors and inorganic electron donors for metabolism. However, some deep subsurface environments have access to oxygen via oxic water originating from the surface [47]. Alternatively, a recent study demonstrated higher than expected concentrations of dissolved oxygen in old groundwaters that may have been produced *in situ* via microbial dismutation, a process termed “dark oxygen production” [48].

Physical space for microorganisms to inhabit the deep subsurface is highly variable, ranging from pore spaces smaller than the size of a microbial cell, to larger fractures and faults that are sometimes interconnected [49]. Rock type influences both pore size and organic carbon availability. Sedimentary rocks are generally more porous than igneous and metamorphic rocks, providing more space for microorganisms to grow and interact [50, 51]. They also generally have not been exposed to the same high temperature and pressure conditions as igneous and metamorphic rocks, thus microbial populations found within them could theoretically have been present since the rock’s deposition [51–53]. In contrast, igneous and metamorphic rocks, which together represent most of the deep subsurface, rely on nutrient and energy source transport via fractures, and are usually void of organic matter [51]. Because the pore spaces of these rocks are usually too small for microbial cells, fractures provide the most likely habitats [54, 55].

#### **1.4.1 Chemical energy for primary production in deep subsurface environments**

In the absence of sunlight, subsurface communities must rely on non-photosynthetic primary production. It was originally thought that subsurface life must be supported by organic carbon deposits that were formed by ancient photosynthetic events. Although subsurface microbial communities that are near organic carbon deposits, such as oil, do take advantage of these carbon sources, other communities rely entirely on chemolithoautotrophic metabolism and

fix their own carbon from inorganic sources available in the subsurface. The first deep terrestrial subsurface microbial community shown to be completely supported by chemolithoautotrophic primary production was discovered in 1995 [46]. Since then, geogenic gases such as dihydrogen ( $\text{H}_2$ ), methane ( $\text{CH}_4$ ), and carbon dioxide ( $\text{CO}_2$ ) were linked with belowground primary production [56].

For primary production to occur, microorganisms must have the capacity to fix inorganic carbon into biomass. Several different carbon fixation pathways exist in microorganisms, but perhaps the most important for deep subsurface metabolism is the reductive acetyl-CoA pathway, or Wood-Ljungdahl pathway, because it is the preferred process for microorganisms living in low-energy environments near the thermodynamic limit of life [57]. Commonly adopted by acetogens, methanogens, and sulfate-reducing microorganisms, this pathway is used for energy production and for fixing inorganic carbon into biomass [57]. Metagenomic studies have demonstrated that the reductive acetyl-CoA pathway dominates within deep terrestrial subsurface microbial communities [58–60].

#### **1.4.2 Hydrogen-driven deep subsurface ecosystems**

A common feature of deep subsurface microbial communities is a reliance on  $\text{H}_2$  for energy (Figure 1.6). Hydrogen gas is present in subsurface environments through processes like radiolysis of water and serpentinization [61]. Although hydrogen-fuelled microbial metabolisms in the deep terrestrial subsurface were demonstrated prior to the availability of metagenomics [56], subsequent metagenomic studies have further reinforced a prevalence of genes involved in  $\text{H}_2$  oxidation associated with deep subsurface samples [58–60, 62]. For example, metagenomes generated from samples of three different borehole depths showed a significant enrichment of hydrogenases in borehole samples from 2.3 km compared to those from 0.6 or 1.5 km [45], suggesting that hydrogen becomes increasingly important with distance below the Earth's surface. Hydrogen gas can be coupled to the reduction of many different electron acceptors that are relevant to deep terrestrial subsurface metabolism, supporting methanogenesis [45, 63–65], homoacetogenesis [45, 66], sulfate/sulfite reduction [11, 14, 36, 63, 67–75], and iron reduction [63, 69, 76–78] (Figure 1.6).

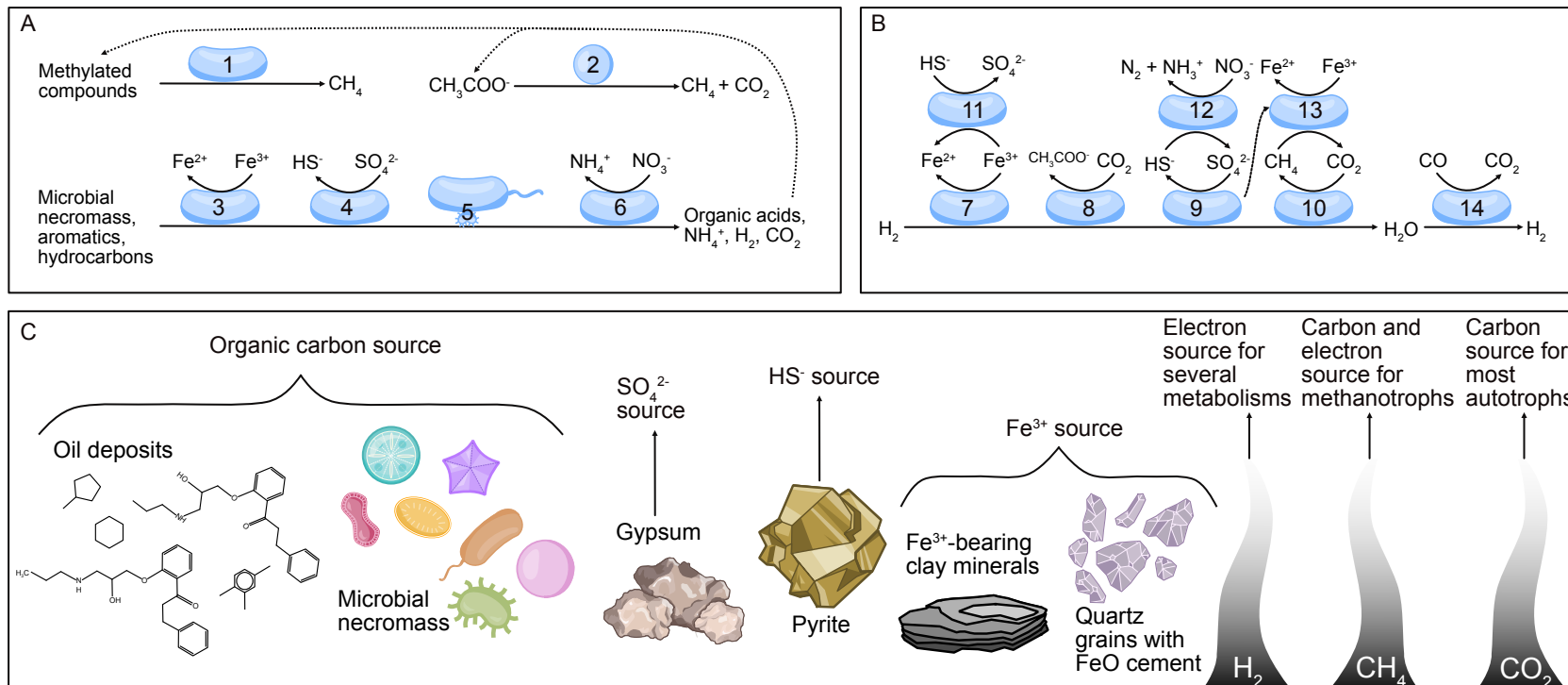


**Figure 1.6** Schematic of deep subsurface microbial metabolisms fueled by organic carbon (A) and  $\text{H}_2$  gas (B), and the sources of electron donors and acceptors in subsurface environments (C). The oxidation of organic carbon compounds can support methylotrophic methanogenesis (1), acetoclastic methanogenesis (2), iron reduction (3), sulfate reduction (4), fermentation, potentially involving episymbiotic relationships (e.g., CPR bacteria and DPANN archaea and their hosts; 5), and nitrate reduction (6). The oxidation of  $\text{H}_2$  gas can support iron reduction (7), acetogenesis (8), sulfate reduction (9), and methanogenesis (10), and the reduced iron, sulfide, and carbon dioxide, and electrons (dashed arrow) produced through these processes can act as electron donors for sulfide oxidation (11), nitrate reduction (12), and anaerobic oxidation of methane (13). Water can be reduced to  $\text{H}_2$ , coupled to the oxidation of  $\text{CO}$  to  $\text{CO}_2$  through the process of carboxydutrophy (14). The metabolisms presented within have been predicted from metagenomic studies of the subsurface [44, 45, 59, 61, 62].

### 1.4.3 Common microbial subsurface communities

Prior to high-throughput sequencing and metagenomics, deep terrestrial subsurface microbial community characterization generally involved either culturing approaches or clone library analysis (16S rRNA gene amplicon sequencing of selected clones). Using such traditional approaches, deep subsurface communities were commonly reported to be dominated by iron-reducing bacteria, SRB, methanogenic archaea, and acetogens [64, 80–83]. When subsurface samples were taken from locations near hydrocarbon reservoirs, fermentative microorganisms were also detected [84, 85].

Because a subset of microorganisms is favoured by cultivation conditions, microbial abundance estimates obtained by these techniques can be much lower than those from microscopy-based techniques, sometimes by orders of magnitude [84]. With the advent of high-throughput amplicon and metagenomic sequencing, it has been possible to study deep biosphere microbial communities with increased resolution, circumventing the biases of culture-based approaches. A survey of existing amplicon sequencing data from global terrestrial deep subsurface environments showed a universal dominance of phyla *Proteobacteria* and *Firmicutes*. It was proposed that the vast metabolic diversity within these phyla could account for their dominance in deep terrestrial subsurface environments, seemingly independent of underlying geology and environmental factors [86]. Metagenomic studies of deep terrestrial subsurface environments commonly reveal microbial communities with diverse metabolisms. For example, a study of deep subsurface samples from the Horonobe Underground Research Laboratory (Japan) found a diverse microbial community consisting of 29 phyla, including 13 uncultured representatives that had never been detected at this site [63]. The most abundant metabolic functions encoded by the metagenomes were sulfate reduction, sulfur oxidation, nitrate reduction, iron reduction, methane oxidation, and methanogenesis. Almost all reconstructed genomes showed the potential for fermentation, several had genes for nitrogen fixation, many encoded the Calvin-Benson-Basham or reductive acetyl-CoA pathways for carbon fixation, and more than half had genes involved in hydrogen oxidation. The functions detected in these metagenomes are common for deep terrestrial subsurface microorganisms [11, 60, 72, 87–90]; however, not all deep subsurface environments host such diversity (Figure 1.6).

#### 1.4.4 Low diversity subsurface microbial communities

Some deep terrestrial subsurface microbial communities have very low diversity. For example, water circulating within igneous rocks ~200 m belowground in Idaho was sampled and shown to be dominated by methanogens, at >90% of all detected taxa [56]. A similarly low-diversity community was discovered in groundwater from 2.8 km belowground in the Mponeng gold mine in South Africa, which had a microbial community dominated by a single SRB population belonging to the *Firmicutes* phylum [91]. Metagenomic sequencing of fracture fluid recovered from this same environment revealed a metagenome with >99% of reads belonging to this same population's genome [36]. Additional reads in the metagenome were considered to be laboratory or drilling contaminants. Named *Candidatus Desulforudis audaxviator*, which in Latin means “bold traveler in search of sulfur,” the assembled genome suggested complete self-sufficiency for this subsurface bacterium. In addition to the ability to couple sulfate reduction with H<sub>2</sub> (e.g., derived from radioactive decay of uranium) or formate oxidation for energy metabolism, the genome for *Ca. D. audaxviator* contains all genes necessary for carbon and nitrogen fixation and encodes all necessary amino acid biosynthesis pathways. Metabolically flexible, *Ca. D. audaxviator* can switch from heterotrophy to autotrophy as conditions change. Adaptations such as this could help to explain its ability to thrive in such a harsh environment independently [59]. Since its discovery, *Ca. D. audaxviator* has been reported in other global subsurface samples [92]. A similarly low diversity microbial community was later discovered in porous sandstone near an oil deposit, dominated (>98%) by *Halomonas sulfidaeris*, a heterotroph capable of using aromatic organic compounds [44].

#### 1.4.5 Factors influencing subsurface microbial community composition

The factors that influence microbial community composition and diversity of deep terrestrial subsurface environments remain poorly understood. Although the least diverse microbial communities discovered have been in some of the deepest sampled environments [36], other deep subsurface environments host relatively diverse microbial communities [89]. Decreasing diversity with depth is likely a combination of related factors that influence microbial community composition, such as water recharge and origin, water activity (e.g., salinity), organic matter availability, and electron donor and acceptor diversity.

Several 1-5 km deep samples taken from boreholes in South Africa had microbial communities dominated by either *Firmicutes* or *Proteobacteria* phyla [93–97]. In general, *Proteobacteria* taxa tend to dominate fracture fluids that have more recently mixed with meteoric (i.e., associated with precipitation) waters, which are relatively shallow subsurface fluids. In contrast, representatives of the *Firmicutes* dominate deeper subsurface communities, which tend to be fed from deep groundwaters with little or no meteoric water input [59]. This trend could be explained by the selection for microorganisms, often *Firmicutes* members, capable of using the reductive acetyl-CoA pathway for carbon fixation in lower energy deep environments, with less fluid input from meteoric sources. Indeed, a metagenomics study observed a higher relative abundance of *Firmicutes* members in fracture fluids with little mixing of meteoric waters, which was associated with a higher abundance of protein encoding genes associated with the reductive acetyl-CoA pathway [59]. A correlation between water origin and microbial community composition has been reported for other environments, including the Fennoscandian Shield and serpentinite springs in Canada [98, 99]. Water recharge, as well as organic matter availability, are also reported to be positively correlated with subsurface microbial community diversity [59]. Although not addressed by these experiments, additional factors could favour the persistence of certain microorganisms at greater depths compared to others, such as the ability of some microorganisms, including members of *Firmicutes*, to form spores and withstand unfavourable conditions.

In addition to carbon fixation pathways, other adaptations for nutrient-poor conditions of the deep subsurface could help explain persistence of certain microorganisms in these environments. For example, *Halomonas sulfidaeris* dominates (>98%) a microbial community in sandstone and is well adapted to use various aromatic organic compounds available from nearby oil deposits [44]. It also has adaptations for survival in the hypersaline subsurface, including transmembrane transporters for ions, heavy metal and ion efflux pumps, and various other osmotic regulators. As a facultative anaerobe, it can also adapt to changes in oxygen availability and is tolerant to high temperature and pressure [44]. The microorganisms detected at the deepest depth sampled in a borehole in Finland had similar adaptations to the high salt and metal concentrations [45]. Further, some obligate fermenting microorganisms can use the osmoprotectant compounds produced by other organisms as a carbon and energy source. It was observed that the microbial community composition in 2.5 km deep shale wells in Pennsylvania

shifted, in response to increasing salt concentration associated with hydraulic fracturing of shale, to favour halotolerant bacterial and archaeal species: *Candidatus* Frackibacter, which was discovered at the site, *Halanaerobium*, *Halomonadaceae*, *Marinobacter*, *Methanohalophilus*, and *Methanlobus* [100]. All genomes had evidence of an osmoprotectant strategy, including use of the molecule glycine betaine, proposed to be produced by other microorganisms present to fuel their fermentative metabolisms [100]. Another proposed adaptation to oligotrophic deep subsurface conditions is small cell size [101]. Approximately 50% of the cells in microbial communities of groundwater collected from the Äspö Hard Rock Laboratory passed through a 0.22- $\mu\text{m}$  filter. These small cells often had genomes that were assigned to phylum *Proteobacteria*, and all had matches to known representative species reported to have cell sizes larger than 0.3  $\mu\text{m}$  [101].

Another factor known to influence subsurface microbial community composition is the surrounding geology of deep terrestrial environments. Microorganisms often make use of the molecules and ions available in the rocks they inhabit, either as electron sources or acceptors, or as sources of limiting minerals (Figure 1.6). This includes metal sulfides like pyrite [102, 103], metals such as iron and manganese and their oxides [104], silicate rocks like feldspar that provide a source of phosphorus [105, 106], and gypsum-derived sulfate [63], which are not evenly distributed in all rocks [103]. Profiles of available electron donors in subsurface ecosystems correlate with microbial community composition [107], but host rock lithology has rarely been directly linked to microorganisms living within that rock. Nonetheless, one study compared the lithology and microbial community compositions of 15 types of host rock taken from many different locations and showed that host rock lithology was a primary driver of microbial community structure [108]. A study out of the Deep Mine Microbial Observatory (South Dakota) looking at biofilms in fluid-filled fractures supported these results and suggested that the types of minerals present could be an important factor for which microorganisms colonize the rock surfaces [109]. Similarly, microbial communities within granite were dependent on elements found within the granite, especially aluminum, silica, and calcium [110]. Another study showed that aquifer fluid type (e.g., gabbro, hyperalkaline peridotite, and alkaline peridotite) was correlated with microbial community composition [111]. Although a single geochemical parameter accounted for the correlation, differing pH, Eh, and availability of carbon and electron acceptors among rock types were predicted to be key factors [111]. As

microorganisms use the minerals present in the rock, they chemically transform them. While this process has been studied in surface environments such as clay minerals in soil [112, 113], it is an important consideration in deep subsurface environments, especially when those environments will be modified, and potentially amended with non-native materials (e.g., clay, concrete), through the construction of underground repositories, such as for long-term storage of used nuclear fuel.

A recent study showed that stochastic geological activity may also play a role in microbial community structure and succession, with a stronger influence than environmental selection, for deep hard rock aquifer systems [114]. The findings suggest that geological activity causing or changing fractures, which leads to the isolation or mixing of fracture fluids, and the nutrients and microbial communities within, plays a significant role in microbial community turnover and the establishment of new microbial communities when environmental conditions and underlying geology of the rock formation remain unchanged [114]. Further understanding the factors that determine microbial community composition and drive succession in deep terrestrial subsurface environments will be critical for the planning of deep subsurface activities that could be impacted by microbial activity, such as the construction of underground repositories for used nuclear fuel storage.

### **1.5 Physical properties expected to change over the lifetime of a DGR**

The construction of a DGR will impact the natural deep subsurface environment and its naturally occurring microbial communities through the introduction of oxygen, water, space, nutrients, and exogenous microorganisms, which may initially favour growth of aerobes and facultative anaerobes [115]. Within a DGR, oxygen is eventually expected to be depleted. Initial models suggested that oxygen may persist within a DGR for several decades due to slow diffusion of oxygen into the host rock [116]. More recent models that additionally take microbial oxygen consumption into account predict that oxygen may be depleted within a few months to one and a half years of DGR closure [20, 117]. At this point, the DGR will return to reducing conditions. In the absence of oxygen, light, and readily available organic matter, chemolithoautotrophic forms of primary production common to natural deep subsurface microbial communities may be favoured.

Apart from oxygen, temperature, saturation, and swelling pressure are expected to change throughout the lifetime of a DGR. Prior to the introduction of used nuclear fuel, the subsurface at repository depth is between 10 and 17°C (Figure 1.7) [118, 119]. Once placed, used nuclear fuel, which releases energy in the form of heat, will increase the temperature of the DGR (Figure 1.7) [118, 119]. The heat at any given area in a DGR is impacted by proximity to used fuel containers, but also by the thermal conductivity of the surrounding materials (HCB, GFM, host rock). A range of temperatures are expected in the DGR both over time and spatially at any given time. At temperatures above 125°C, the electrochemical behaviour of copper changes such that corrosion occurs at an increased rate, and above a temperature of about 140°C, bentonite begins converting to the non-swelling clay illite [120]. Both situations increase the susceptibility of the system to corrosion. As such, DGRs are being designed so that the temperature within the system remains sufficiently low that the barriers are not altered or destroyed. The Canadian DGR specifically is being designed such that the temperature at the surface of the used fuel containers never exceeds 100°C [120].

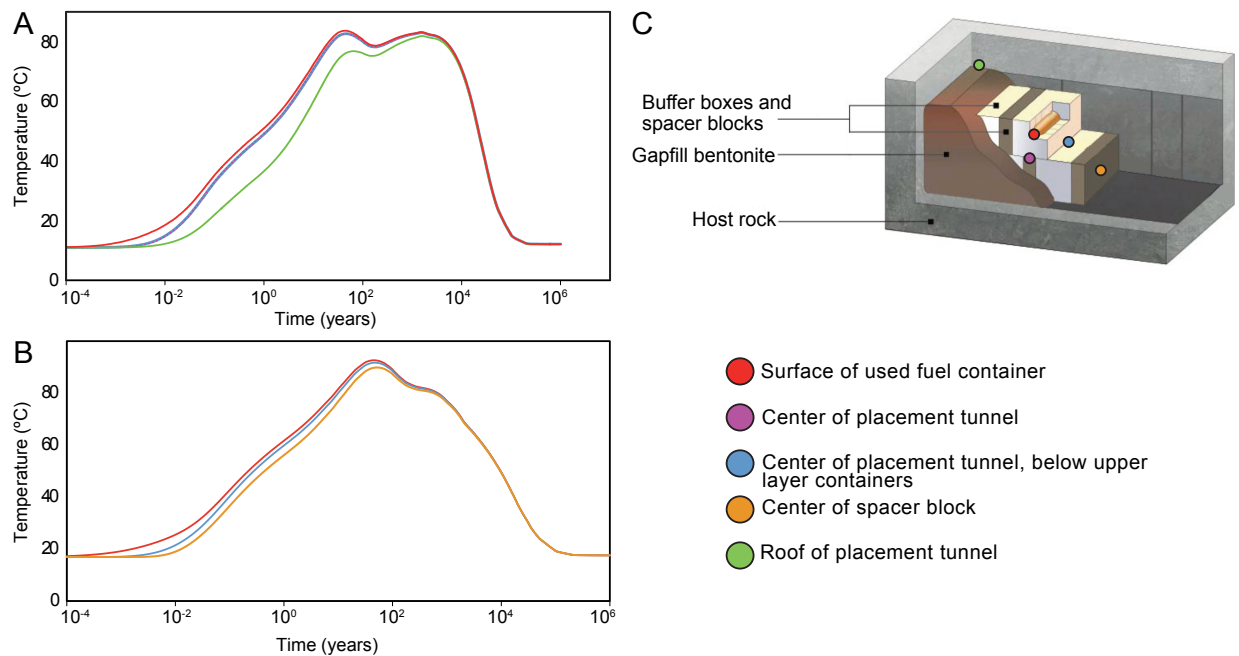


**Figure 1.7** Predicted temperature fluctuations in different locations (C) inside of a DGR hosted in a crystalline (A) or sedimentary (B) host rock over one million years after used fuel placement. Modified from several other sources [20, 118, 119].

In a DGR hosted in a crystalline rock formation, models show that the temperature at the surface of a UFC (the hottest part of the DGR) and in the highly compacted buffer boxes surrounding the UFCs will climb from about 12°C to about 50°C within the first year of emplacement and will continue to rise until the maximum temperature of 84°C is reached 45 years after emplacement (Figure 1.7). Temperatures will then remain elevated (i.e., >70°C) until about 1600 years after emplacement, at which point the temperature will begin decreasing (Figure 1.7). The bentonite at the outer edges of the placement rooms would be slightly cooler, reaching a temperature of approximately 35°C within the first year after emplacement and reaching a maximum of about 75°C about 50 years after emplacement (Figure 1.7). In a DGR hosted in a sedimentary rock formation, slightly higher temperatures are expected. The temperature at the UFC surface and in the bentonite buffer boxes surrounding the UFC would rise from about 17°C to about 50°C within the first year after emplacement and would reach a maximum temperature of 93°C, 47 years after emplacement, after which it would slowly decrease over more than 10,000 years (Figure 1.7).

The saturation of bentonite within a DGR is driven by the heat produced by used nuclear fuel. The initial saturation of the highly compacted bentonite buffer boxes and of the GFM are expected to be 64-90% and 6%, respectively [20]. As heat is produced by used nuclear fuel, water will be pushed away from the area surrounding UFCs (Figure 1.8). During this period of low saturation, bentonite will not swell to its full capacity, and so the swelling pressure within the system will be reduced, as will the rate of solute transport through bentonite [20]. As temperature decreases, moisture will move back towards the UFCs (Figure 1.8) [20]. The saturation pattern within a DGR will depend on the specific hydraulic conductivity of the host rock and is therefore expected to differ for crystalline or sedimentary host rock (Figure 1.8) [20]. In the case of a crystalline host rock, full saturation may occur in fewer than 100 years, whereas it is expected to take more than 1000 years in a sedimentary rock-hosted DGR (Figure 1.8) [20].

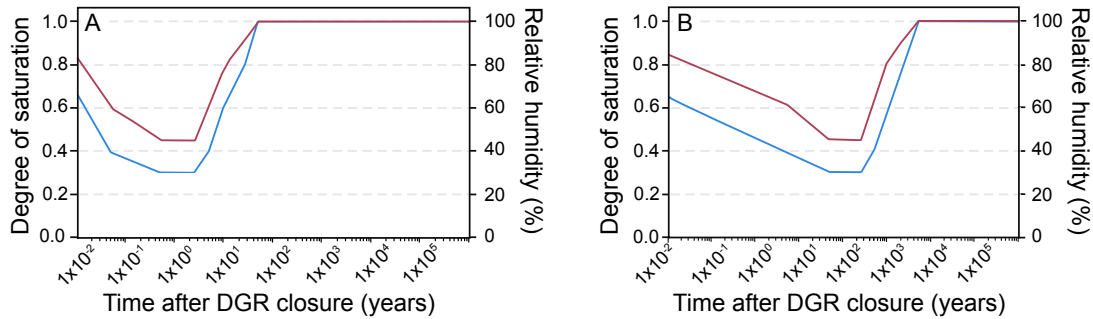


**Figure 1.8** Predicted saturation (blue) and relative humidity (red) of a DGR hosted in a crystalline (A) and sedimentary (B) host rock over a one-million-year timespan after DGR closure. Modified from elsewhere [20].

### 1.6 Study of microbiology in the context of long-term used nuclear fuel storage

Different microorganisms are adapted to a wide variety of physical conditions, including oxygen concentration, temperature, water availability, pressure, pH, and salinity, which are all expected to fluctuate throughout the lifetime of a DGR (Figure 1.9). Due to the potential detrimental role that some microorganisms could have within a DGR, it is important to study their viability, growth, and activity within bentonite. There are two broad approaches to study bentonite microbiology in this context. These include relatively short-term microcosms, which allow for the control of specific physical properties of interest, and natural analogue studies, which are limited in terms of control of conditions but have naturally existed for DGR-relevant timeframes.

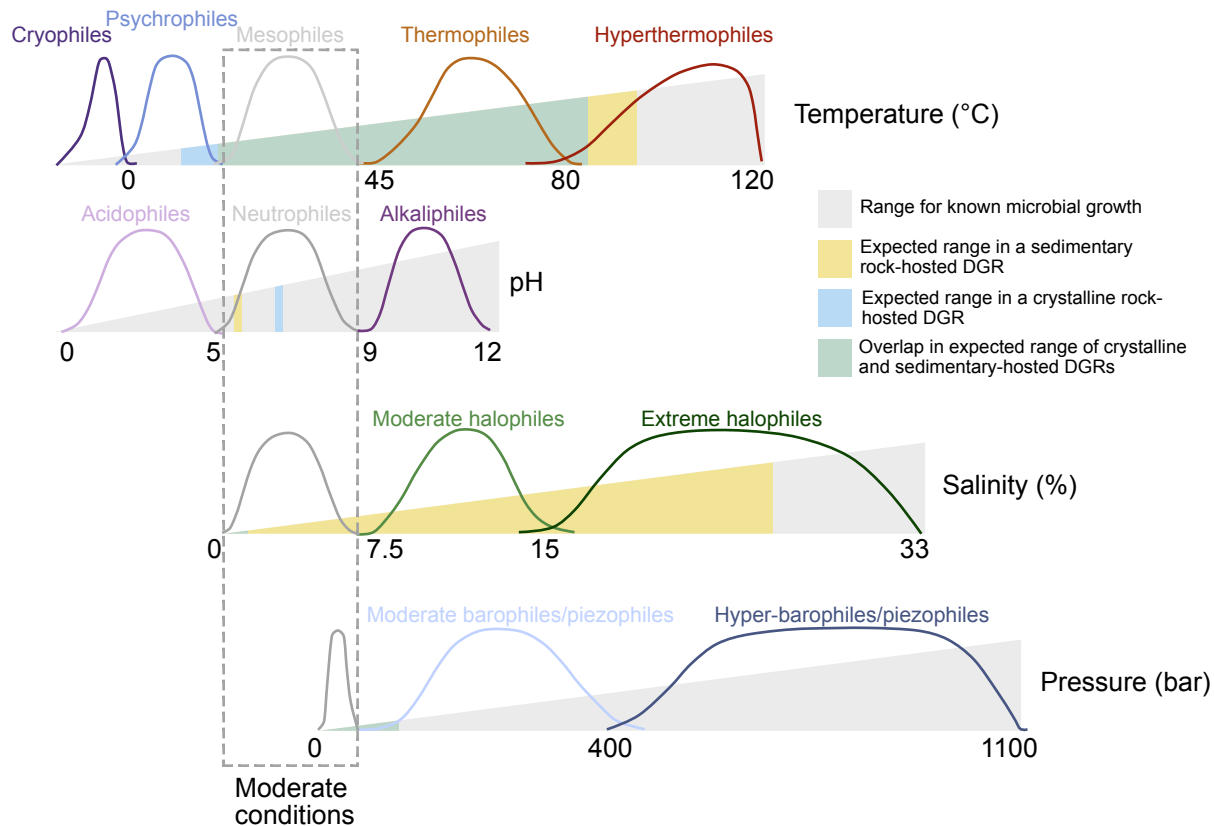


**Figure 1.9** Range of temperature, pH, salinity, and pressure that can support known microbial life, and the ranges expected in a crystalline or sedimentary rock-hosted DGR. Salinity range is based on an initially low salinity of as-received bentonite to a maximum salinity based on that of reference groundwater. Maximum pressure is based on the swelling pressure of ~100 bar associated with the planned dry density of highly compacted bentonite buffer boxes of 1.7 g/cm<sup>3</sup>. Figure adapted from a previous source [121], with overlay of DGR-relevant temperature [118, 119], pH [2], salinity [20], and pressure [122, 123].

### 1.6.1 Study of microbiology in bentonite compacted to varying dry densities

Due to the potential detrimental impact that some microorganisms could have on DGR longevity, a goal in designing a DGR is to identify conditions that prevent microbial growth. In the Canadian design, the objective is to identify the minimum bentonite dry density necessary to suppress microbial growth in bentonite. Initial work looking at powdered Wyoming MX-80 bentonite saturated with distilled water under oxic conditions identified a dry density of 1.6 g/cm<sup>3</sup> as a conservative dry density target at or beyond which microbial growth is confidently suppressed (i.e., the abundance of culturable heterotrophs and SRB do not increase beyond that of the initial powdered bentonite starting material) [123]. The same study measured the abundance of culturable microorganisms in bentonite plugs compacted to lower dry densities of

1.3 g/cm<sup>3</sup>, in which similar abundances of microorganisms were cultured compared to the starting bentonite material, and 0.8 g/cm<sup>3</sup>, in which the abundance of culturable aerobic heterotrophs was orders of magnitude higher than that of the starting bentonite material. Given that this experiment only ran for up to 90 days, a second experiment was prepared using the same methods and bentonite compressed to 2.0 g/cm<sup>3</sup> and was conducted for eight years to confirm that microbial growth suppression was maintained over longer time frames [124]. In this second experiment, an increase in abundance of culturable microorganisms was only observed for aerobic heterotrophs in the outer layer (and not the inner layer) of the clay plug (i.e., the layer in contact with the inside of the pressure vessel). Growth of culturable anaerobic heterotrophs and SRB was suppressed in both layers of the clay plug. A follow-up experiment demonstrated that high dry density had a static effect rather than killing microorganisms. Although abundance of culturable microorganisms did not increase beyond that of the starting material in bentonite compacted to a dry density of 1.6 g/cm<sup>3</sup>, when that same clay plug was then allowed to expand to fill a larger volume (i.e., the dry density was effectively increased), the abundance of culturable microorganisms increased, demonstrating the importance of not only achieving but also maintaining a high bentonite dry density in a DGR [125].

The combined effect of bentonite dry density and anoxic conditions was tested in two *in situ* experiments using Wyoming bentonite-packed modules deployed in boreholes. For one experiment, a significant increase in the abundance of culturable aerobic and anaerobic heterotrophs compared to the bentonite starting material was observed after 1 and 1.5 years of incubation in a 1.25 g/cm<sup>3</sup> (i.e., low) dry density module in all tested layers of the module, whereas microbial growth was suppressed in a parallel 1.55 g/cm<sup>3</sup> (i.e., high) dry density module. By the 5.5-year time point, abundances of culturable heterotrophs were the same or lower than those of the starting material regardless of bentonite dry density [126]. For the second experiment, in contrast, there was a significant difference in abundance of culturable heterotrophs between the inner and outer layers of the module, with a significant increase in abundance of culturable aerobic heterotrophs compared to the starting material in the outer layer of a 1.25 g/cm<sup>3</sup> (i.e., low) dry density module after 5 years of *in situ* incubation, but suppression of microbial growth in the inner layer of this low dry density module and in both layers of a parallel 1.50 g/cm<sup>3</sup> (i.e., high) dry density module [127]. In both experiments, the abundance of culturable SRB never increased in abundance beyond the starting material regardless of dry

density or incubation time [126, 127]. Similarly, in two studies using a type of Spanish bentonite compacted to a high dry density of  $1.7 \text{ g/cm}^3$ , survival of culturable SRB was observed after one year of anoxic incubation at  $30^\circ\text{C}$  [128] or at  $60^\circ\text{C}$  [129], but final culturable abundances of SRB remained within an order of magnitude of the abundance of culturable SRB in the clay starting material [128, 129]. Together, current literature suggests that under both oxic and anoxic conditions, maintenance of a sufficiently high dry density (i.e.,  $1.50\text{--}1.70 \text{ g/cm}^3$ ) suppresses microbial growth, whereas heterotrophs in lower dry density bentonites (i.e.,  $\leq 1.25 \text{ g/cm}^3$ ) tend to initially increase in abundance prior to eventual suppression of growth given sufficient time. Regardless of oxygen and dry density conditions, the abundance of culturable SRB in bentonite has not been observed to increase in abundance over time.

### **1.6.2 Study of microbiology in bentonite exposed to different temperatures**

Within a year after placement of used nuclear fuel, the temperature within the DGR is expected to surpass the temperature range for mesophilic microbial growth (Figure 1.9), which is usually the focus of study; most experiments are conducted between room temperature and  $30^\circ\text{C}$  [118, 119]. Although barrier components (copper, bentonite, host rock) are expected to be physically unaltered by the range of temperatures expected in a DGR, microbial communities within and surrounding the DGR would be expected to change in response to temperature, and there are different microorganisms adapted to survive in all temperatures expected within a DGR (Figure 1.9).

Because a DGR is expected to experience a range of elevated temperatures for thousands of years after emplacement, it is important to study the effect of temperature on microbial community abundance and composition in bentonite clay. To date, very few studies have explored microbial community composition and abundance in bentonite clay exposed to high temperature. In one study, bentonite collected from El Cortijo de Archidona (Spain) was dried (moisture content of 12%) and compacted to a high dry density of  $1.7 \text{ g/cm}^3$ . After a year of anoxic bentonite plug incubation at  $60^\circ\text{C}$ , aerobic heterotrophs, anaerobic heterotrophs, and SRB grew in cultures incubated at  $30^\circ\text{C}$  but not  $60^\circ\text{C}$ , suggesting that at least some mesophilic microbial community members were able to withstand the harsh conditions and proliferate once conditions became more favourable (e.g.,  $30^\circ\text{C}$  with excess water and nutrients), but that thermophilic microorganisms either did not proliferate within the bentonite clay plugs or were

not culturable [129]. When bentonite was first spiked with potential electron donors and acceptors (acetate, lactate, and sulfate), there was a small increase in the abundance of culturable mesophilic microorganisms throughout the one-year 60°C incubation. Even in bentonite that was first treated with a Tyndallization (i.e., repeated heating and cooling) process prior to compaction, culturable microorganisms were still detected after the year-long high temperature incubation, further demonstrating that some microorganisms can withstand very harsh temperature exposure, at least for a relatively short time, and proliferate once temperature and dry density are reduced [129]. A second study explored microbial activity in a slurry of Bavarian bentonite and Opalinus porewater solution, also over a year of anoxic 30 or 60°C incubation. In this study, there was evidence of *Desulfosporosinus*-driven sulfate reduction in 30°C microcosms amended with hydrogen, but not for amended 60°C microcosms or unamended 30°C or 60°C microcosms [130]. Although sulfate reduction was undetectable for 60°C microcosms, DNA sequencing suggested that a change in microbial community composition occurred. In microcosms with or without amendment with electron donors, 16S rRNA gene profiles were dominated by sequences associated with the thermophile *Caldinitratiruptor microaerophilus*, which was not a dominant member of the Bavarian bentonite prior to microcosm setup [130].

### 1.6.3 Study of microbiology in natural analogue samples

Laboratory and *in situ* experiments are ideal for testing how microorganisms in bentonite respond to DGR-relevant conditions like dry density, swelling pressure, and temperature. One major limitation of lab-scale experiments is a lack of realistic timeframes. Even if microbial activity is low in a DGR, DGRs are being designed to persist for hundreds of thousands of years, and so the cumulative activity of microorganisms over this time scale must be considered. Although modelling can help, this is not easily achieved with laboratory or *in situ* experiments. To overcome this limitation, laboratory-scale experiments can be coupled to the study of natural analogue sites. Certain sites have been identified as suitable analogues for different DGR barriers, including bentonite, copper, and rock. In this context, natural analogues are naturally occurring bentonite deposits that have existed for times comparable to or longer than the projected lifetime of a DGR. Natural analogues serve as a “snapshot” of microbial communities that have had the chance to establish in subsurface bentonite clay over long timeframes, and could be used to infer the microbial communities that may be expected in DGR bentonite in the long term. The study of natural analogues comes with its own set of challenges, including that it

is challenging to find a natural analogue site that adequately mimics all conditions of a DGR. Additionally, microbial growth and activity that may occur during the early phases of a DGR, when conditions (e.g., temperature, saturation, and swelling pressure) are expected to fluctuate, cannot be assessed readily. Nonetheless, coupling lab-scale and *in situ* experiments with the study of natural analogues can help to achieve a more holistic view of the range of microbial population sizes, community compositions, and activities expected to occur in a DGR.

Two natural analogue sites have been identified and studied in Japan: the Kawasaki bentonite mine in Miyagi prefecture, and the Tsukinuno bentonite mine in Yamagata prefecture. Kawasaki mine is a strip mine with layers of Ca-type bentonite that are 25-30 m thick [131]. The Tsukinuno mine includes 35 layers of Na-type bentonite within the mining area, up to 7 m thick and located between parallel strata of shale [132]. These sites are suitable as analogues for a DGR because they consist of ancient bentonite with relatively high concentrations of montmorillonite. The Tsukinuno mine is additionally relevant given that the bentonite at this site is a Na-type (swelling) bentonite, as are powdered and gapfill bentonites proposed for use in DGRs. In two cores drilled from each site, the highest counts of culturable aerobic and anaerobic heterotrophs on R2A agar were nearest the drilling mouth and decreased to less than  $10^2$  cells/gdw by 1 m into the cores [131]. In this study, the authors measured several geochemical parameters for comparison to culture counts. Montmorillonite content, cation exchange capacity, degree of saturation, leached cation composition, and pH were not correlated with abundances of culturable heterotrophs. Moisture content and dry density were both weakly to moderately correlated with counts of culturable aerobic heterotrophs, anaerobic heterotrophs, and CFDA-AM cell counts [131].

A second study at the Tsukinuno mine built on initial research by incorporating cultivation of SRB as well as DNA-based techniques that allow for the study of microorganisms beyond those capable of growing on the selected growth medium under a specific set of conditions [133]. In this study, abundances of culturable heterotrophs were low (i.e., between  $10^1$  and  $10^4$ ) in the ten hand samples studied, and culturable SRB were only detected in a single sample at the lowest abundance measurable by the MPN assay used [133]. However, the samples were taken from tunnel walls exposed to oxygen, which is not the bentonite condition expected to host populations of putatively anaerobic SRB. The 16S rRNA gene profiles were often quite

different between samples but were generally dominated by a single genus within the *Proteobacteria*, *Actinobacteria*, or *Euryarchaeota* phyla. It was suggested that at least a portion of the DNA detected in 16S rRNA gene profiles could be relic as there was a poor overlap between sequences associated with cultured microorganisms and taxa detected directly from the clay via DNA-based techniques [133].

Several bentonite deposits in the Almeria region of Spain have been studied for their physical properties, including their high compaction densities which make them suitable as both sealing materials and as analogue samples [134]. Of these, three have been further studied from a microbiology perspective: El Cortijo de Archidona, El Toril, and Los Trancos [135]. These bentonite samples had montmorillonite concentrations between 71 and 96% [135]. Similar to the study of bentonite cores in the Tsukinuno mine, no SRB were detected and microbial communities varied between samples, but were generally dominated by sequences associated with *Proteobacteria*, *Bacteroidetes*, and *Actinobacteria*. A range of chemical species and geochemical parameters were measured to attempt to explain differences in microbial community composition between samples, but no correlation was found [135].

Although not bentonite specifically, the analogue site perhaps most similar to a DGR is Cigar Lake, because it serves as an analogue to multiple components of the multi-barrier DGR design. Cigar Lake is a 1.3-billion-year-old high-grade uranium deposit located within the sandstone of the Athabasca Basin (Saskatchewan, Canada). At this site, uranium ore is situated 450 m below the surface, at the interface of sandstone and underlying Canadian shield basement rock, and is surrounded by a clay-rich halo between 5 and 30 m thick [136]. Nearest the uranium deposit, the clay is a mixture of illite and chlorite, with concentrations of clay minerals between 80-100%, and it shifts to a mixture of illite and kaolinite with decreasing overall concentrations of clay minerals as distance from the uranium deposit increases [136]. There is no radioactivity from the uranium deposit detectable at the surface, demonstrating the capacity of bentonite and overlying rock, even without additional engineered barriers, to effectively contain radionuclides over very long timeframes [136]. From a microbiology perspective, Cigar Lake provides a unique opportunity to study microorganisms present in ancient subsurface clay with elevated levels of uranium, and the associated groundwater. Initial study of microorganisms within Cigar Lake groundwater samples demonstrated the presence of microorganisms via cultivation on R2A

agar and microscope counts using DAPI stain [137]. Much of the microbiological information that exists for this site comes from a large-scale multidisciplinary study of the site that was conducted through a collaboration between Atomic Energy of Canada Limited (AECL) and the Swedish Nuclear Fuel and Waste Management Company (SKB) between 1989 and 1994. In this study, modelling was used to predict the limiting nutrient in the groundwater and estimate the maximum microbial community size that could be supported by the concentration of that nutrient. For both groundwater samples taken from the clay halo, nitrogen was identified as the limiting nutrient and a maximum microbial population density of between  $5.4 \times 10^5$  and  $5.4 \times 10^7$  cells/mL was estimated. Measurements of microbial abundance (i.e., ATP assay, biological activity reaction tests, or direct microscopic cell count) all yielded estimates of total microbial abundance lower than the proposed carrying capacity of the environment, on the order of  $10^3$  to  $10^5$  cells/mL [136]. For most samples, the number of anaerobes measured was upwards of an order of magnitude greater than the number of aerobes. Sulfate-reducing bacteria were detected in at least one groundwater sample from all layers of the sandstone, the clay zone, the ore, and the basement, but were not detected in the shallower overburden or surface samples [136].

Although the study of the microbiology of natural bentonite analogues began more than 30 years ago, relatively few natural bentonite deposits have been studied, and even fewer using modern DNA-based analyses that allow for a more in-depth assessment of microbial community composition than cultivation alone. More recent studies that did make use of DNA-based techniques identified sequences most often associated phyla *Proteobacteria* and *Actinobacteria* [133, 135]. Comparisons between geochemical parameters and microbial community abundance or composition have so far yielded weak or no correlations, except for moisture content and swelling pressure that can be weakly correlated with microbial abundance [131, 135]. One commonality among the few previous natural analogue bentonites studied is that SRB are typically undetectable or at very low abundance. Existing natural analogue data suggest that a future DGR could host relatively low abundance microbial communities, at least once DGR conditions stabilize, potentially dominated by the *Proteobacteria* and *Actinobacteria* phyla and largely associated with low SRB abundances.

## 1.7 Thesis outline

A focus of existing microbiology research in the context of long-term used nuclear fuel storage has been on the effect of dry density on microbial viability. A minimum dry density for suppression of microbial growth (i.e., 1.6 g/cm<sup>3</sup>) has been proposed for Wyoming MX-80 bentonite, one of two types of bentonite in the Canadian DGR design, under oxic conditions [123], but similar thresholds do not exist for GFM, the second type of bentonite in the Canadian DGR design, under oxic or anoxic conditions. Further, little is known about microbial growth that may occur during the saturation phase when swelling pressure is relatively low, or about how microbial communities in bentonite respond to increased temperature. Although several bentonite natural analogue sites have been studied [131, 133, 135, 136], there remain too few natural analogue data to make robust predictions about the microbial community size and composition that may be expected of a future DGR. The goal of this thesis was to address these gaps in the literature.

This thesis includes five chapters focusing on the microbiology of different types of bentonite exposed to repository-relevant conditions. Chapter 2 aimed to identify a minimum dry density necessary to suppress microbial growth in Wyoming MX-80 and GFM bentonite under oxic conditions and included early sampling time points prior to complete saturation to study the microbial community composition and abundance throughout the saturation phase. Chapter 3 had a similar focus as Chapter 2, but the pressure vessel experiments were conducted under anoxic conditions. Chapter 4 explored the responses of bentonite microbial communities to seven different DGR-relevant temperatures, using both as-received and hydrated bentonite. Chapter 5 describes the study of a bentonite natural analogue site in Japan, which builds on previous research conducted at the same site by analyzing pristine core samples with a combination of cultivation, DNA, and geochemical analyses. Throughout Chapters 2-4, as well as in previous studies, there are unresolved observations related to the growth of aerobic heterotrophs and SRB in bentonite. Consequently, Chapter 6 consists of several related experiments that aimed to address some of these uncertainties. The first set of experiments was related to growth of aerobic heterotrophs in bentonite. Specifically, they aimed to test whether observed increases in abundance of culturable aerobic heterotrophs in hydrated bentonite compared to as-received material are due to microbial growth or rather a recovery from the VBNC state, and why measured abundances of aerobic heterotrophs in replicate bentonite samples are often highly

variable. The second set of experiments was related to observed growth of SRB in Chapter 3. These experiments aimed to compare SRB medium and experimental setup conditions used in Chapter 3 to those used in previous studies where growth of SRB was not observed, and further test the oxygen sensitivity of representative bentonite SRB.

## **Chapter 2: Impact of dry density and incomplete saturation on microbial growth in bentonite clay for nuclear waste storage**

### **2.1 Introduction**

Nuclear power is an important global source of energy and an integral component of a fossil-fuel independent future. After a fuel bundle is removed from a nuclear reactor, it remains highly radioactive and must be stored safely until radiation levels return to those of naturally occurring uranium ore, essentially indefinitely [138]. To ensure isolation of used nuclear fuel from the environment, several countries are in the process of designing a deep geological repository (DGR). While the details vary from country to country, generally DGR designs propose to seal fuel bundles within long-lived used fuel containers (UFC), which will be surrounded in a buffer material, often bentonite clay, that has low hydraulic conductivity and is inhospitable to microorganisms. These UFCs and the buffer backfill will be stored in DGR placement rooms, which will be situated deep belowground in suitable host rock. In the Canadian DGR design, UFCs will be placed into highly compacted bentonite (HCB) buffer boxes which will be placed underground as a package. After emplacement, the space between the HCB boxes and host rock will be packed with a granulated “gapfill material” (GFM) bentonite [139]. Both the HCB and GFM are types of processed bentonite which are formed from the application of high isostatic pressure (~100 MPa) or a roll compacting/milling process, of powdered Wyoming MX-80 bentonite, respectively. Wyoming MX-80 bentonite is a sodium bentonite with a mineral composition of approximately 81% montmorillonite, 3.4% muscovite, 3.5% plagioclase, 0.6% pyrite, and 3.0% quartz [7].

To ensure the long-term sustainability of a DGR, there are several important safety considerations. Within the context of microbiology, an important concern is the potential for microbiologically influenced corrosion (MIC), which is a process where corrosion of a surface is initiated or propagated by corrosive compounds produced by microbial metabolism. The process of MIC is well studied for a wide range of environments where metals, such as pipes, tubes, and bridge foundations [140–144], or non-metals, including concrete sewer systems [145] are placed underground or underwater. For a DGR, MIC is important because it could lead to corrosion of the copper-coated used fuel containers via sulfide induced corrosion. Sulfate-reducing bacteria (SRB) can contribute to MIC because sulfide reacts directly with copper and copper oxides to

produce copper sulfides and hydrogen gas, potentially fuelling additional SRB activity. Previous studies show that SRB are dominant members of anoxic subsurface microbial communities when sulfate is available [14, 16–19, 67, 71, 72, 146] and they have additionally been identified in the commercially available MX-80 bentonite proposed for use in a future DGR design [9, 10]. Further, Canadian groundwaters relevant to DGR placement contain sulfate [147]. Although SRB are a focus of microbiology research related to long-term nuclear waste storage, due to their potential to cause MIC, other microorganisms could also potentially influence a DGR, for example through the formation of gases that could lead to permeability fissures in clay or host rock [71].

A focus of microbiology research related to long-term nuclear waste storage has involved identifying conditions necessary to suppress microbial growth. In addition to nutrient availability, which is expected to be low in the subsurface and for engineered barrier components (e.g., clay, metal), factors expected to influence microbial growth in bentonite clay are water activity, dry density, and swelling pressure [123, 148–150]. Previous research demonstrated that the abundance of culturable heterotrophs and SRB did not increase when powdered Wyoming MX-80 bentonite samples were packed to a dry density of  $1.6 \text{ g/cm}^3$  when pore water (i.e., water used to saturate the bentonite plugs) salinity was  $\leq 50 \text{ g L}^{-1}$ , which corresponded to a water activity of 0.96 and a swelling pressure of 2 MPa [123]. Stable phospholipid fatty acid (PLFA) abundances reported in this same study led to an interpretation that, rather than being killed, microorganisms persisted as dormant cells or inactive endospores [123]. Another study investigating powdered Wyoming MX-80 bentonite clay under pressure showed similar non-lethal suppression of microbial growth at a dry density of  $1.7 \text{ g/cm}^3$  [124]. An additional study made use of bentonite-containing modules placed in boreholes, showing that bentonite-associated microbial communities were stable within the first year of incubation [151]. However, the added variable of saturation with groundwater that had its own microbial community, separate from the bentonite, makes interpretations of these results more challenging.

Although dry densities necessary to suppress microbial growth in Wyoming MX-80 bentonite clay have been proposed, how transferrable these same dry densities are to other product forms (e.g., GFM) such that the same suppression of microbial growth is achieved is not as well known. Previous studies have also focused on longer term incubations, which miss the

changes in microbial abundance and community composition during the process of saturation that can be observed using a temporal short-term assessment approach. Although the saturation process is relatively short in a small pressure vessel, it is expected to span for 50-5000 years in a DGR [20] and thus any increased microbial abundance and activity during this time could have important implications in DGR evolution. Because DNA is difficult to extract from bentonite clay in sufficient quantities for downstream analyses, DNA-based techniques were rarely used to directly profile and quantify clay-associated microbial communities until recently. However, applying DNA-based techniques directly to clay from pressure vessels is an important complement to culture-dependent analyses for comprehensive characterization of the types of microorganisms capable of growing in bentonite at various dry densities.

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

## **2.2 Materials and methods**

### **2.2.1 Oxidic pressure vessel setup and disassembly**

Pressure vessels were filled with bentonite clay that was supplied by the Nuclear Waste Management Organization (NWMO) and compacted to dry densities of 1.25 and 1.6 g/cm<sup>3</sup> for gapfill bentonite and to dry densities of 1.1, 1.4, and 1.6 g/cm<sup>3</sup> for powdered bentonite (Figure 2.1). One batch of powdered bentonite was used for all powdered bentonite pressure vessels. Two different batches of GFM were used for gapfill pressure vessels; all 1.6 g/cm<sup>3</sup> 1-, 3-, and 18- month vessels, one of the two 1.6 g/cm<sup>3</sup> 6-month vessels, and one of the two 1.25 g/cm<sup>3</sup> 6-month vessels were filled with gapfill material one, and the remaining vessels were filled with gapfill material two. The mineral composition of each as-received bentonite sample used to fill pressure vessels was determined using quantitative X-ray diffraction (XRD) analysis performed by Activation Laboratories. The XRD analysis was performed in a Bruker D8 Endeavour

diffractometer equipped with a Cu X-ray source, at 40 kV and 40 mA over a  $2\theta$  range of 4–70°, with a step size of 0.02° and a time per step of 0.5 seconds, a fixed divergence slit at 0.30°, and sample rotation at 15 rpm. A portion of each sample was dispersed in distilled water and the <25  $\mu\text{m}$  size fraction was separated by gravity settling for clay speciation. The PDF4 minerals database (International Centre for Diffraction Data) was used for mineral quantification and the Rietveld method [152] was used to determine the quantity of each crystalline mineral. The proportion of clay minerals was calculated using the ratios of their basal-peak areas.

The diameter and height of the clay plugs were 5.3 cm and 14.8 cm, respectively. The water activity and moisture content of the gapfill bentonite prior to saturation in a pressure vessel were measured to be 0.38 and 6.1% respectively. The water activity and moisture content of the powdered bentonite were 0.42 and 10.5% respectively. The pressure vessel casings were fabricated from 316 stainless steel, with detachable stainless-steel filter stones and end caps. Each pressure vessel was lined with a polytetrafluoroethylene (PTFE) inner casing and all vessels were sterilized by exposure to 99.5% (v/v) acetone then to 70% (v/v) ethanol. Pressure vessels were assembled and disassembled in an oxic environment using a hydraulic press and sterilized bentonite sampling tools, and were sealed for the duration of incubation. To accelerate saturation of bentonite clay with water during the experiments, vessels were connected to an AZURA P6.1L HPLC pump and pressurized to 10 MPa with naturally aerated Type I water (resistivity = 18.2  $\text{M}\Omega/\text{cm}$ ) from a purification system (Thermo Scientific). The bentonite was not amended with any nutrients for microorganisms.

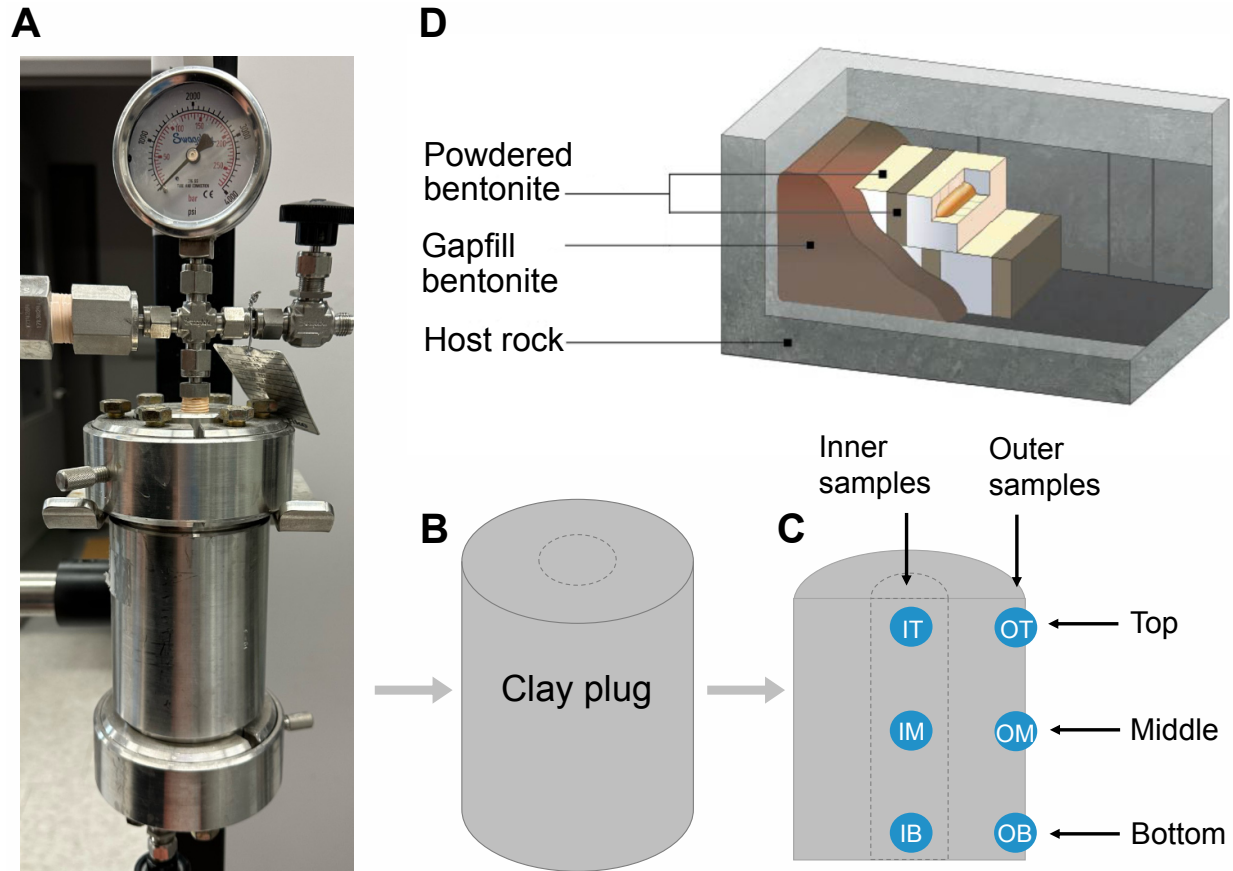


**Figure 2.1** Photograph of a pressure vessel (A) and schematic of the clay plug within the pressure vessel (B) and the samples layers sampled: top, middle, and bottom of the inner core and the outer layer (C). Pressure vessels were filled with powdered or gapfill bentonite clay, the former being the raw material which was processed to produce highly compacted bentonite and gapfill material, which are both components of the theoretical design of a deep geological repository (D). Modified from a previous publication [20].

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

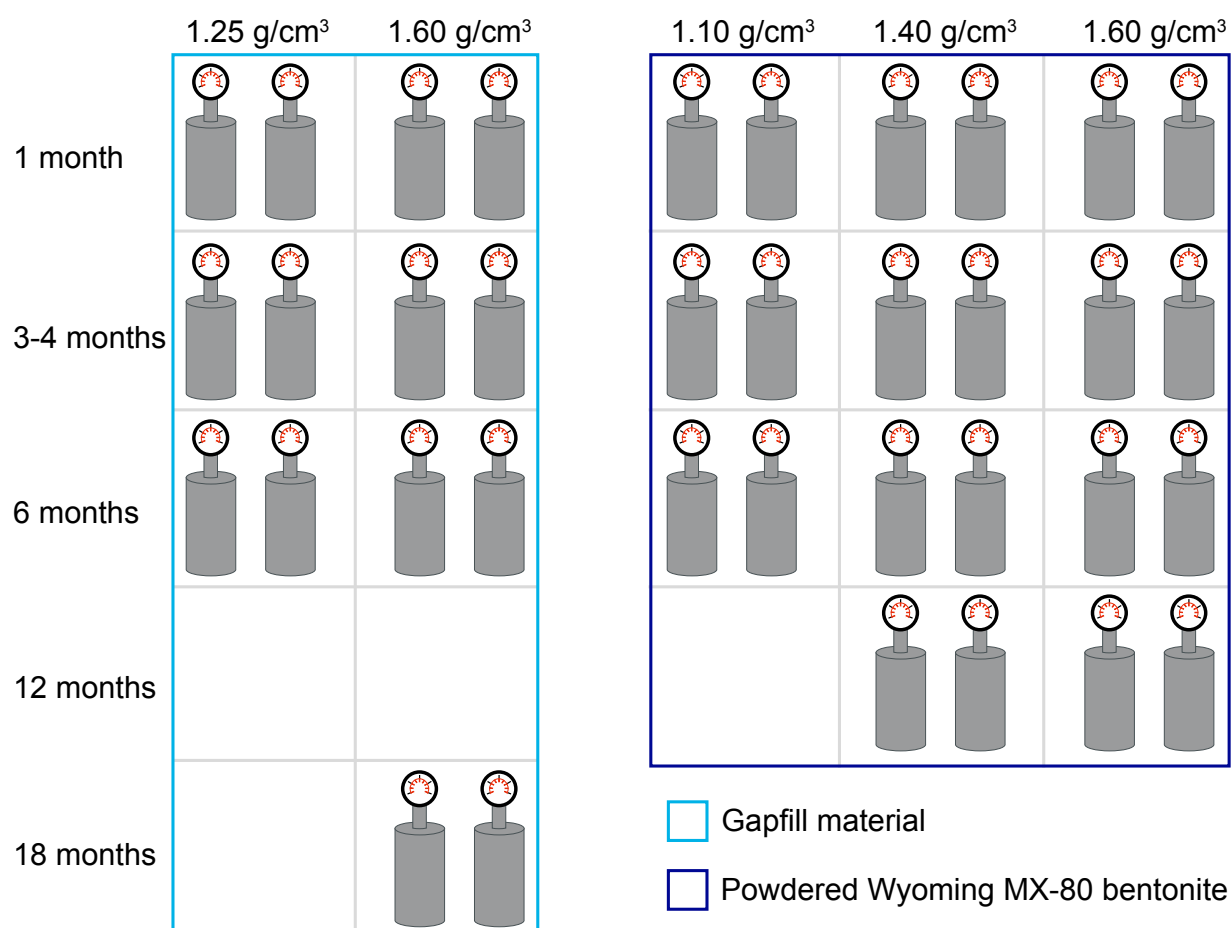


**Figure 2.2.** Experimental design schematic for anoxic pressure vessels.

### 2.2.2 Oxic pressure vessel bentonite sample storage and preparation

After pressure vessel disassembly and bentonite coring at the University of Western Ontario (Figure 2.1A, 2.1B), bentonite samples were shipped to the University of Waterloo on ice and transferred to 4°C storage upon arrival. Sampling of bentonite took place next to a Bunsen burner on sterile aluminum foil. The outer surfaces (i.e., surfaces in contact with the inside of the pressure vessel) of the three outer-layer bentonite samples (i.e., top, middle, and bottom, based on position in the pressure vessel) were removed with a sterile scalpel and are referred to throughout as “outer layer” samples (Figure 2.1C). The outer surfaces of the inner core bentonite samples were removed with a sterile scalpel to avoid potential contamination introduced during the coring process. The inner core was then split into three separate samples (i.e., top, middle, and bottom, based on position in the pressure vessel). These core samples were cut into smaller pieces using a sterile scalpel and are referred to throughout as “inner layer” samples.

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

### **2.2.3 Water activity and moisture content measurements of oxic pressure vessel samples**

A WP4C Water Potential Meter (Decagon) was used to measure the water activity of each bentonite sample using the fast mode of the instrument. The same bentonite aliquots were then weighed and placed in an oven at 110°C for 24-48 hours. Samples were weighed again when removed from the oven and the difference in weight, before and after baking, was used to calculate sample moisture content (equation 2.1).

$$2.1 \quad \text{Moisture content} = \frac{\text{mass before baking} - \text{mass after baking}}{\text{mass before baking}} \times 100\%$$

### **2.2.4 Cultivation of oxic pressure vessel bentonite**

A 2 g aliquot of each bentonite sample was added to 18 mL of phosphate-buffered saline (PBS; 8 g NaCl, 0.2 g KCl, 1.44 g Na<sub>2</sub>HPO<sub>4</sub>, and 0.24 g KH<sub>2</sub>PO<sub>4</sub> in a final volume of 1 L Type I water, with pH adjusted to 7.4 using HCl) solution in a 50 mL conical tube, then vortexed at low speed for 30 minutes at room temperature (approximately 21°C). This clay suspension is referred to as “dilution A” throughout. After vortexing, two ten-fold serial dilutions were made in PBS from dilution A and are referred to as “dilution B” and “dilution C”. Dilution A (100 µL) was added to six Reasoner’s 2A (R2A; M1687, HiMedia Laboratories) agar plates and spread using a sterile glass spreader until dry. Dilutions B and C (100 µL) were added to three R2A agar plates each and spread using the same method as dilution A. Three dilution A, B and C plates were incubated under oxic conditions in a 30°C incubator for 5-6 days. The remaining three dilution A plates were placed in a vacuum chamber (BVV3GSSW, BVV), which was flushed five times with a 90% N<sub>2</sub> and 10% CO<sub>2</sub> gas mixture. Each vacuum chamber contained a GasPak EZ anaerobe sachet (BD), to help remove residual oxygen present after flushing, and an anaerobic indicator strip (BD), to verify anoxic conditions throughout the incubation. Vacuum chambers were placed in a 30°C incubator for 28 days. After incubation, colonies were counted on each

plate and the number of colony forming units per gram (CFU g<sup>-1</sup>) was calculated for each sample. A sterile foam-tipped applicator (Puritan) was used to swab colonies from each plate and swabs were stored at -20°C until DNA extraction. This culture biomass was collected for DNA sequencing for the purpose of identifying taxa that are viable in the clay samples. Not all viable microorganisms in the clay can necessarily grow in the cultures, but comparing the culture 16S rRNA gene profiles to the clay profiles can provide information about the viability of the microorganisms associated with the clay profiles, which would not otherwise be possible through sequencing of clay DNA alone.

One mL of dilution A, B, and C for each bentonite sample was added to each of 5 test tubes (i.e., 15 test tubes total for each bentonite sample) filled with 9 mL of SRB enrichment medium (M803, HiMedia Laboratories). Test tubes were placed in a vacuum chamber and the vacuum chambers were flushed as described above and incubated under anoxic conditions at 30°C. After 28 days of incubation, positive tubes were identified by the presence of black ferrous sulfide precipitate and the most probable number (MPN) method was used to estimate the number of SRB present per gram of original clay sample [153]. From each positive SRB tube, 1 mL was collected and centrifuged at 8000 x g for 10 minutes to pellet cells. Supernatants were discarded and cell pellets stored at -20°C prior to DNA extraction.

Using the moisture content measured for each sample, each value of CFU g<sup>-1</sup> and MPN g<sup>-1</sup> was converted to a value of CFU or MPN per gram dry weight (gdw) (equation 2.2).

$$2.2 \quad \text{abundance gdw}^{-1} = \frac{\text{abundance g}^{-1}}{(100 - \text{moisture content}) \times 0.01}$$

An ANOVA was used to test the effect of dry density, pressure vessel layer, and incubation time on abundance of culturable SRB, aerobic heterotrophs, and anaerobic heterotrophs. Each sample group included the six replicate pressure vessel samples for a given time point, layer (inner or outer), and dry density. After a significant *p* value was observed for the initial ANOVA, Tukey's HSD test was performed with a *p* value threshold of 0.1 to determine which time points had samples with significantly higher abundances of culturable SRB, aerobic heterotrophs, or anaerobic heterotrophs compared to the starting material.

### **2.2.5 DNA extraction from bentonite, swabs, and culture biomass**

Genomic DNA was extracted from a 2 g aliquot of each bentonite sample using the DNeasy PowerMax Soil Kit (Qiagen), from control swabs using the DNeasy PowerSoil Kit (Qiagen), and from culture biomass using the DNeasy UltraClean Kit (Qiagen). A “kit control” containing no sample was included for each extraction batch. The manufacturer’s instructions were followed with the following changes. After addition of lysis solution, PowerBead Tubes were incubated at 70°C for 10 minutes for the PowerSoil and UltraClean extractions, and at 65°C for 30 minutes for the PowerMax extractions. This was followed by beadbeating using a FastPrep-24 Classic Instrument (MP Biomedicals) at 5.5 m/s for 45 seconds for the PowerSoil and UltraClean Kit extractions, and beadbeating using a MM 400 Mixer Mill (Retsch) at 30 Hz for 10 minutes for the PowerMax extractions. Final elution volumes for the PowerSoil, UltraClean, and PowerMax extractions were 60 µL, 50 µL, and 2 mL, respectively. The PowerMax DNA extraction method used here was validated previously for clay samples [154]. DNA samples were stored at -20°C until further analysis.

### **2.2.6 Quantitative PCR of oxic pressure vessel bentonite samples**

Bacterial 16S rRNA genes were quantified in duplicate for each bentonite sample using the CFX96 Real-Time PCR Detection System (Bio-Rad). The total volume of each PCR was 15 µL, which consisted of 1x Sso Advanced Universal SYBR Green Supermix (Bio-Rad), 0.3 µM each of forward primer 341F and reverse primer 518R [155], 7.5 µg of bovine serum albumin (BSA), and 2 µL of template DNA. The following thermocycler conditions were used: 98°C initial denaturation for 3 minutes, followed by 40 cycles of 98°C for 15 s and 55°C for 30 s. For the standard curve, the V3-V5 region of the *Thermus thermophilus* 16S rRNA gene was previously cloned into the pUC57-Kan plasmid, flanked by M13F/R primer binding sites. The M13 primers were used to amplify the 16S rRNA gene fragment, which was then gel purified and stored at -20°C prior to use for the qPCR standard curve. Each aliquot of the standard DNA was thawed and quantified using the Qubit dsDNA High Sensitivity Assay Kit (Invitrogen) with 4 µL of sample per Qubit quantification immediately prior to preparing the qPCR standard curve.

Bacterial 16S rRNA gene copy numbers were calculated using linear regression of the standard curve based on the Qubit quantification of the standard. The starting quantity (SQ)

results from the qPCR assay for each bentonite DNA sample were converted to values of 16S rRNA gene copies  $\text{gdw}^{-1}$ , and SQ values of no template controls (NTCs) on the same plate were subtracted (equation 2.3).

### 2.3 16S rRNA gene copies $\text{gdw}^{-1}$

$$= \frac{(\text{average SQ sample} - \text{average SQ of NTCs}) \times 2000 \mu\text{L elution in DNA extraction}}{2 \mu\text{L template in qPCR reaction} \times 2 \text{ g clay in DNA extraction}}$$

#### 2.2.7 Amplicon sequencing of bentonite, swab, and culture biomass samples

The V4-V5 region of the 16S rRNA genes from each genomic DNA extract were amplified in triplicate in 96 well plates with universal primers 515F-Y[156] and 926R [157], modified to contain a unique six-base index sequence and flow cell binding and sequencing sites [158]. The PCR setup was performed in a PCR hood (AirClean Systems) with ISO 5-HEPA-filtered air and surfaces treated with UV light for 15 minutes before each use. All tubes, PCR water, and BSA were UV treated (302 nm) for 20 minutes on a transilluminator (ProteinSimple). The final volume of each PCR was 25  $\mu\text{L}$  and consisted of 1x ThermoPol Buffer (New England BioLabs), 0.2  $\mu\text{M}$  of each primer, 200  $\mu\text{M}$  dNTPs, 15  $\mu\text{g}$  BSA, 0.625 U hot start Taq DNA polymerase (New England BioLabs), and 1  $\mu\text{L}$  of template DNA. The following thermocycler conditions were used: 95°C initial denaturation followed by 35 or 50 cycles of 95°C denaturation for 30 s, 50°C annealing for 30 s, and 68°C extension for 1 min, followed by a final extension at 68°C for 7 minutes. Bentonite DNA samples were amplified for 50 cycles, and culture DNA samples were amplified for 35 cycles. Negative PCR controls containing no template DNA (NTCs) were included in each 96 well plate to test for cross contamination between wells of the plate and NTCs were also prepared in tubes separate from the plate for each master mix to test for contamination.

Triplicate uniquely indexed PCR products were visualized on a 1% agarose gel containing ethidium bromide and were then pooled and quantified on a 1% agarose gel containing GelRed (Biotium). Based on gel quantification, equimolar volumes of each amplicon were pooled into a single tube. Negative PCR controls (in plate and out of plate NTCs) and PCR-amplified DNA kit controls were added to the pool (5  $\mu\text{L}$  each), even if amplification was not detected on the agarose gel. Pooled 16S rRNA gene amplicons were excised from a 1.5%

agarose gel and purified using the Wizard SV Gel and PCR Clean-Up System (Promega). The purified library was quantified using the Qubit dsDNA High Sensitivity Assay Kit (Invitrogen) and by qPCR. The library was denatured, diluted to 8 pM, and mixed with 15% PhiX control v3 (Illumina) following Illumina guidelines (Document No. 15039740 v01). All samples were sequenced across five runs on a MiSeq System using a 2 x 250 cycle MiSeq Reagent Kit v2 (Illumina) with an average cluster density of 799,000 clusters mm<sup>-2</sup> and average clusters passing filter of 83.8%.

### **2.2.8 Oxidic pressure vessel sequence analysis**

Sequences were demultiplexed using Local Run Manager version 4.0.0.1769 and analyzed using Quantitative Insights Into Microbial Ecology 2 [159] and Divisive Amplicon Denoising Algorithm 2 (DADA2; Callahan et al., 2016), managed by Automation, Extension, and Integration Of Microbial Ecology (AXIOME3; Min et al., 2021). Paired-end reads were imported into Quantitative Insights into Microbial Ecology 2 (QIIME2) and DADA2 was used to remove low quality reads and primer sequences, and to truncate forward and reverse reads to 250 bases. The DADA2 pipeline was additionally used to denoise and dereplicate paired-end reads and generate amplicon sequence variants (ASVs). Taxonomy was assigned to ASVs using a naïve Bayes classifier pre-trained with SILVA database release 138 [162]. Potential contaminant ASVs were identified using the Decontam R package [163] with a threshold of 0.5. For each MiSeq System run, clay samples and culture samples were processed through Decontam separately. The list of potential contaminants produced by Decontam was analyzed manually and likely contaminants were removed from the ASV table; this final ASV table was used for all subsequent analyses (Supplementary File S1). Samples were normalized to 5000 reads using the scaling with ranked subsampling (SRS) package in R [162], and this normalized ASV table was used to compute Bray-Curtis distances in Python (Figure 2.7).

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

## 2.3 Results

### 2.3.1 Mineral composition of as-received bentonite used in pressure vessels

The mineral composition of each as-received bentonite sample used to fill pressure vessels was determined using quantitative XRD analysis (Figure 2.1). Montmorillonite, the primary clay mineral in bentonite, varied from 73.7 to 87.0%. Other minerals were detected at similar abundances in the three bentonite samples.

**Table 2.1** Mineral composition (wt %) of the three bentonites used. “n.d.” indicates that the mineral was not detected. and when detected in amounts too low to quantify is reported as “trace.”

|                       | Montmorillonite<br>( $(\text{OH})_2\text{Si}_2\text{Al}_2\text{O}_{10} \cdot n\text{H}_2\text{O}$ ) | Quartz<br>( $\text{SiO}_2$ ) | Cristobalite<br>( $\text{SiO}_2$ ) | Plagioclase<br>( $\text{NaAlSi}_3\text{O}_8$ -<br>$\text{CaAl}_2\text{Si}_2\text{O}_8$ ) | K feldspar<br>( $\text{KAlSi}_3\text{O}_8$ ) | Mica ( $\text{XY}_2\text{-}$<br>$3\text{Zr}_4\text{O}_{10}(\text{OH})_2$ ) | Clinoptilolite<br>( $(\text{Na}_2,\text{K}_2,\text{Ca})_3\text{Al}_6\text{Si}_8\text{O}_{72} \cdot 24\text{H}_2\text{O}$ ) | Calcite<br>( $\text{CaCO}_3$ ) | Gypsum<br>( $\text{CaSO}_4 \cdot 2\text{H}_2\text{O}$ ) |
|-----------------------|-----------------------------------------------------------------------------------------------------|------------------------------|------------------------------------|------------------------------------------------------------------------------------------|----------------------------------------------|----------------------------------------------------------------------------|----------------------------------------------------------------------------------------------------------------------------|--------------------------------|---------------------------------------------------------|
| Gapfill<br>Material 1 | 83.4                                                                                                | 1.0                          | n.d.                               | 7.4                                                                                      | 3.7                                          | 1.8                                                                        | n.d.                                                                                                                       | 2.7                            | trace                                                   |
| Gapfill<br>Material 2 | 73.7                                                                                                | 1.6                          | 2.7                                | 10.4                                                                                     | 2.9                                          | 2.0                                                                        | 6.7                                                                                                                        | trace                          | trace                                                   |
| Powdered<br>bentonite | 87.0                                                                                                | 2.3                          | n.d.                               | 5.3                                                                                      | 2.8                                          | 1.2                                                                        | n.d.                                                                                                                       | 1.4                            | trace                                                   |

### 2.3.2 Microbial abundance and growth

Prior to saturation in a pressure vessel, both gapfill and powdered bentonite had similar mineral compositions (Table 1), had relatively low abundances of culturable heterotrophs and SRB, and had 16S rRNA gene abundances greater than the combined numbers of culturable microorganisms detected (Figure 2.3). The number of 16S rRNA gene copies derived from qPCR, culturable aerobic heterotrophs derived from plate counts, and culturable SRB derived from MPN tests were not significantly different between the powdered starting material and the gapfill starting material (Tukey’s HSD,  $p < 0.10$ ).

Within the first month of pressure vessel incubation, there was a significant increase in the average abundance of culturable aerobic heterotrophs in the outer layer of each pressure vessel at all dry densities less than  $1.6 \text{ g/cm}^3$  (Tukey’s HSD,  $p < 0.10$ ). The number of culturable aerobic heterotrophs measured in each of the  $1.6 \text{ g/cm}^3$  dry density pressure vessels after one month of incubation varied. Although the average number of culturable aerobic heterotrophs across the six sampling locations of these  $1.6 \text{ g/cm}^3$  pressure vessels was not significantly greater than the

abundance in the starting materials, several individual samples had significantly higher abundances of culturable aerobic heterotrophs than the starting material. After the increase in abundance of culturable aerobic heterotrophs within the first month of pressure vessel incubation, the average abundance decreased to the level of the starting material in subsequent time points for the 1.4 and 1.6 g/cm<sup>3</sup> dry density pressure vessels (Figure 2.3). The early time points where an increased abundance of aerobic heterotrophs was observed were also associated with incompletely saturated bentonite. Although saturation was not measured directly, the moisture content and water activity of the six sampled locations within each pressure vessel varied at early time points, especially in pressure vessels with a high dry density (Figure 2.3, Figure 2.4

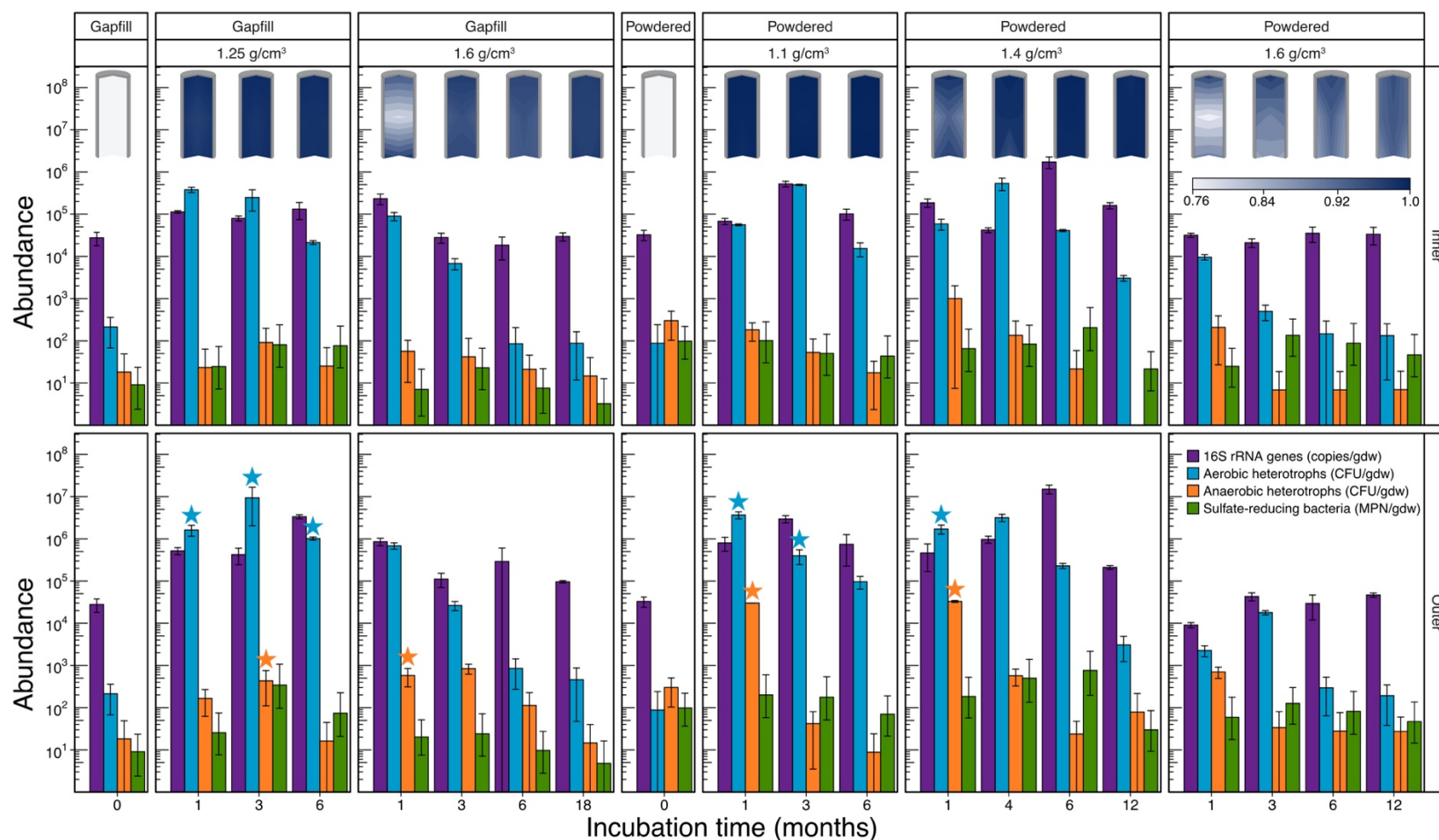


**Figure 2.3.** Abundance of 16S rRNA gene copies/gdw, aerobic and anaerobic heterotroph CFU/gdw, and SRB MPN/gdw for inner core (top) and outer layer (bottom) bentonite samples. Measures of CFU/g less than 2000 are below the limit of quantification for this method (as fewer than 20 colonies on a plate is considered statistically insignificant). Error bars represent the standard deviation of replicates for values of 16S rRNA gene copies/gdw and CFU/gdw, and represent average 95% confidence intervals specified by the MPN table for values of MPN/gdw. The contour plots (top of the figure) represent the water activity throughout the pressure vessel, generated using the average measured water activity values from the six sampled locations within each pressure vessel. The stars above bars representing culture counts indicate average abundances that were significantly greater than the corresponding abundance in the initial gapfill bentonite (Tukey's HSD,  $p < 0.1$ ).

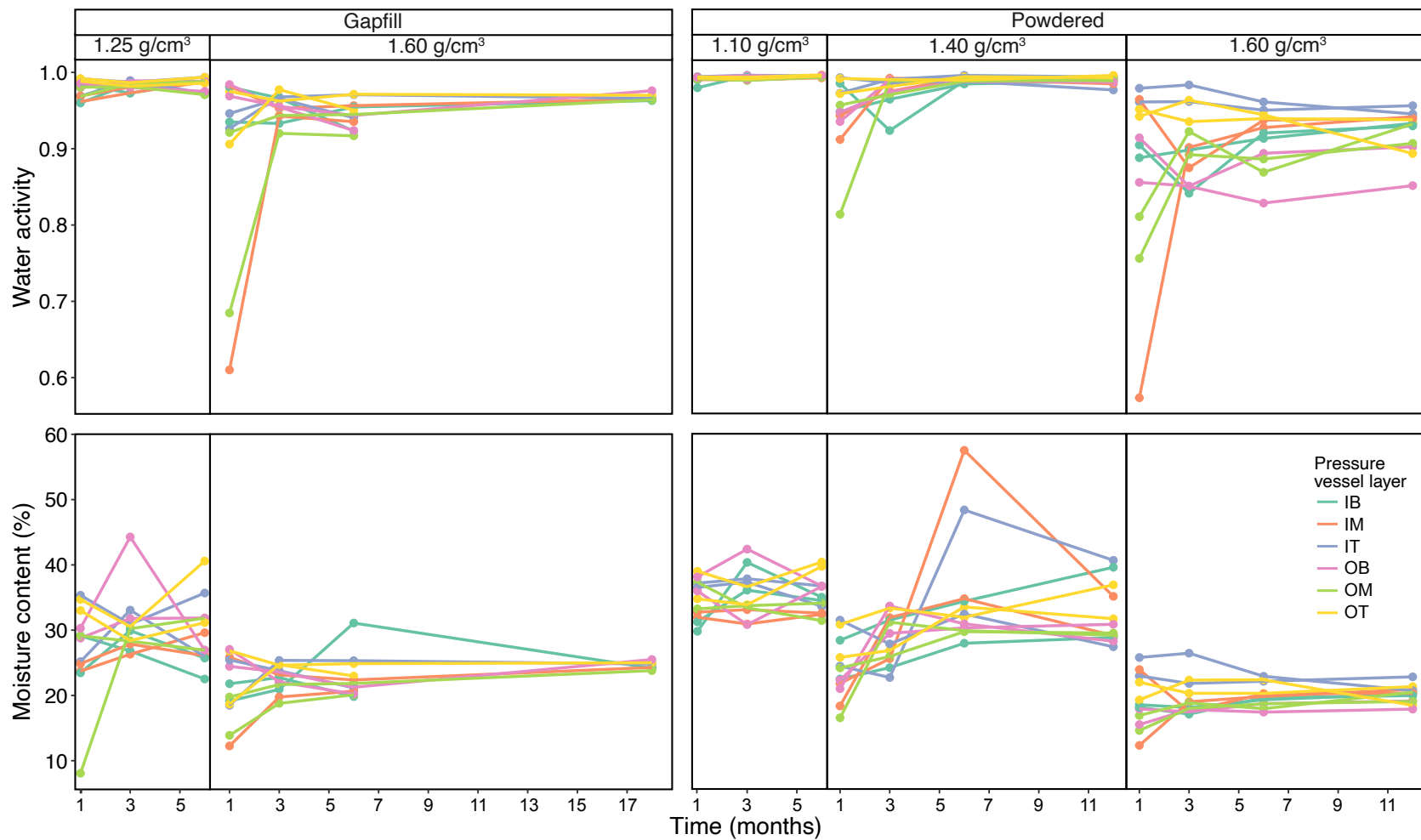


**Figure 2.4** Temporal changes in water activity and moisture content of each pressure vessel layer.

Fewer anaerobic heterotrophs were cultured from the pressure vessel bentonite samples compared to aerobic heterotrophs, and most of these values were below the limit of quantification (2000 CFU/g), which corresponds to 20 colonies counted on each replicate agar plate). The average number of anaerobic heterotrophs cultured from the outer layer of the 3-month 1.25 g/cm<sup>3</sup> and from the 1-month 1.6 g/cm<sup>3</sup> gapfill pressure vessels were significantly higher than starting material (Tukey's HSD,  $p < 0.10$ ). The average increases in the abundance of anaerobic heterotrophs in the outer layer of both the 1-month 1.1 g/cm<sup>3</sup> and 1.4 g/cm<sup>3</sup> powdered bentonite pressure vessels were both significantly higher than the starting material (Tukey's HSD,  $p < 0.10$ ). By the 6-month time point, the average numbers of culturable anaerobic heterotrophs in the powdered and gapfill pressure vessels returned to the same number as for starting material (Tukey's HSD,  $p < 0.10$ ). For most samples, MPN estimates for SRB abundances were lower than culturable heterotroph abundances (Figure 2.3). The average number of culturable SRB never exceeded that of the as-received bentonite starting material. (Tukey's HSD,  $p < 0.10$ ).

### **2.3.3 Microbial characterization of pressure vessel clay samples**

Very few taxa were detected in more than one replicate of the powdered bentonite starting material (Figure 2.5), reflecting a low biomass and heterogeneous starting material. The genera detected in more than one replicate were *Bryobacter*, *Nocardioides*, *Bacillus*, PIR4 lineage, and *Thiobacillus*, with *Thiobacillus* being the only genus detected in all replicates. The gapfill bentonite starting material also had replicate 16S rRNA gene profiles with few overlapping taxa detected (Figure 2.6). Genera detected in more than one replicate were *Amycolatopsis*, *Arthrobacter*, *Glycomyces*, *Micrococcaceae*, *Nocardioides*, *Streptomyces*, *Comamonadaceae*, and *Paracoccus*. Lack of consistency among replicates could be due to low read counts for certain replicates or could reflect heterogeneity in the distribution of microorganisms in the clay starting material. Microbial community profiles associated with the starting material were distinct from the one-month time point samples for all dry densities and both clay types, after which similar dominant taxa were detected (Figures 2.3 and 2.4).

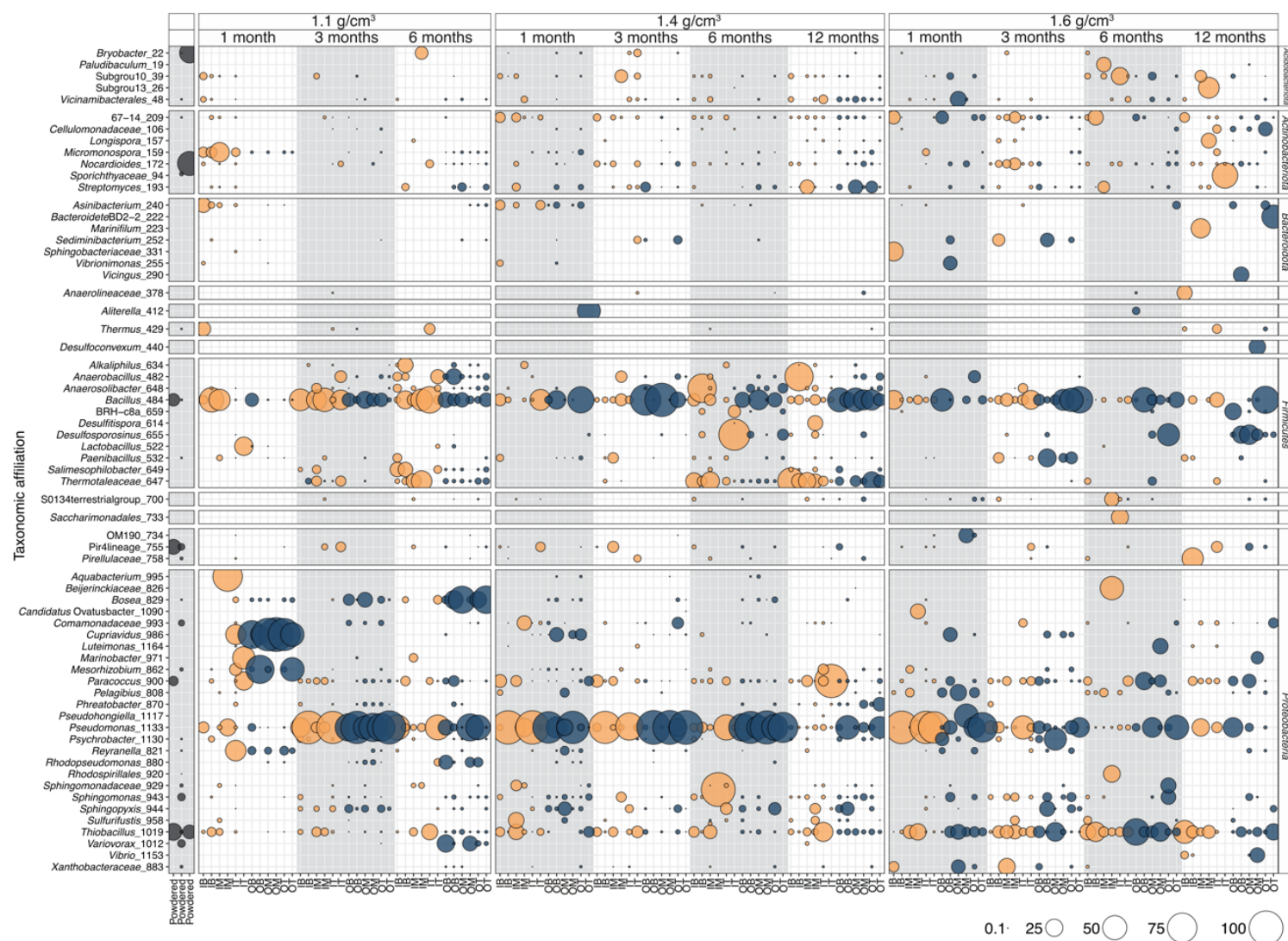


**Figure 2.5** Bubble plot showing the 16S rRNA gene profiles of the powdered bentonite pressure vessel samples. Only genera at or above 15% relative abundance are shown. IB, IM, IT, OB, OM, and OT (x-axis) refer to the location in the pressure vessel (inner bottom, inner middle, inner top, outer bottom, outer middle, outer top; see Figure 1). Bubbles are coloured by inner layer (orange) and outer layer (blue). Pressure vessel target dry density and incubation time are indicated at the top of the figure.

Microbial community profiles associated with the starting material were distinct from the one-month time point samples for all dry densities and both clay types, after which similar dominant taxa were detected (Figures 2.3 and 2.4). Most pressure vessel samples had high relative abundances of ASVs associated with *Bacillus* and *Pseudomonas*, especially the powdered bentonite pressure vessel samples. Sequences associated with other genera of primarily aerobic heterotrophs, including *Cupriavidus* and *Streptomyces*, were also detected in several samples, especially from the 1.1 and 1.25 g/cm<sup>3</sup> dry density pressure vessels at relative abundances greater than the starting material (Figures 2.3 and 2.4).

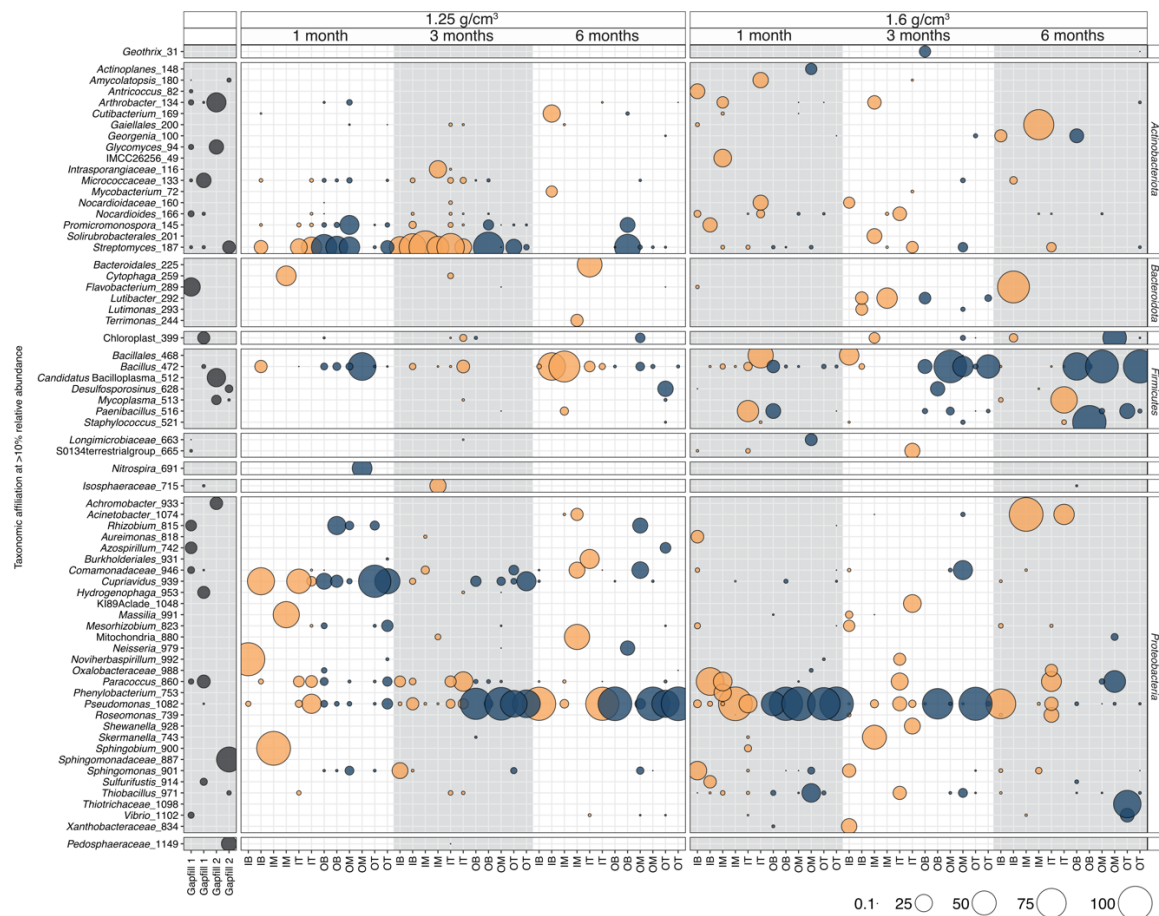


**Figure 2.6** Bubble plot showing the 16S rRNA gene profiles of the gapfill bentonite pressure vessel samples. Only genera at or above 10% relative abundance are shown. IB, IM, IT, OB, OM, and OT (x-axis) refer to the location in the pressure vessel (inner bottom, inner middle, inner top, outer bottom, outer middle, outer top; see Figure 1). Bubbles are coloured by inner layer (orange) and outer layer (blue). Pressure vessel target dry density and incubation time are indicated at the top of the figure.

The only genera of SRB detected in powdered bentonite pressure vessel samples at a minimum relative abundance of 10% were *Desulfosporosinus*, *Desulfitispora*, and

*Desulfoconvexum*, which were detected in 20, 1, and 1 sample, respectively (Figure 2.5). *Desulfosporosinus* was the only genus of SRB detected in more than one gapfill pressure vessel sample and was the only putative SRB present at higher than 10% relative abundance in any sample (Figure 2.6). The only other genus of putative SRB found in both gapfill and powdered bentonite was *Halodesulfovibrio*, which was detected in one gapfill and one powdered bentonite pressure vessel sample at 1.8% and 0.58% relative abundance, respectively. The remaining genera of SRB detected in a single powdered pressure vessel sample each were affiliated with *Desulfobacca*, *Desulfoconvexum*, *Desulfuromonadaceae*, *Desulfuromonas*, *Desulfitobacterium*, *Desulfurispora*, and *Desulfitibacter*, which were each detected at relative abundances less than 1%. Additional genera of SRB detected in a single gapfill pressure vessel sample each were *Desulfobacter*, *Desulfatitalea*, and *Desulfovibrio*, which were detected at relative abundances between 0.39% and 8.82%.

#### **2.3.4 Microbial characterization of pressure vessel culture samples**

The 16S rRNA gene profiles for anaerobic heterotroph cultures were dominated by very few ASVs, mostly from the genera *Pseudomonas*, *Cupriavidus*, *Deftia*, and *Staphylococcus* (Figure 2.7). The 16S rRNA gene profiles of aerobic heterotroph and SRB cultures were dominated by sequences associated with genera similar to those detected in the clay samples from direct DNA extraction and sequencing. The most abundant genera detected in these cultures were *Pseudomonas* and *Bacillus* (Figure 2.7). Because of how SRB biomass was collected, and that most tubes positive for SRB were from the lowest dilutions, it is likely that at least a portion of the DNA sequenced from these cultures came from the clay itself rather than the microorganisms that grew in the culture.

The dominant genus detected in SRB cultures was *Desulfosporosinus*, which was represented by 120 ASVs in the 16S rRNA gene profiles of 70% of the SRB cultures at an average combined relative abundance of 29%. Three other genera of known SRB were detected in the SRB culture 16S rRNA gene profiles: *Desulfurispora*, which was represented by 2 ASVs and was detected in 11% of SRB cultures at an average relative abundance of 0.5%, *Desulfotomaculum*, which was represented by 5 ASVs and was detected in 5% of SRB cultures at an average relative abundance of 0.6%, and *Desulfallus*, which was represented by three ASVs and was detected in a single SRB culture at a relative abundance of 9%. In total, 35 out of the

140 positive SRB culture tubes had no known SRB detected in the 16S rRNA gene profiles. Several of these samples had very few sequences due to low biomass, and likely represent culture tubes that had relatively small amounts of black precipitate and consequently low relative abundances of SRB.

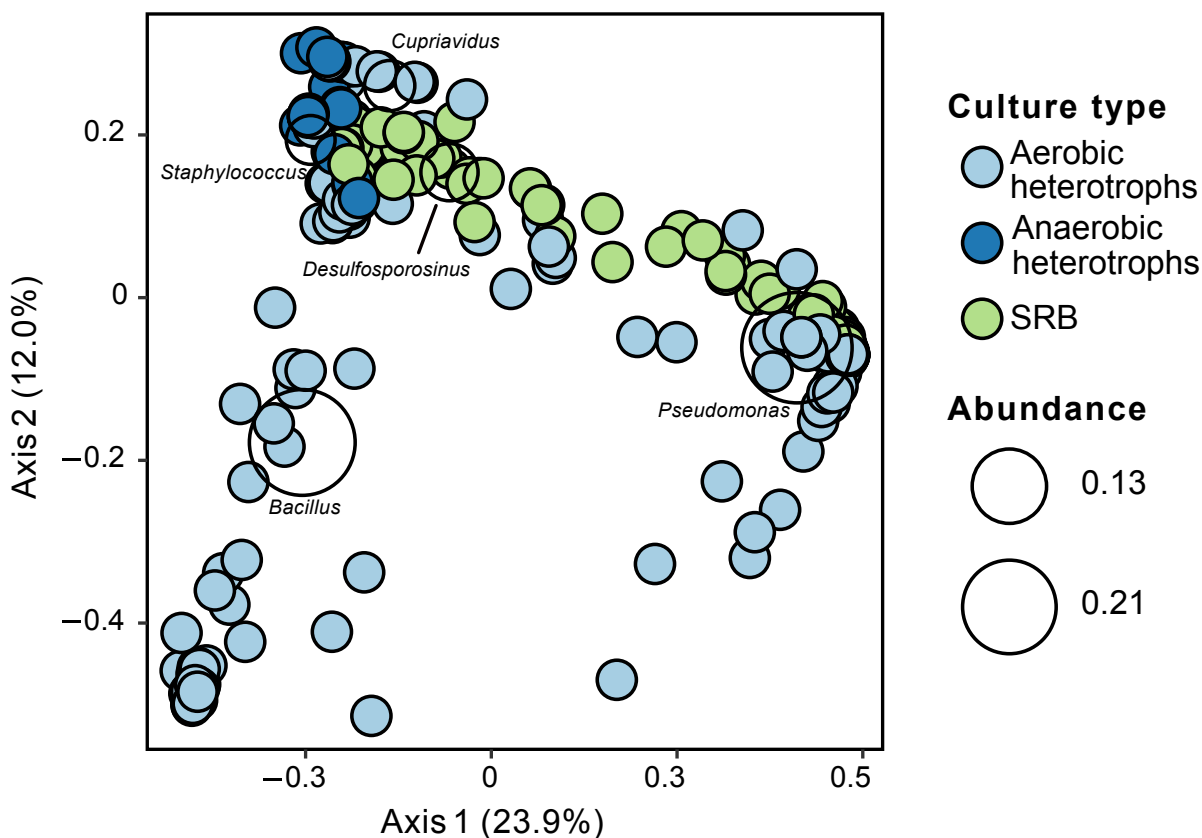


**Figure 2.7** Principal coordinate analysis (PCoA) biplot based on Bray-Curtis distances of SRB, aerobic heterotrophs, and anaerobic heterotroph 16S rRNA gene profiles. Genera greater than or equal to 5% relative abundance are displayed as clear circles. Samples were normalized to 5000 reads using the scaling with ranked subsampling (SRS) package in R.

## 2.4 Discussion

An initial (i.e., by the one-month time point) increase in average abundance of culturable aerobic heterotrophs was observed in all pressure vessels with dry densities less than  $1.6 \text{ g/cm}^3$ , and in several individual samples from the  $1.6 \text{ g/cm}^3$  pressure vessels (Figure 2.3). The variation in moisture content and water activities of the six sampled locations within the  $1.6$  and  $1.4 \text{ g/cm}^3$  density one- and three-month time points indicates that these high dry density pressure vessels were not fully saturated at the one, three, and possibly six-month time points (Figure 2.3 contour plots, Figure 2.4). As such, the results indicate that the initial increase in abundance of culturable

microorganisms for the high dry density samples likely occurred as water became available prior to complete pressure vessel saturation, when the pressure within the pressure vessel was still relatively low (Figure 2.3). A previous study using powdered MX-80 bentonite clay in pressure vessels showed a suppression of microbial growth at measured dry densities of at least 1.6 g/cm<sup>3</sup>, defined as no increase in the abundance of culturable microorganisms throughout the 40-90 day experiment [123]. This difference may be explained by the smaller size of clay plugs in that study (2-2.5 x 1.6 cm) that would be expected to saturate more quickly than those used in the present study (5.3 x 14.8 cm). For the Canadian design, a DGR is expected to take 50-5000 years to fully saturate, depending on whether the host rock is crystalline or sedimentary [20], and so these results suggest that a similar increase in abundance of heterotrophs following the saturation front, in areas with available water and below-target swelling pressure, may be expected. By the final time point, the average abundance of culturable aerobic heterotrophs was statistically the same as the starting material in 1.4 and 1.6 g/cm<sup>3</sup> dry density pressure vessels, suggesting that microbial growth is eventually suppressed by a dry density of at least 1.4 g/cm<sup>3</sup>.

Although an initial growth of aerobic heterotrophs was observed in several pressure vessel samples, the results of this study do not show an increase in the abundance of SRB throughout the saturation process. Oxygen in the DGR is predicted to be consumed within 1.5 years [20], well before the DGR is fully saturated. Because known SRB are strictly anaerobic, pressure vessel experiments conducted under anoxic conditions (Chapter 3) will provide additional insight into potential pre-saturation increases in SRB abundance during the anoxic phase of the DGR.

Most pressure vessel clay samples had high relative abundances of ASVs associated with *Bacillus* and *Pseudomonas*, especially the powdered bentonite pressure vessel samples. Members of the *Pseudomonas* genus have previously been identified within bentonite profiles retrieved from borehole modules, although these were suggested to derive from the groundwater rather than clay itself [151]. Because the pressure vessels were saturated with pure water, the results of the present study suggest that viable *Pseudomonas* cells are present in both powdered and gapfill MX-80 bentonite clay and proliferate during bentonite saturation. In a previous study, *Bacillus* was the dominant genus cultured directly from dry clay samples on both oxic R2A, as well as a denitrifying bacteria enrichment medium [10]. Most *Pseudomonas* and *Bacillus* representatives are putative aerobic chemoheterotrophic bacteria and a previous study demonstrated that spore-

forming species of *Bacillus* survived better than other types of bacteria in spiked and compacted bentonite samples (Pedersen et al., 2000). Others have demonstrated that specific *Pseudomonas* representatives can withstand desiccation by producing internal solutes to increase cell turgor pressure, and montmorillonite within bentonite has additionally been shown to increase the survivability of desiccation-resistant microorganisms in low water content environments [165]. Sequences associated with other genera of primarily aerobic heterotrophs, including *Cupriavidus* and *Streptomyces*, were detected in several samples, especially from the 1.1 and 1.25 g/cm<sup>3</sup> dry density pressure vessels at relative abundances greater than the starting material. These results suggest that *Pseudomonas*, *Bacillus*, and to a lesser degree *Cupriavidus* and *Streptomyces* may be amongst the taxa expected to grow initially in a DGR as saturation occurs and while oxygen remains available.

Previous work demonstrated that most microorganisms that grew in cultures from dry as-received clay did not overlap with the dominant microorganisms detected in the 16S rRNA gene profiles of the clays [10]. However, in the present study, the dominant members of the 16S rRNA gene profiles of the culture and clay samples often did overlap. The overlap of dominant genera detected in the 16S rRNA gene profiles of the clays and the cultures suggests that the culturing methods applied are targeting the dominant microorganisms present, and that the dominant microorganisms detected in the clay 16S rRNA gene profiles are viable.

Although SRB were not dominant members of the pressure vessel clay 16S rRNA gene profiles, the genera *Desulfosporosinus*, *Desulfitispora*, and *Desulfoconvexum* were all detected at relative abundances of greater than 10% in at least one clay sample. *Desulfosporosinus* was the dominant genus of SRB detected in the SRB cultures, although genera *Desulfurispora*, *Desulfotomaculum*, and *Desulfallus* were all also detected in some SRB cultures at low relative abundance. These four genera of SRB are all spore-forming members of the *Firmicutes* phylum [166], and all but *Desulfosporosinus* are known to be thermophilic or moderately thermophilic [166–168]. The dominance of *Desulfosporosinus* in the cultures and the clays compared to the other genera could be attributed to the pressure vessel incubation (ie., room temperature) and culture (ie., 30°C) conditions that were more suited to its growth than to that of the other SRB detected. Taxa affiliated with *Desulfosporosinus* have also previously been identified as dominant SRB representatives in as-received dry bentonite and bentonite clay exposed to high

swelling pressures. Because the temperature of a DGR is expected to change, temporally and spatially, there is potential for different types of SRB to occupy different temperature niches. For example, in a study looking at the response of bentonite-associated microbial communities to heat, the dominant SRB switched from *Desulfosporosinus* to *Desulfotomaculum* before and after a 24-hour heat incubation at 90°C [169]. The four genera of SRB detected in the cultures were also detected in the 16S rRNA gene profiles of bentonite in a study where bentonite was incubated in an excess of sterile groundwater, sometimes as dominant members of the 16S rRNA gene profiles [170]. Future research should explore the viable SRB in MX-80 hydrated bentonite clays incubated at a range of DGR-relevant temperatures.

## 2.5 Conclusion

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

persisted in the pressure vessels. The next step of this research will be to conduct the experiments under anoxic conditions to investigate if SRB do increase in abundance in bentonite at high dry densities when their preferred oxygen requirements are met.

## **Chapter 3: Suppression of microbial growth by increased dry density of anoxic bentonite**

### **3.1 Introduction**

Nuclear energy is critical for achieving a fossil-fuel independent future. One consideration that must be made with this form of energy is the highly radioactive used nuclear fuel that is produced. After a fuel bundle is removed from a nuclear reactor, it must be stored safely until its radioactivity level dissipates to that of naturally occurring uranium ore (i.e., approximately a million years) [138]. To isolate used nuclear fuel from Earth's surface for such long timescales, many countries are in the process of designing deep geological repositories (DGR) for the underground storage of used nuclear fuel. The DGR design specifications vary from country to country, but generally involve identification of a suitable host rock within which used fuel, placed in an engineered used fuel container, is added to placement rooms that are eventually filled with a buffer material after emplacement.

Bentonite clay is a commonly proposed buffer material for DGR designs because it has several qualities that make it suitable for use in a DGR, including its ability to swell when hydrated, leading to a low hydraulic conductivity and a relatively inhospitable environment for microorganisms [171]. The Canadian design includes two types of bentonite. The first is a powdered Wyoming MX-80 bentonite that will be highly compacted through application of high isostatic pressure (~100 MPa) to form buffer boxes and spacer blocks. The second is a granulated gapfill material (GFM), produced from powdered Wyoming MX-80 through a roll compaction/milling process, to fill in spaces between buffer boxes/spacer blocks and the host rock. Wyoming MX-80 bentonite is a sodium bentonite that has a high concentration of the primary bentonite mineral montmorillonite, which is what gives bentonite its swelling property, as well as lower concentrations of other minerals like muscovite, plagioclase, pyrite, and quartz [7].

To ensure long-term integrity of a deep geological repository, safety must be assessed from several perspectives. In the context of microbiology, a key consideration is the potential for microbiologically influenced corrosion (MIC), a process through which corrosion is initiated or propagated by corrosive compounds produced by microbial metabolisms. The process of MIC is a widespread problem that for over a century has troubled industries that use underground or

underwater components like pipes, tubes, and bridge foundations. For a DGR, MIC is relevant to the longevity of used fuel containers, which in the Canadian design are made of carbon steel and coated in copper. Under anoxic conditions expected for most of a DGR lifetime, sulfate-reducing bacteria (SRB) are expected to dominate MIC contributions [12]. Sulfate-reducing bacteria reduce sulfate to sulfide, which can corrode copper. Not only are SRB prevalent members of deep subsurface microbial communities [11, 60, 72, 87–90], but they can be detected in commercially available bentonites proposed for use in a DGR [9, 10]. Although SRB are a focus of microbiology research in relation to DGR design, other microorganisms could impact the longevity of a DGR. For example, many microorganisms produce gas through their metabolism, which in large enough quantities could produce permeability fissures in the clay and lead to increased groundwater flow through the system [71].

Due to the potential impacts that some microorganisms could have on DGR longevity, a goal in designing a DGR is to identify conditions that prevent microbial growth altogether. For the Canadian design, the objective is to identify the minimum bentonite dry density required to suppress microbial growth. Initial work tested powdered Wyoming MX-80 bentonite saturated with distilled water under oxic conditions and identified  $1.6 \text{ g/cm}^3$  as a conservative dry density target at or beyond which microbial growth was fully suppressed (i.e., the abundance of culturable heterotrophs and SRB do not increase beyond that of the initial powdered bentonite starting material) [123]. In a more long-term (i.e., 8 years) version of this study, using bentonite compacted to  $2.0 \text{ g/cm}^3$ , microbial growth was similarly suppressed in the inside of the clay plug [124]. A more recent study (Chapter 2) built on this previous work by testing the effect of dry density on microbial growth in both powdered and gapfill bentonite, and additionally tested a range of shorter incubation times prior to complete saturation to observe microbial growth during the saturation process. In this study, after an initial increase in average abundance of culturable heterotrophs in bentonite compacted to dry densities  $<1.6 \text{ g/cm}^3$ , by the 6-month time point microbial growth was suppressed in bentonite with dry densities of at least  $1.4 \text{ g/cm}^3$ , presumably as the bentonite neared saturation and the associated target swelling pressure. The effect of dry density under anoxic conditions was tested with two in situ experiments using borehole modules packed with Wyoming bentonite. Both experiments demonstrated suppression of microbial growth within clay plugs after five years of borehole incubation when compacted to low (i.e.,  $1.25 \text{ g/cm}^3$ ) and high (i.e.,  $1.50$  or  $1.55 \text{ g/cm}^3$ ) dry density [126, 127]. In one study, this

was following an initial increase in abundance of heterotrophs in the first 1.5 years of incubation in low (but not high) dry density modules [126]. In all experiments discussed, and additionally two other studies that measured the abundance of culturable SRB but not heterotrophs in bentonite compacted to 1.7 g/cm<sup>3</sup>, the abundance of culturable SRB did not increase significantly beyond that of the starting clay material, regardless of oxygen and dry density conditions [123, 124, 126–128, 172]; Chapter 2]

Although it has been demonstrated that microbial growth can be suppressed in sufficiently compacted bentonite clay, a minimum dry density necessary to suppress microbial growth in gapfill bentonite under anoxic conditions has not yet been identified. Further, most studies have either used bentonite pre-saturated before compaction, or sufficiently long incubation times to achieve complete bentonite saturation, and thus overlook microbial growth that may occur during the saturation phase. In the context of a small clay plug exposed to a pressurized pump, the saturation process is relatively quick. However, in a DGR, the saturation phase is expected to take 50-5000 years depending on the host rock selected [20]. During the saturation phase, areas of bentonite would have access to water but experience below-target swelling pressure, thus establishing more favourable conditions for microbial growth. As such, understanding the changes in microbial community composition and abundance during the saturation phase is critical. The objective of this study, thus, is to analyze microbial community composition and abundance in gapfill bentonite compacted to various DGR-relevant dry densities and incubated for one month (i.e., early in the saturation process) to one year (i.e., nearing or at complete saturation) under anoxic conditions, with the goal of identifying the minimum dry density necessary to suppress microbial growth in anoxic gapfill bentonite.

## **3.2 Materials and methods**

### **3.2.1 Anoxic pressure vessel setup and disassembly**

Pressure vessel casings were made of 316 stainless steel with detachable stainless-steel filter stones and end caps at the top and bottom of the inside of the vessel. Each pressure vessel was also lined with a polytetrafluoroethylene (Teflon; PTFE) inner casing. All vessels were sterilized with 99.5% (v/v) acetone followed by 70% (v/v) ethanol. The diameter and height of the clay plugs that fit within each vessel were 5.3 cm and 14.8 cm, respectively. Pressure vessels were filled with gapfill bentonite clay, supplied by the NWMO, to dry densities of 1.25 g/cm<sup>3</sup>,

1.40 g/cm<sup>3</sup>, 1.45 g/cm<sup>3</sup>, 1.50 g/cm<sup>3</sup>, or 1.60 g/cm<sup>3</sup> (Figure 3.1). Mineral composition of the bentonite was previously determined using XRD analysis (Activation Laboratories; Table 2.1). The average water activity and moisture content of six replicate gapfill bentonite aliquots prior to saturation in a pressure vessel were measured to be 0.45 +/- 0.0008 and 5.8% +/- 0.14%, respectively. Prior to assembly, gapfill bentonite was transported into an anaerobic chamber at least 48 hours prior to pressure vessel assembly to allow for residual oxygen to diffuse from the bentonite.

To accelerate bentonite saturation, pressure vessels were connected to an AZURA P6.1L HPLC pump and pressurized to 10 MPa with Type I water (resistivity = 18 MΩ cm<sup>-1</sup>). Type I water was autoclaved and filter sterilized using a 0.20 μm Stericup Vacuum Filter (Millipore), then flushed with argon gas for one hour in a volumetric flask. A second water sample, sterilized with the same procedure, was flushed in parallel in a beaker. At the end of the flushing period, water from the beaker was used to top up the water in the volumetric flask as the gas line was removed, and the overflowing volumetric flask was quickly stoppered to minimize oxygen in the headspace. The volumetric flask was then quickly transported into an anaerobic chamber.

Pressure vessels were assembled and disassembled in an anaerobic chamber. Assembly involved the use of a hydraulic press to pack the bentonite into pressure vessels. The hydraulic press and bentonite sampling tools were sterilized using 70% ethanol. For each dry density, duplicate pressure vessels were incubated for 1, 4, or 6 months (Figure 3.1). All pressure vessels were assembled, incubated, and disassembled at room temperature. During disassembly, sterile foam-tipped applicators (Puritan) were used to swab the top, middle, and bottom of the Teflon layer, and top and bottom filter stones, which were all in contact with the bentonite, as well as the insides of top and bottom lids.

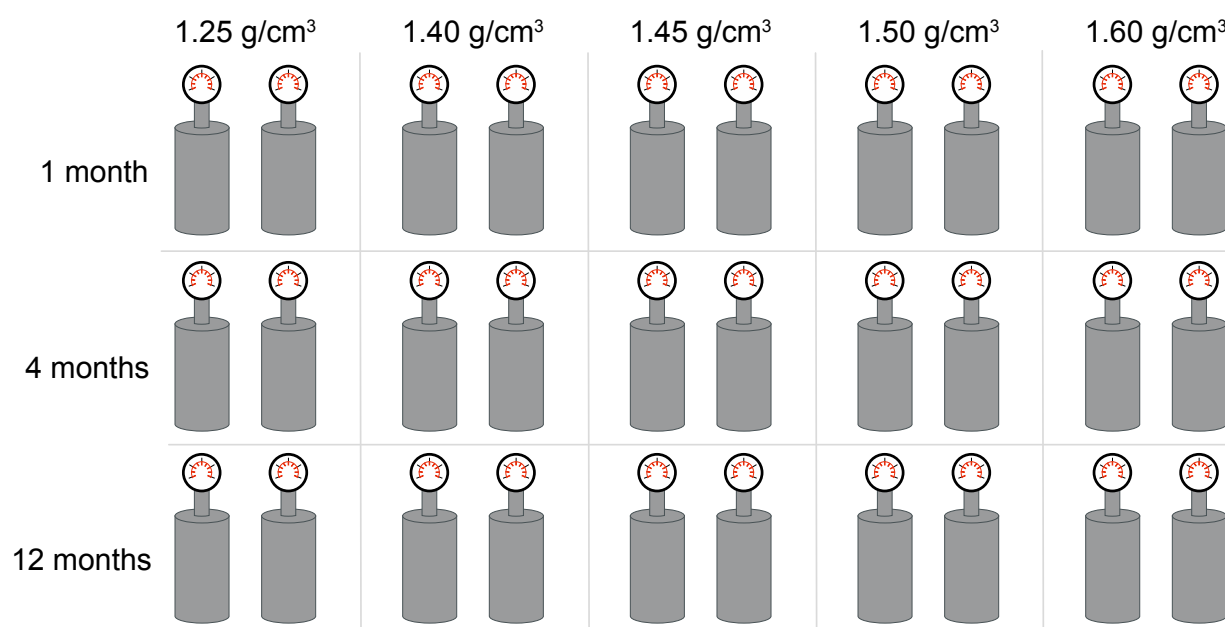


**Figure 3.1** Experimental design schematic for anoxic pressure vessels.

### 3.2.2 Anoxic pressure vessel bentonite sample storage and preparation

After pressure vessel disassembly and bentonite coring at the University of Western Ontario, bentonite samples were packed in airtight, heat-sealed bags (Uline), while still in the anaerobic chamber, and were then shipped to the University of Waterloo on ice where they were transferred to 4°C storage upon arrival. Individual samples were moved to an anaerobic chamber immediately before processing. Inside the anaerobic chamber, the outer layer of the bentonite was removed with a sterile scalpel on sterile aluminum foil. Samples were separated into top, middle, and bottom layers based on their location in the pressure vessel. These samples are referred to throughout as “outer layer” samples (Figure 2.1C). The outer surfaces of the inner core bentonite samples were removed with a sterile scalpel to remove any contamination introduced during the coring process. The inner core was then split into top, middle, and bottom layer samples based on position in the pressure vessel. These samples are referred to throughout as “inner layer” samples (Figure 2.1C).

Working in the anaerobic chamber, from each of the six samples per pressure vessel, a 2 g aliquot was weighed into a sterile 25 mL conical tube and stored in an airtight heat-sealed bag (Uline) at 4°C for up to 48 hours before cultivation. A second 2 g aliquot was weighed into a

sterile 5 mL tube and stored at -20°C prior to DNA extraction. A final portion of each sample was immediately used to measure water activity and moisture content.

### **3.2.3 Water activity and moisture content measurements of anoxic pressure vessel samples**

An Aqualab 4TE water activity meter was used to analyze each pressure vessel sample. After water activity measurement, the same sample of bentonite was transferred to a pre-weighed aluminum foil container, weighed, and dried at 110°C for at least 24 hours. Samples were weighed a second time upon removal from the oven and the difference in mass was used to calculate moisture content (equation 2.1).

### **3.2.4 Cultivation of anoxic pressure vessel bentonite**

All cultivation was completed in the anaerobic chamber. Liquid media was flushed with N<sub>2</sub> gas prior to being transported into the anaerobic chamber. For phosphate-buffered saline solution, 1L bottles were flushed with N<sub>2</sub> gas for 30 minutes using sterile tubing downstream of a 0.20 µm PES filter (Sarstedt). The PBS consisted of 8 g NaCl, 0.2 g KCl, 1.44 g Na<sub>2</sub>HPO<sub>4</sub>, and 0.24 g KH<sub>2</sub>PO<sub>4</sub> in a final volume of 1 L Type I water, with pH adjusted to 7.4 using an HCl solution. For SRB medium (M803, HiMedia Laboratories), 1 L bottles containing part A of the medium were flushed with N<sub>2</sub> gas for 20 minutes, and then an additional 15 minutes after adding parts B and C of the medium, also using sterile tubing and a filter. After flushing, bottles were immediately capped and transported into the anaerobic chamber and lids were briefly opened to allow any oxygen trapped in the headspace to escape. Agar was not flushed, but agar plates were transported into the anaerobic chamber at least 24 hours before use. Resazurin was added to all SRB media and agar at a final concentration of 0.5 mg/L to visibly detect if oxygen was present in the media.

To each 25 mL tube containing 2 g of bentonite, 18 mL of PBS was added, then the tubes were vortexed at low speed for 30 minutes at room temperature. This clay suspension is referred to as “dilution A” throughout. After vortexing, two ten-fold serial dilutions were made from dilution A in a final volume of 10 mL, and are referred to as “dilution B” and “dilution C” throughout.

Dilution A (100 µL) was added to six Reasoner’s 2A (R2A; M1687, HiMedia Laboratories) agar plates and spread using 2 mm sterile glass beads until dry. Dilutions B and C

(100  $\mu$ L) were each added to three R2A plates and spread using the same method as dilution A. Three dilution A and all dilution B and C plates for each sample were incubated under oxic conditions at 30°C for 5 days. The remaining three dilution A plates for each sample were placed in an airtight, heat-sealed bag containing a GasPak EZ anaerobe sachet (BD) to help remove residual oxygen, and an anaerobic indicator strip (BD) to verify anoxic conditions throughout incubation. Anaerobic bags were packed and sealed in the anaerobic chamber. Anaerobic bags containing plates were incubated at 30°C for 28 days. After incubation, colonies were counted and a value of CFU/g was calculated for each sample. Culture biomass was collected on sterile foam-tipped applicators (Puritan) and stored at -20°C until DNA extraction.

Sulfate-reducing bacteria were enumerated using the most probable number (MPN) method [153]. For each sample, 15 test tubes containing 9 mL of SRB medium each were inoculated with 1 mL of either dilution A (five tubes), B (five tubes), or C (five tubes). Test tubes were placed in vacuum chambers containing a GasPak EZ anaerobe sachet (BD) and an anaerobic indicator strip (BD), then flushed five times with a 90% N<sub>2</sub> and 10% CO<sub>2</sub> gas mixture. Vacuum chambers containing SRB cultures were incubated at 30°C for 28 days. After incubation, each test tube was assessed for evidence of SRB growth. Positive tubes were identified by the presence of black ferrous sulfide precipitate produced by a reaction between SRB-derived sulfide and the ferric ammonium sulfate present in the medium. The number of tubes positive for SRB growth was compared to an MPN table to obtain a value of MPN/g of clay [153]. For each sample, 1 mL of each positive SRB tube was pooled separately for each dilution in 5 mL tubes, which were centrifuged at 8,000 x g for 10 minutes to pellet cells. Supernatants were discarded and cell pellets were stored at -20°C prior to DNA extraction. For ten four-month samples, all 15 cultures were positive for SRB growth, and in this situation an MPN table does not yield a specific MPN value. As such, for these ten samples, new aliquots of clay were used to inoculate MPN tubes using nine serial dilutions as opposed to three. Using the moisture content measured for each sample, each value of CFU/g or MPN/g was converted to a value of CFU or MPN per gram dry weight (gdw; equation 2.2).

An ANOVA was used to test the effect of dry density, pressure vessel layer, and incubation time on abundance of culturable SRB, aerobic heterotrophs, and anaerobic heterotrophs. Each sample group consisted of the six replicate pressure vessel samples for a

given time point, layer (inner or outer), and dry density. The Tukey's HSD test was then performed with a *p* value threshold of 0.1 to determine which time points had samples with significantly higher abundances of culturable SRB, aerobic heterotrophs, or anaerobic heterotrophs compared to the starting material. The majority of anaerobic heterotroph counts were 0, making any statistical test that relies on a normal distribution challenging. As such, for anaerobic heterotrophs, the Tukey's HSD test only compared samples where anaerobic heterotrophs were detected.

### **3.2.5 DNA extraction from bentonite, swabs, water filters, and culture biomass**

Genomic DNA was extracted from the 180 pressure vessel clay samples and six aliquots of gapfill bentonite starting material. Genomic DNA was also extracted from the highest dilution of SRB culture that had at least one positive tube for all four-month pressure vessel samples, and from all Teflon layer and filter stone swabs from the four-month pressure vessels.

Genomic DNA was extracted from a 2 g aliquot of each bentonite sample using the DNeasy PowerMax Soil Kit (Qiagen), from Teflon layer, filter stone, and lid swabs using the DNeasy PowerSoil Kit (Qiagen), and from culture biomass using the DNeasy UltraClean Kit (Qiagen). At least one kit control containing no sample was included for each extraction batch. The manufacturer's instructions were followed with the exception of the cell lysis and elution steps. After addition of lysis solution, PowerBead tubes were incubated at 65°C for 30 minutes for PowerMax extractions, and at 70°C for 10 minutes for PowerSoil and UltraClean extractions. This was followed by bead beating using an MM 400 Mixer Mill (Retsch) set to 30 Hz for 10 minutes for PowerMax extractions and a FastPrep-24 Classic Instrument (MP Biomedicals) set to 5.5 m/s for 45 seconds for PowerSoil and UltraClean extractions. Final elution volumes were reduced to concentrate DNA. For PowerMax extractions, PowerSoil extractions, and Ultraclean extractions, elution volumes of 2 mL, 60 µL, and 50 µL were used, respectively. The PowerMax DNA extraction method used here was validated previously for bentonite clay samples [154]. After extraction, DNA samples were stored at -20°C until further analysis.

### **3.2.6 Quantitative PCR of anoxic pressure vessel bentonite samples**

For each bentonite sample, bacterial 16S rRNA genes were quantified in duplicate using the CFX Opus Real-Time PCR System (Bio-Rad). The total volume of each PCR was 15 µL, which consisted of 1x Sso Advanced Universal SYBR Green Supermix (Bio-Rad), 0.3 µM each

of forward primer 341F and reverse primer 518R [155], 7.5 µg of bovine serum albumin (BSA), and 2 µL of template DNA. The following thermocycler conditions were used: 98°C initial denaturation for 3 minutes, followed by 40 cycles of 98°C for 15 s and 55°C for 30 s. For the standard curve, M13 forward and reverse primers were used to amplify a region of a previously constructed plasmid containing the full length 16S rRNA gene from *Thermus thermophilus* (Figure 3.1). This amplicon was purified, quantified, and used as the standard for generation of the standard curves. Samples were split among six 96 well plates that were processed on the same day using a single tube of master mix and set of standard dilutions. The aliquot of the standard DNA was thawed and quantified using the Qubit dsDNA High Sensitivity Assay Kit (Invitrogen) with 4 µL of sample immediately prior to preparing the qPCR standard curve.

Bacterial 16S rRNA gene copy numbers were calculated using linear regression of the standard curve based on the Qubit quantification of the standard. DNA extraction kit controls were included in the qPCR. The average starting quantity (SQ) value from all kit controls from kits with the same lot number was calculated. For each sample SQ value, the average kit control SQ corresponding to the same lot number as the kit the sample was extracted using was subtracted from the sample SQ. Adjusted SQ values for samples were converted to values of 16S rRNA gene copies/gdw (equation 3.1).

### 3.1 16S rRNA gene copies $gdw^{-1}$

$$= \frac{(\text{average SQ sample} - \text{average SQ of kit controls}) \times 2000 \mu\text{L elution in DNA extraction}}{2 \mu\text{L template in qPCR} \times 2 \text{ g clay in DNA extraction}}$$

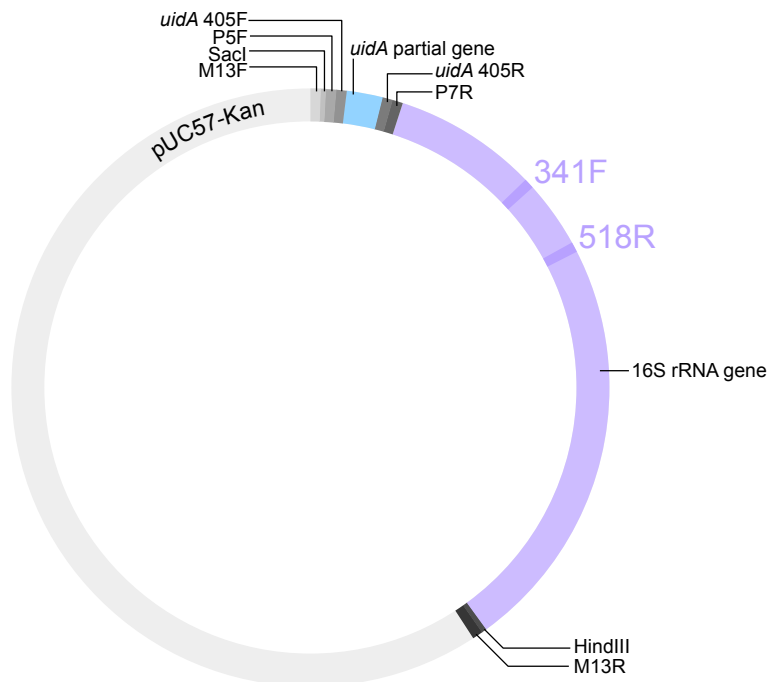


**Figure 3.2** Plasmid used for making qPCR standard. The *uidA* partial gene comes from *Escherichia coli* and the 16S rRNA full length gene from *Thermus thermophilus*.

### 3.2.7 Amplicon sequencing of bentonite, swab, water filter, and culture biomass samples

For each genomic DNA sample (i.e., pressure vessel clay, culture biomass, or swab), the V4-V5 region of the 16S rRNA gene was amplified in triplicate with universal primers 515F-Y [156] and 926R [157], modified to contain a unique six-base index sequence, flow cell binding, and sequencing primer binding sites [158]. The PCR setup was performed in a PCR hood (AirClean Systems) with ISO 5-HEPA-filtered air. Surfaces within the PCR hood were treated with UV light for 15 minutes before each use. All tubes, PCR water, and BSA were pre-treated with UV (302 nm) for 20 minutes on a transilluminator (ProteinSimple). The final volume of each PCR was 25  $\mu$ L and consisted of 1 x ThermoPol Buffer (New England BioLabs), 0.2  $\mu$ M of each primer, 200  $\mu$ M dNTPs, 15  $\mu$ g BSA, 0.625 U Hot Start Taq DNA polymerase (New England BioLabs), and 1  $\mu$ L of template DNA. The following thermocycler conditions were used: 95°C initial denaturation, followed by 35 (cultures) or 40 (swabs and bentonite) cycles of 95°C denaturation for 30 s, 50°C annealing for 30 s, and 68°C extension for 1 min, followed by a final extension at 68°C for 7 min. Negative PCR controls containing no template DNA (NTCs) were included in each 96 well plate to test for cross contamination between wells of the plate, and both NTCs and positive controls were also prepared in tubes separate from the plate for each master mix.

Triplicate PCR products were visualized on a 1% agarose gel stained with ethidium bromide and were then pooled and quantified on a second 1% agarose gel stained with GelRed (Biotium). Based on gel quantification, equimolar volumes of each amplicon were pooled into a single tube. For samples with no visible amplification, 20  $\mu$ L of sample was added. Kit controls from DNA extraction as well as NTCs were added to the pool, even if amplification was not detected on an agarose gel, at a volume of 5  $\mu$ L each. Pooled amplicons were excised from a 1.5% agarose gel stained with ethidium bromide and were purified using the Wizard SV Gel and PCR Clean-Up System (Promega). The purified library was quantified using the Qubit dsDNA High Sensitivity Assay Kit (Invitrogen) and qPCR using P5 and P7 primers (Illumina). The library was denatured and diluted to 10.5 pM following Illumina guidelines (Document No. 15039740 v01), then spiked with PhiX Sequencing Control v3 (Illumina) to a final concentration of 15% (v/v). All samples were sequenced on a MiSeq System using a 2 x 250 MiSeq Reagent Kit v2 (Illumina).

All clay and culture DNA samples were sequenced on a single MiSeq run with an average cluster density of 668,000 clusters/mm<sup>2</sup>, average clusters passing filter of 93.2%, and average Q30 of 91.5%. Swab DNA samples were sequenced on a second MiSeq run with an average cluster density of 774,000 clusters/mm<sup>2</sup>, average clusters passing filter of 91.4%, and average Q30 of 90.0%.

### **3.2.8 Anoxic pressure vessel sequence analysis**

Sequences were demultiplexed using Local Run Manager version 4 and further analyzed using Quantitative Insights Into Microbial Ecology 2 [159] and Divisive Amplicon Denoising Algorithm 2 (DADA2; Callahan et al., 2016). Paired-end reads were imported into QIIME2, and DADA2 was used to remove low quality reads and primer sequences and to truncate forward and reverse reads to 250 bases. The DADA2 pipeline was additionally used, through QIIME2, to denoise and dereplicate paired-end reads and generate amplicon sequence variants (ASVs). Taxonomy was assigned to ASVs using a naïve Bayes classifier pre-trained with SILVA database release 138. Potential contaminant ASVs were identified using the Decontam R package [163] with a threshold of 0.5, selected by examining histograms of Decontam score distribution. Clay, culture, and swab samples were processed through Decontam separately. ASVs flagged as contaminants were removed from the ASV table for subsequent analysis.

All sequence data were deposited in the European Nucleotide Archive with accession number PRJEB79405.

### **3.3 Results**

#### **3.3.1 Cultivation from pressure vessel bentonite samples**

Prior to saturation in a pressure vessel, gapfill bentonite had very few culturable microorganisms. Six as-received bentonite clay replicates yielded an average abundance of 360 CFU/gdw for aerobic heterotrophs, 159 CFU/gdw for anaerobic heterotrophs, and 8 MPN/gdw for SRB (Figure 3.3). After incubation in pressure vessels, the abundance of culturable microorganisms increased beyond the abundances measured in the gapfill starting material for most samples (Figure 3.3). The average abundance of culturable microorganisms for each set of replicate pressure vessels was compared to the average abundance of culturable microorganisms in the gapfill starting material. For inner layer samples, only two average abundances were significantly higher than the corresponding starting material: anaerobic heterotrophs in the 1-month 1.40 g/cm<sup>3</sup> pressure vessels, and SRB in the 12-month 1.40 g/cm<sup>3</sup> pressure vessels (Tukey's HSD;  $p < 0.10$ ). For outer layer samples, the average abundances of culturable aerobic and anaerobic heterotrophs were higher than the corresponding starting material only for the 1 month 1.25 and 1.40 g/cm<sup>3</sup> pressure vessels (Tukey's HSD,  $p < 0.10$ ). The average abundance of SRB in most outer layer pressure vessel samples was significantly higher than the abundance measured in the starting material (Tukey's HSD,  $p < 0.10$ ). However, after the initial increase observed in the one-month pressure vessels, the abundance of culturable SRB did not increase further at later time points in pressure vessels with a minimum dry density of 1.45 g/cm<sup>3</sup> (Anova,  $p < 0.10$ ). Abundance of 16S rRNA gene copies in bentonite samples ranged from below detection limit to 10<sup>5</sup>.

Moisture content and water activity within many pressure vessels were inconsistent, presumably because the bentonite was not pre-hydrated and saturation progressed slowly, especially for high dry density pressure vessels (Figure 3.3). On average, the higher the dry density, the lower the average moisture content and water activity, and the range of measured water activities also increased with increasing dry density (Figure 3.4). For individual samples, higher moisture content was typically associated with increased abundances of culturable aerobic heterotrophs, though the highest measured abundances of culturable heterotrophs and SRB often

had relatively low moisture content (Figure 3.4). The trend of high water activity leading to overall higher abundances of aerobic heterotrophs was less clear for abundances of anaerobic heterotrophs and SRB. Although water activity overall had lower correlations to abundances of culturable microorganisms compared to moisture content, all samples with greater than  $10^3$  CFU or MPN/gdw of aerobic heterotrophs, anaerobic heterotrophs, or SRB had a minimum water activity of 0.91 (with one outlier for aerobic heterotrophs). Although this water activity may be a threshold below which growth of microorganisms was minimal, having a water activity greater than 0.91 was not always associated with microbial growth. For all three types of culturable microorganisms, samples with a minimum water activity of 0.91 were associated with the full range of measured abundances of culturable microorganisms (Figure 3.4).

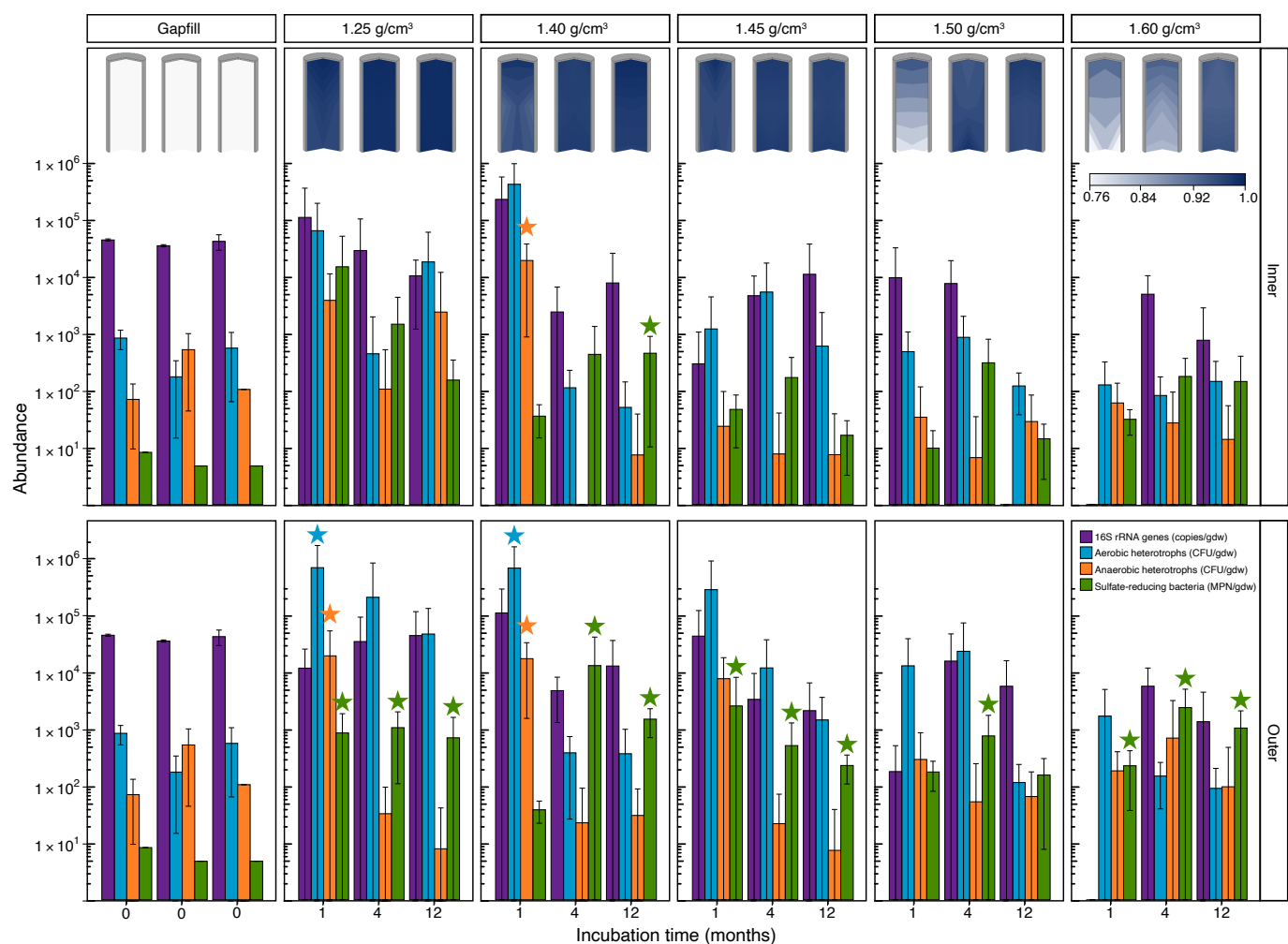


**Figure 3.3** Bar plot depicting abundance of 16S rRNA genes, and culturable aerobic heterotrophs, anaerobic heterotrophs, and SRB. Each bar represents the average of six biological replicate samples and their technical replicates. Error bars represent the standard deviation of 18 replicate plates for heterotrophs, the standard deviation of six replicate MPN/gdw values for SRB, and the standard deviation of 12 replicate qPCR counts. The stars above bars representing culture counts indicate average abundances that were significantly greater than the corresponding abundance in the initial gapfill bentonite (Tukey's HSD,  $p < 0.10$ ). Contour plots are based on the measured water activity at the six sampled locations in each pressure vessel.

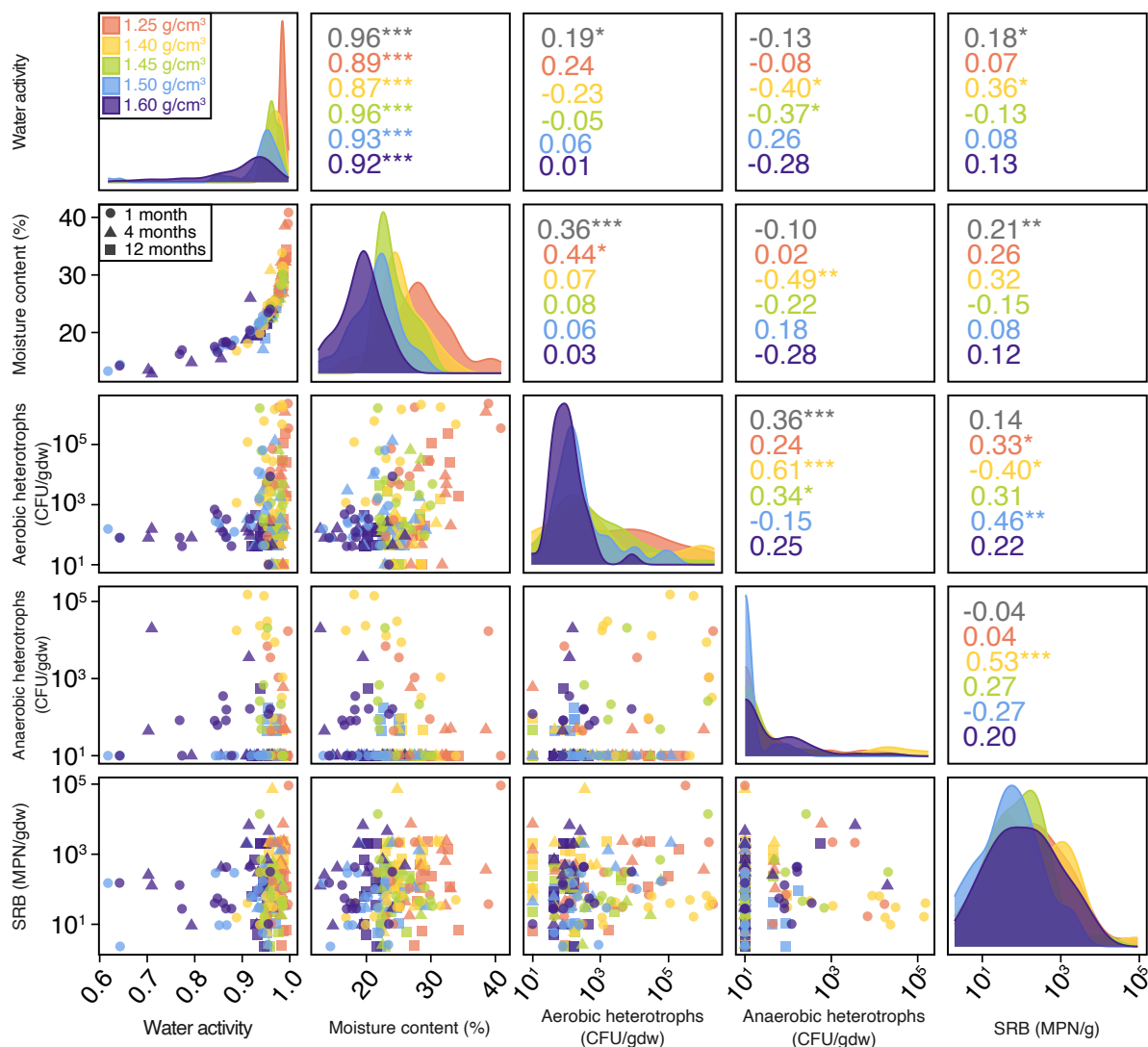


**Figure 3.4** Scatterplot matrix showing relationship between water activity, moisture content, and culturable abundances of aerobic heterotrophs, anaerobic heterotrophs, and SRB. The diagonal includes density plots, and the top panels include correlations. For all comparisons including water activity, the Spearman correlation is reported, and for all other comparisons, the Pearson correlation is reported. The top correlation in each set (grey) is of all dry densities combined, and the remaining correlations are grouped and coloured by dry density. The significance of the correlation is indicated with asterisks ( $p < 0.5$  is depicted by \*,  $p < 0.01$  by \*\*, and  $p < 0.001$  by \*\*\*).

### 3.3.2 16S rRNA gene analysis of pressure vessel bentonite, cultures, and swabs

Genomic DNA was extracted from six samples (top, middle, and bottom of the inner and outer layers) from each pressure vessel and the V4-V5 region of the 16S rRNA gene was amplified and sequenced. Overall, sequencing depth was very low for most samples (Figure 3.5), likely due to the low concentrations of DNA present (usually below the detection limit of Qubit).

The most prevalent genera detected (i.e., present in at least half the samples) were *Vibrio*, *Mycoplasma*, *Acinetobacter*, and *Pseudomonas* (Figure 3.5). The most abundant putative SRB detected in the 16S rRNA gene profiles was *Desulfosporosinus*, which was detected in 32 of the 180 pressure vessel clay samples and none of the gapfill starting material replicates (Figure 3.5). For samples with a minimum sequencing depth of 10 reads (the remaining 30 samples had sequencing depth between 54 and 8790 reads), the average relative abundance of sequences associated with *Desulfosporosinus* was 5.0%.

Given the large number of samples with abundances of culturable SRB significantly greater than those of the gapfill starting material, the SRB cultures from all four-month pressure vessels were selected for sequencing to ensure that sequences associated with SRB were present in the cultures, and that observed SRB activity (i.e., black precipitate) was not erroneous. At least one ASV (and in most samples four ASVs) associated with *Desulfosporosinus* was present in each SRB culture from both the pressure vessel clay and the gapfill starting material clay (Figure 3.6). For most samples, ASVs associated with *Desulfosporosinus* made up most of the reads (Figure 3.6). Apart from *Desulfosporosinus*, many cultures had ASVs associated with *Pseudomonas*, and SRB cultures from gapfill starting material in particular had several ASVs associated with *Bacillus* and *Sporacetigenium*.

All swabs of the Teflon layer, filter stone, and lids (i.e., the pressure vessel surfaces in contact with the bentonite) from the four-month pressure vessels were also selected for DNA extraction and sequencing to assess if the SRB that grew in the cultures might be associated with the pressure vessel-bentonite interfaces. For comparison, Teflon layer and filter stone swabs from four-month pressure vessels from previously conducted oxic experiments (Chapter 2) were included. Sequences associated with *Desulfosporosinus* were detected in 47 of the 62 anoxic pressure vessel swab samples, often at high relative abundance (Figure 3.7). In contrast, sequences associated with *Desulfosporosinus* were only detected in 1 of 23 oxic pressure vessel swabs at very low relative abundance (Figure 3.7). In addition to SRB, anoxic pressure vessel 16S rRNA gene profiles were similar to each other and distinct from oxic pressure vessel swab 16S rRNA gene profiles (Figure 3.7). The most prevalent ASVs (i.e., detected in at least half the samples) in the anoxic pressure vessel 16S rRNA gene profiles were associated with *Pseudomonas*, *Anaerobacillus*, *Cellulomonadaceae*, *Desulfosporosinus*, *Bacillus*, *Mycoplasma*,

*Escherichia-Shigella*, *Alkalibacter*, and *Anaerosolibacter* (Figure 3.7). Most swabs from oxic pressure vessels had 16S rRNA gene profiles dominated by sequences associated with the *Pseudomonas* genus (Figure 3.7).

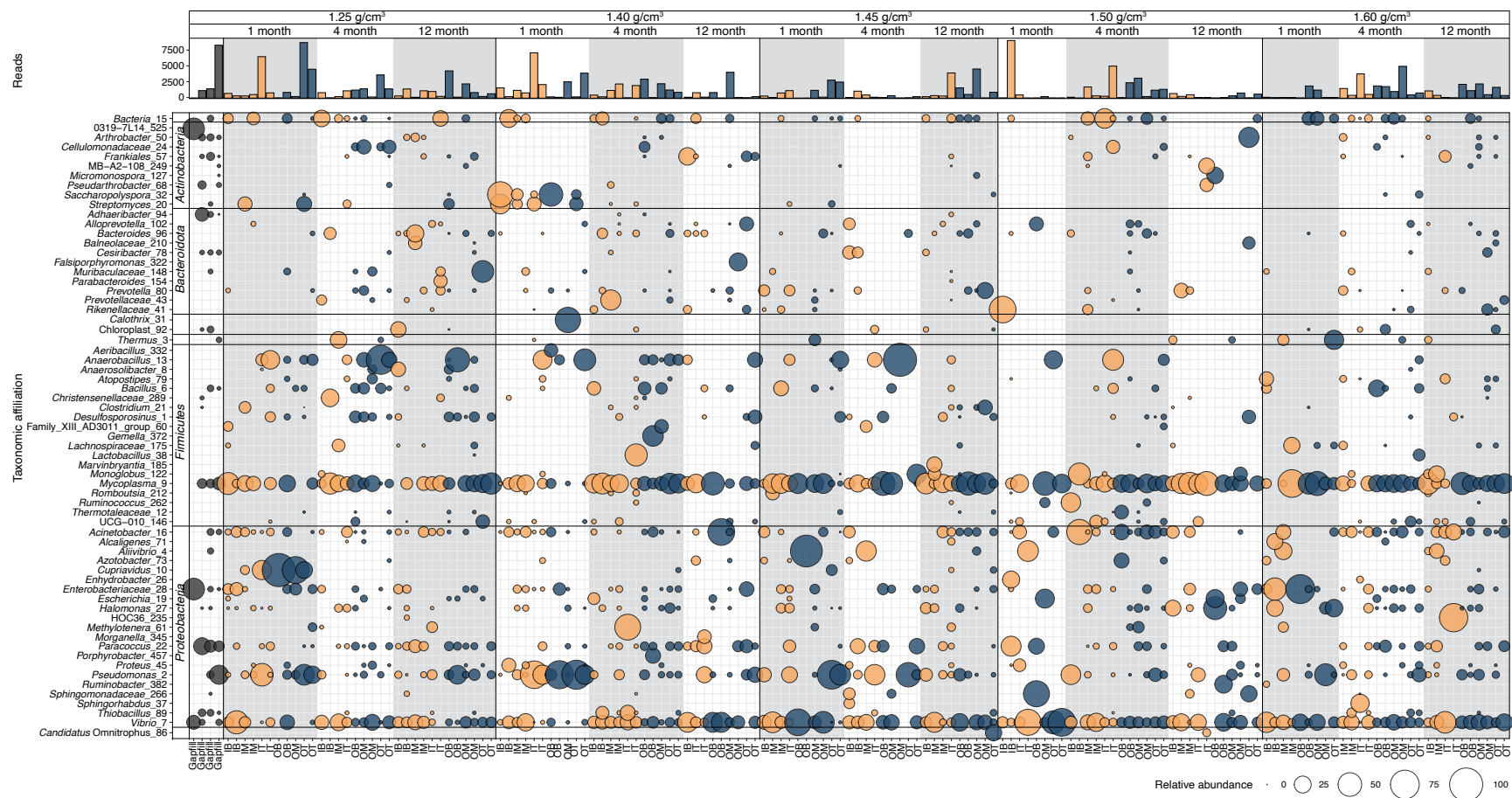


**Figure 3.5** Bubble plot of 16S rRNA gene profiles from pressure vessel inner (orange) and outer (blue) layer clay, as well as gapfill bentonite starting material (grey) that was never exposed to a pressure vessel. Genera with a minimum relative abundance of 15% in at least one sample are included. The bar graph shows the number of sequencing reads obtained for each sample.

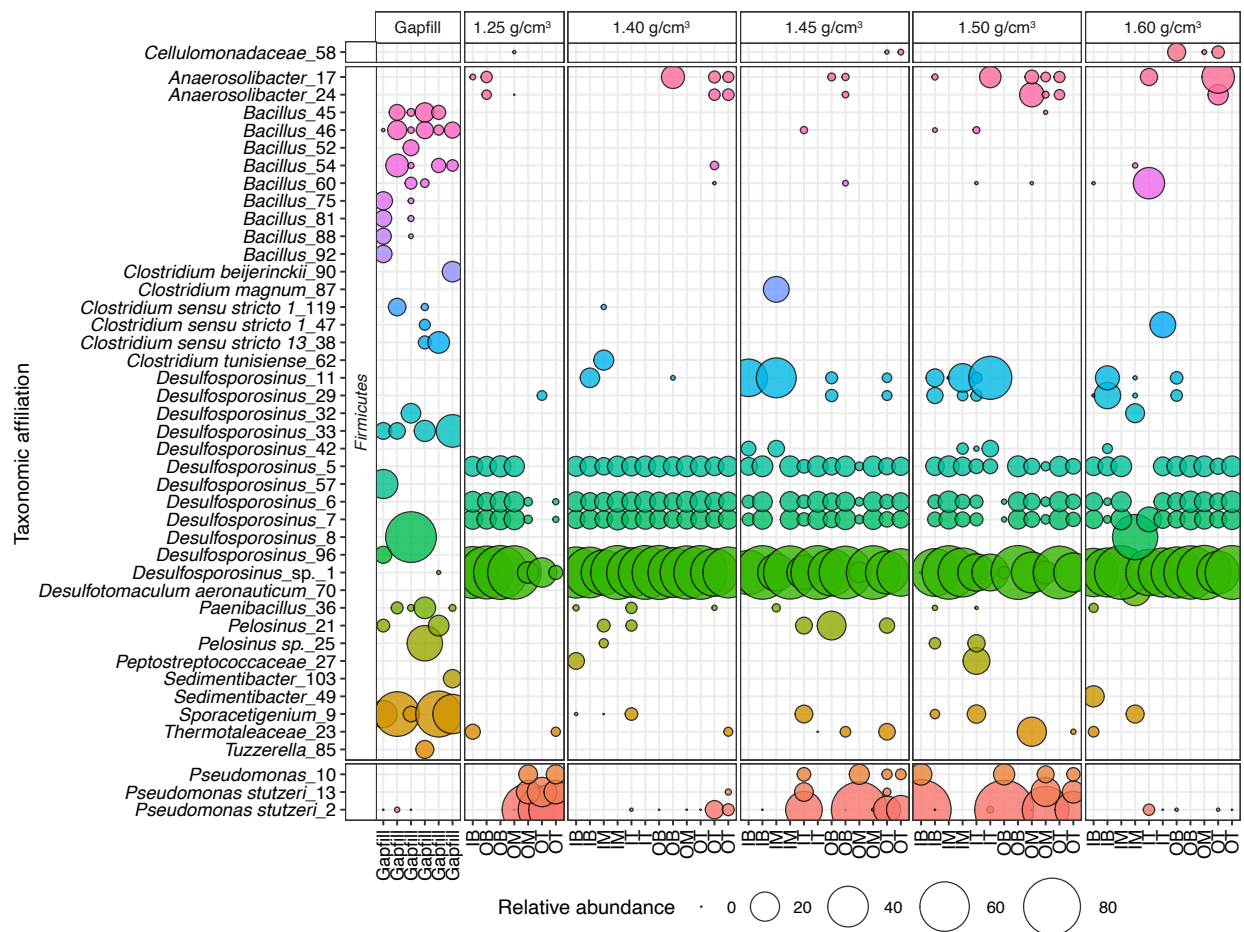


**Figure 3.6** Bubble plot of 16S rRNA gene profiles from 4-month pressure vessel and gapfill starting material SRB culture biomass. Only ASVs with a minimum relative abundance of 5% in at least one sample are included.

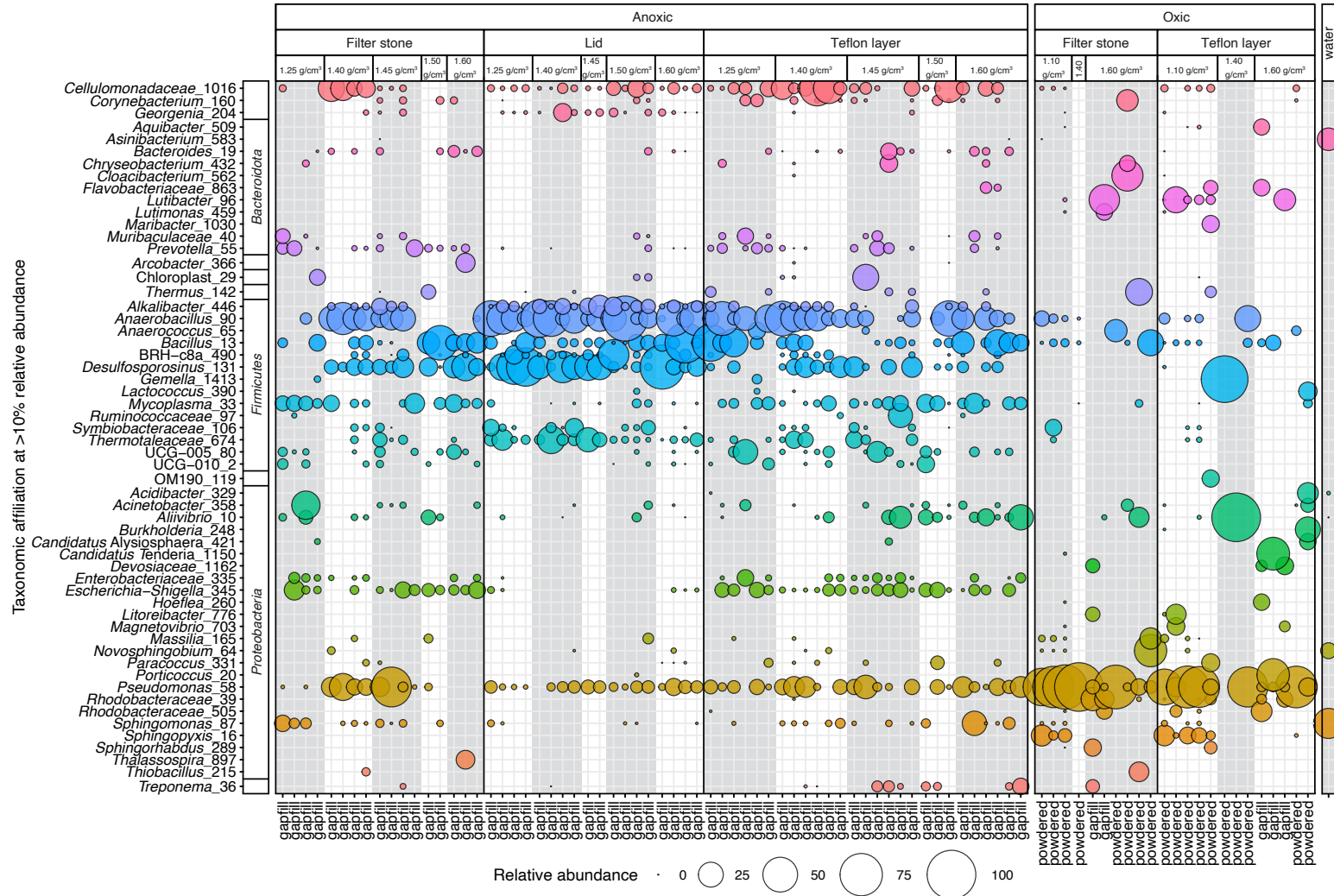


**Figure 3.7** Bubble plot of 16S rRNA gene profiles from four-month pressure vessel swabs and from a filter of the water used to saturate pressure vessels, at the end of all pressure vessel setup. The left panel includes swabs of filter stones, lids, and Teflon layers from anoxic pressure vessels. The middle panel includes swabs of filter stones and Teflon layers from previous oxic pressure vessels, for comparison. Only genera with a minimum relative abundance of 10% in at least one sample are included.

### 3.4 Discussion

#### 3.4.1 Cultivation from pressure vessel bentonite samples

For anoxic pressure vessels, the initial abundance of culturable aerobic heterotrophs, anaerobic heterotrophs, and SRB were similar to those reported in previous experiments using Wyoming bentonite (Figure 3.3) [10, 123, 126]. When water is added to as-received bentonite, an initial increase in abundance of culturable aerobic heterotrophs has previously been reported for both oxic [123, 125] and anoxic experiments [126]. Presumably as the bentonite saturates and swells to its full capacity, microbial growth is eventually suppressed given a sufficiently high dry density (Chapter 2). Even in lower dry densities, incubations that are long enough have additionally resulted in suppression of microbial growth in bentonite, especially in “inner layer” bentonite [126, 127]. In experiments set up under anoxic conditions, an initial increase in abundance of anaerobic heterotrophs has been reported in bentonite compacted to both low (i.e., 1.25 g/cm<sup>3</sup>) and high (i.e., 1.55 g/cm<sup>3</sup>) dry densities [126]. For culturable heterotroph abundances, the results of the present study support previous literature [10, 125, 126]. By the one-month time point, the abundance of culturable aerobic and anaerobic heterotrophs was occasionally greater than that of the gapfill starting material, especially in low dry density and outer layer samples (Figure 3.3). At these early time points, water activity and moisture content within the pressure vessels were inconsistent, suggesting that the pressure vessels were not fully saturated and thus likely had regions of bentonite with available water and below-target swelling pressures (Figure 3.3).

Different from previous literature, the average abundance of culturable SRB increased in several outer layer samples and one inner layer sample between the initial gapfill bentonite and the one-month time point, and these abundances often persisted throughout the one-year experiment (Figure 3.3). However, after the initial (i.e., in the one-month pressure vessels) increase in abundance of culturable SRB in pressure vessels with a minimum dry density of 1.45 g/cm<sup>3</sup>, the abundance did not further increase throughout the remainder of the one-year experiment, suggesting that cells were no longer dividing. The abundance of culturable SRB was poorly correlated with water activity and moisture content, and was elevated in some bentonite samples from all dry densities and incubation times tested (Figure 3.4). For the oxic version of the work presented here (Chapter 2), as well as foundational research also conducted under oxic conditions, no increase in abundance of SRB was reported regardless of dry density [123]. This

was hypothesized to be at least in part due to the presence of oxygen, which would be expected to preclude the growth of obligately anaerobic SRB. However, SRB have similarly not been observed to increase in abundance in other experiments where compacted bentonite clay was incubated under anoxic conditions. The present experiment is perhaps most similar to *in situ* experiments where “modules” were packed with as-received bentonite clay to various dry densities and incubated in DGR-relevant underground environments. Under these conditions, SRB did not increase in abundance from the initial abundance in the bentonite starting material, regardless of dry density, even when modules were sampled at sufficiently early time points to observe increased abundances of culturable heterotrophs [126, 127]. Additional anoxic lab-scale experiments have studied SRB in compacted bentonite and have similarly not observed growth. In another experiment, a goal was to identify conditions that encouraged growth of SRB in bentonite [173]. Specifically, bentonite was hydrated with both water and SRB medium to achieve a high water activity, in the absence of pressure, and was incubated under anoxic conditions at 30°C. Even under such conditions predicted to be ideal for growth of SRB, the abundance of culturable SRB never increased beyond that of the starting bentonite [173].

Although SRB have not previously been reported to increase in abundance in bentonite clay, there were some differences between the present study and previous studies. This study used gapfill bentonite, which has not been used in previous such studies. Pressure vessels were incubated at room temperature, unlike *in situ* experiments that involved incubations at colder temperatures associated with the underground environments for modules. The relatively large size of pressure vessels, and the fact that the bentonite was not pre-saturated, makes it such that the saturation process, when some microorganisms are suspected to grow, is elongated compared to other experiments. Further, although several experiments use “anoxic conditions,” perhaps there were trace amounts of oxygen present that impacted the ability of obligate anaerobes like SRB to survive and proliferate. Additional experiments are needed to better understand the factors that allow for the growth of SRB in compacted bentonite clay.

Even though abundances of culturable SRB were higher in this experiment than in previous research, the abundances detected were still relatively low (Figure 3.3). Ongoing work studying the rate of corrosion on copper coupons embedded within the bentonite clay of anoxic pressure vessels will additionally help to demonstrate whether the SRB present in the clay were

sufficient in number and activity to generate detectable copper corrosion. Further, although many average abundances of culturable SRB were greater than those of the starting gapfill material in the outer layers of the pressure vessel, this was only true for one set of inner layer bentonite samples. In pressure vessels with a minimum dry density of 1.45 g/cm<sup>3</sup>, abundances of all three types of cultured microorganisms were not significantly different than the starting material in inner layer bentonite, suggesting that this dry density is sufficient to suppress microbial growth in the inner layer (Figure 3.3).

### 3.4.2 16S rRNA gene analysis of pressure vessel bentonite, cultures, and swabs

A second major goal of this research was to study the microbial community composition of bentonite clay from pressure vessels. The most prevalent genera detected in 16S rRNA gene profiles were putative heterotrophs, including *Vibrio*, *Mycoplasma*, *Acinetobacter*, and *Pseudomonas* (Figure 3.5). The most dominant and prevalent genus of SRB detected in pressure vessel clay samples was *Desulfosporosinus*, which is a commonly detected genus of SRB in bentonite [10, 127, 151, 173]. Given that culturable SRB were detected in 178 of the 180 pressure vessel bentonite samples, it was hypothesized that ASVs associated with *Desulfosporosinus* would be present in the 16S rRNA gene profiles of most samples, but they were only detected in 32 of the 180 samples (Figure 3.5). It is possible that this is due at least in part to the low sequencing depth of most samples given very low DNA concentrations.

To confirm that SRB cultures included SRB and were not counted as being positive for SRB growth (i.e., blackness in the medium) due to an unknown abiotic reaction, the most dilute set of positive SRB cultures from each 4-month pressure vessel was selected for DNA extraction and 16S rRNA gene sequencing. All 16S rRNA gene profiles from the 4-month SRB cultures included at least one, and usually multiple, ASVs associated with *Desulfosporosinus* (Figure 3.6). This genus often made up the majority of reads for a sample. The water used to saturate pressure vessels was also filtered at the end of the experiment, and DNA was extracted from this filter and sequenced to confirm that SRB were not introduced to the system from the water. Although there was detectable DNA in the water sample by the end of the experiment, no sequences associated with known SRB were found in this sample (Figure 3.7).

With confirmation that there were sequences associated with known SRB growing in the SRB cultures, DNA was extracted and sequenced from swabs taken from pressure vessel

components (Teflon layers, filter stones, and lids) in contact with the bentonite to identify if putative SRB were present at greater relative abundance at the pressure vessel-bentonite interface as compared to within the bentonite. Indeed, sequences associated with *Desulfosporosinus* were detected in most swab samples, often at high relative abundances (Figure 3.7). Similar swabs from the oxic version of the current study (Chapter 2), where culturable SRB never increased in abundance beyond the initial abundance of the bentonite starting material, were included for comparison. These swabs were dominated by sequences associated with *Pseudomonas*, which was also a dominant member of the community of most oxic pressure vessel clay samples, and *Desulfosporosinus* was only detected in a single sample at very low relative abundance (Figure 3.7). Consistent with the higher abundance of culturable SRB in outer layer bentonite compared to inner layer bentonite, the 16S rRNA gene analysis results suggest that SRB may predominantly grow at the pressure vessel-clay interfaces rather than within the clay itself.

### **3.5 Conclusion**

Studying abundances and composition of microbial communities in bentonite compacted to different dry densities is critical for informing DGR design. Previous research has focused on testing the effect of dry density on microbial growth suppression in powdered bentonite clay (i.e., the material used to make highly compacted bentonite buffer boxes in the Canadian design), but rarely gapfill bentonite (i.e., the material used to fill spaces between bentonite buffer boxes and the walls of the placement rooms in the Canadian design), and never gapfill under anoxic conditions. The results of this study demonstrate that under anoxic conditions, growth of microorganisms is suppressed by a minimum dry density of 1.45 g/cm<sup>3</sup> in the inner layer, although abundances of culturable SRB greater than the initial abundances in the starting material are maintained throughout the one-year experiment in several outer layer bentonite samples.

The 16S rRNA gene analysis demonstrated that sequences associated with known SRB were detected in many bentonite samples but were even more prevalent and abundant in the 16S rRNA gene profiles of swabs taken from the bentonite-pressure vessel interface. It remains unclear why SRB increased in abundance in anoxic pressure vessels when a similar phenomenon has not been observed in other studies examining growth of SRB in bentonite, even when the bentonite was supplemented with SRB medium and incubated under anoxic conditions in the

absence of pressure. However, the abundances of culturable SRB detected in the pressure vessels were still relatively low, and ongoing work studying the corrosion of copper coupons that were embedded within the bentonite in anoxic pressure vessels will be critical for demonstrating the impact, if any, that the growth and activity that SRB had on copper corrosion.

Future experiments should continue to study the conditions that promote growth of SRB in compacted bentonite clay. For example, it is possible that the porous components of the pressure vessel (i.e., Teflon layer, filter stones, and lids) provided a necessary surface for biofilm formation that was not present in other experiments. In the context of a DGR, bentonite will not be saturated with Type I water, nor will it exist at room temperature for the duration of its lifetime. As such, testing the effect of dry density on microbial growth suppression in groundwater-saturated bentonite, and under a range of DGR-relevant temperatures, is critical. Even though SRB increased in abundance in compacted bentonite in the present study, this may not be true for pressure vessels set up using more DGR-relevant conditions. Finally, with increasing information related to microbial growth and corrosion in compacted bentonite, an important next step will be to incorporate experimentally determined values into models to predict DGR evolution on physical and temporal scales that cannot be tested experimentally.

## **Chapter 4: Effect of temperature on microbial communities in bentonite clay**

### **4.1 Introduction**

Bentonite clay is an important component of many proposed deep geological repository (DGR) designs, where it serves as a buffer between used fuel containers and the rock within which the DGR is hosted. In terms of microbiology, it is important to understand the response of bentonite-associated microbial communities to the conditions they are likely to experience in a DGR to better predict the long-term stability of such a repository. Studies on the microbiology of bentonite clay in the context of DGR design often focus on sulfate-reducing bacteria (SRB) because they commonly dominate occurrences of microbiologically influenced corrosion (MIC) in other anoxic environments [12]. In a DGR, MIC could compromise the integrity of metal used fuel containers (UFCs), which house the used nuclear fuel. Despite focussing on SRB, microorganisms live in communities with other microorganisms, where they may have various symbiotic relationships. As such, it is important to study microbial communities of bentonite clay as a whole rather than by focussing only on specific populations. One important goal in designing DGRs, including the Canadian DGR, is to identify the minimum dry density of saturated bentonite clay that is required to suppress microbial growth. As such, the major area of focus in bentonite microbiology studies has been the effect of dry density on microbial abundance and survival [123–129, 151].

In addition to dry density, an important factor that has been less well studied in bentonite microbiology research is temperature. A DGR is expected to experience a wide range of microbiologically relevant temperatures. Before construction of a DGR, the temperature in the subsurface of the two potential Canadian host sites, at repository depth, is between 12 and 17°C [118, 119]. Within a DGR, used nuclear fuel, which releases energy in the form of heat, will increase the temperature [118, 119]. DGRs are being designed in such a way that the temperature within the system never reaches sufficiently high temperatures to lead to bentonite conversion to non-swelling illite clay (i.e., >140°C) or electrochemical changes in the copper coating of the UFCs (i.e., >125°C) that could increase the rate at which they corrode [120]. The Canadian DGR, specifically, is being designed such that the temperature at the surface of the UFCs never exceeds 100°C [120]. The expected temperature that may be reached within different areas of a DGR depends on the type of rock at the host site. Models show a steady increase in temperature

in the area surrounding the UFCs until the maximum temperature (84°C for crystalline host rock and 93°C for sedimentary host rock) is reached ~45 years after emplacement, after which temperatures are expected to remain elevated (>70°C) for over 1000 years before beginning to cool over several thousand years. Areas of the DGR further from the UFCs are expected to experience slightly cooler conditions. Overall, the expected temperature range within a DGR is anywhere between 12°C and 93°C, depending on the location within the DGR, the type of host rock, and the time after used fuel placement.

To date, very few studies have investigated microbial community composition and abundance in bentonite exposed to elevated temperatures. In bentonite from El Cortijo de Archidona (Spain), heterotrophs and SRB did not increase in abundance in bentonite compacted to 1.7 g/cm<sup>3</sup> and incubated under anoxic conditions at 60°C for a year, but they did remain culturable in similar abundances before and after compaction and incubation [129]. Even in bentonite first sterilized through a “Tyndallization” process (i.e., repeated heating and cooling) prior to compaction, microorganisms from the clay plugs were culturable, though in lower numbers than the starting material [129]. This suggests that native bentonite microbial communities may not increase in number under harsh temperature and dry density conditions, but that they can survive the conditions and proliferate once temperature and dry density are reduced. In a second study exploring the effect of temperature on microbial communities within Bavarian bentonite, in the absence of pressure, sulfate reduction was evident in slurries of Bavarian bentonite and Opalinus clay porewater solution incubated at 30°C, but not in parallel 60°C incubations [130]. However, a shift in microbial community composition was observed through DNA sequencing of the 60°C slurry. The 16S rRNA gene profiles of these high temperature microcosms were dominated by sequences associated with the thermophilic species *Caldinitratiruptor microaerophilus* [130].

No known studies have investigated the effect of temperature on microbial community composition in as-received clay nor clay hydrated to DGR-relevant water activities/moisture contents. Additionally, the effect of temperature has been limited to 60°C and has not been explored in either of the bentonite clays proposed for use in a Canadian DGR: Wyoming MX-80 bentonite, and gapfill material produced from Wyoming MX-80 through a low-temperature roll-compaction method. Thus, the purpose of this study was to explore the impact of a range of

DGR-relevant temperatures (15, 30, 45, 60, 75, 90, and 105°C) on microbial community composition and abundance within five as-received bentonite clays, with and without hydration.

## **4.2 Materials and methods**

### **4.2.1 Bentonite background**

Five different manufactured sodium-type bentonite clays were used in this study, and are referred to throughout as Asha, MX6, MX7, MX9, and GFM. Asha is a bentonite sourced from Kutch, India, and manufactured by Ashapura Minechem Co. This as-received clay was previously characterized using DNA sequencing and cultivation [10]. Both MX6 and MX7 are Wyoming bentonite and were used in borehole modules in the Grimsel Underground Research Laboratory [127, 151]. Clay MX9 is Wyoming MX-80 bentonite that was characterized in the same study as Asha bentonite [10] and separately in a hydrated clay microcosm study [173]. Gapfill material (GFM) was made from Wyoming MX-80 bentonite through a roll compaction process. It was included in two studies that aimed to identify the minimum dry density necessary to suppress microbial growth using pressure vessels (Chapter 2 and Chapter 3). The mineral composition of each as-received bentonite sample used to fill pressure vessels was determined using quantitative XRD analysis performed by Activation Laboratories. The XRD analysis was performed in a Bruker D8 Endeavour diffractometer equipped with a Cu X-ray source, at 40 kV and 40 mA over a  $2\theta$  range of 4–70°, with a step size of 0.02° and a time per step of 0.5 seconds, a fixed divergence slit at 0.30°, and sample rotation at 15 rpm.

### **4.2.2 Microcosm experiment setup**

From each as-received clay, cultivation and DNA analysis were conducted with no hydration nor prior incubation at elevated temperature. Separately, each as-received clay was hydrated and incubated at various temperatures (Figure 4.1). Sample names including “R1,” “R2,” and “R3” refer to biological replicates.

For hydrated clay microcosms, as-received bentonite was mixed with a pre-determined volume of autoclaved Type I water to achieve a water activity of 0.99 (Table 4.2). The hydrated clay was mixed by hand with sterile nitrile gloves until a homogenous mixture was achieved. A WP4 Dew Point Potentiometer (Meter Group, USA) was used to ensure that the water activity of hydrated bentonite was 0.99. For 15–60°C microcosms, three 80 g aliquots of bentonite were mixed with water and each was then split into 4 x 20 g aliquots for each of the four temperatures

of a given replicate. For 75-105°C microcosms, 10 g of bentonite was mixed with water and each hydrated 10 g aliquot was added to an individual jar.

**Table 4.1** Volumes of Type I water added to 10 or 80 g of each type of bentonite to achieve a water activity of 0.99.

| Bentonite | Volume of water (mL) to add to 10 g of bentonite | Volume of water (mL) to add to 80 g of bentonite |
|-----------|--------------------------------------------------|--------------------------------------------------|
| Asha      | 3.5                                              | 28.0                                             |
| MX6       | 4.3                                              | 34.0                                             |
| MX7       | 4.4                                              | 35.0                                             |
| MX9       | 3.0                                              | 24.0                                             |
| GFM       | 4.5                                              | 36.0                                             |

For each type of bentonite, a total of 21 microcosms were set up in sterile 250 mL mason jars. Triplicate jars were incubated at 15, 30, 45, 60, 75, 90, or 105°C for one week (Figure 4.1). Setup occurred on the bench and hydrated clay microcosms were incubated under oxic conditions. After incubation, the jars were shaken to mix the clay, and a subsample was collected for cultivation and for DNA extraction. Cultures were set up immediately, and bentonite intended for DNA extraction was stored at -20°C until DNA extraction.

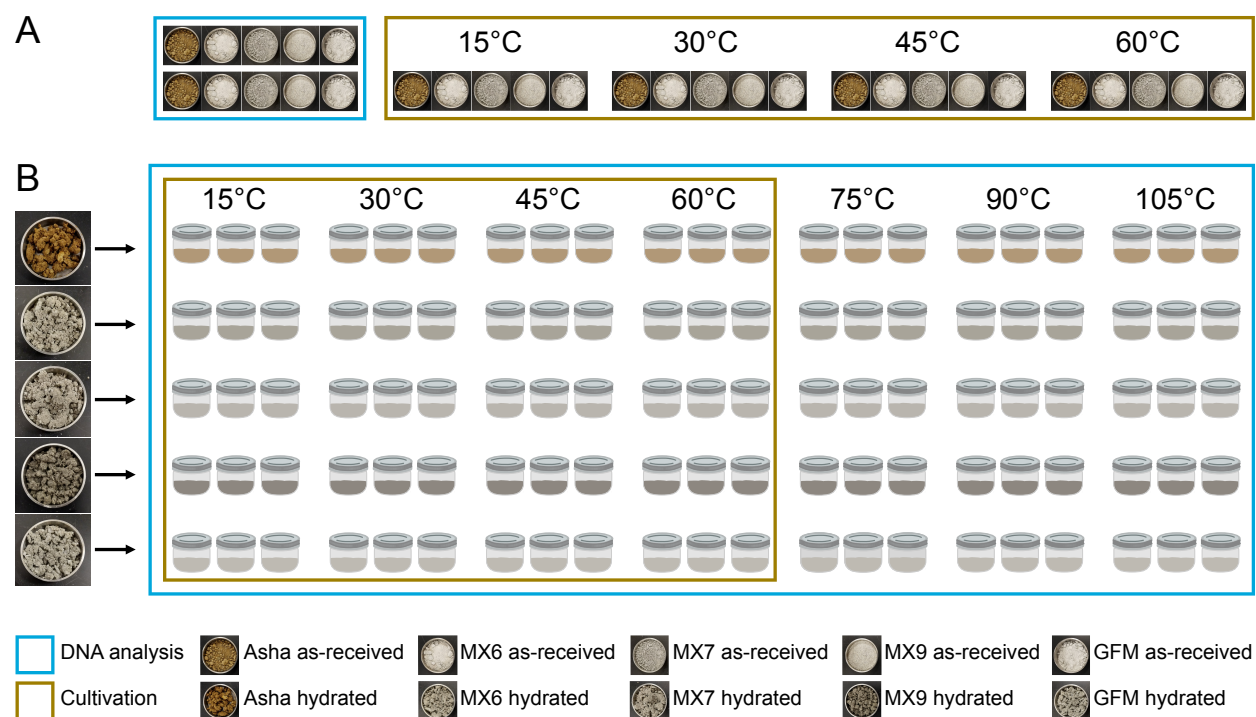


**Figure 4.1** Schematic of experimental set up of as-received bentonite (A) and hydrated bentonite (B) DNA and cultivation-based analyses for five bentonite types.

### 4.2.3 Cultivation from microcosms

For each bentonite sample (as-received or hydrated), 2 g of bentonite was added to 18 mL of phosphate buffered saline solution (PBS; 8 g NaCl, 0.2 g KCl, 1.44 g Na<sub>2</sub>HPO<sub>4</sub>, and 0.24 g KH<sub>2</sub>PO<sub>4</sub> in a final volume of 1 L Type I water, with pH adjusted to 7.4 using HCl) in a 25 mL tube. Tubes were vortexed horizontally at low speed for 30 minutes to disperse the bentonite in the PBS. This is referred to as dilution A throughout. One mL of dilution A was added to 9 mL of PBS and is referred to as dilution B, and 1 mL of dilution B to a separate 9 mL of PBS and is referred to as dilution C.

Three types of cultures were set up: aerobic heterotrophs, anaerobic heterotrophs, and SRB. For heterotrophs, 100 µL of dilution A was added to six Reasoner's 2A (R2A) agar (M1687; HiMedia Laboratories) plates, 100 µL of dilution B to six R2A agar plates, and 100 µL of dilution B to three R2A agar plates. Three dilution A, three dilution B, and three dilution C plates were incubated under oxic conditions for 5 days, and three dilution A and three dilution B plates under anoxic conditions for 28 days. An agar concentration of 1.5% was used for plates incubated at 15 and 30°C, and a concentration of 3% was used for plates incubated at 45 or 60°C as 1.5% agar plates partially melted during incubation at elevated temperatures.

For SRB, five test tubes filled with 9 mL of SRB medium (M803; HiMedia Laboratories) were inoculated with 1 mL of dilution A, five test tubes with 1 mL of dilution B, and five test tubes with 1 mL of dilution C. All SRB cultures were incubated under anoxic conditions for 28 days. For cultures incubated anoxically, tubes or plates were placed in vacuum chambers (BVV3GSSW, BVV) containing a GasPak EZ anaerobe sachet (BD) to help remove residual oxygen and an anaerobic indicator strip (BD) to verify anoxic conditions. Vacuum chambers were flushed five times with a 90% N<sub>2</sub> and 10% CO<sub>2</sub> gas mixture to create an anoxic environment.

For each as-received (un-hydrated) clay, a set of cultures was incubated at each of 15, 30, 45, and 60°C (Figure 4.1). For hydrated clays, cultures were incubated at the same temperature as the hydrated bentonite used to inoculate the culture (Figure 4.1). Overall, for each of the five clay samples, triplicate sets of cultures, each derived from a biological replicate mason jar, were incubated at each of 15, 30, 45, and 60°C. Although hydrated clay microcosms were also

incubated at 75, 90, and 105°C, cultures were not incubated at these temperatures because agar would melt.

After incubation, colonies on R2A agar plates were counted, and these numbers were used to calculate values of CFU/g. For SRB cultures, tubes were examined for SRB activity, which was identifiable by the presence of a black ferrous sulfide precipitate produced by a reaction between SRB-derived sulfide and the ferric ammonium sulfate present in the SRB medium. Biomass was collected from each culture where growth was evident. For heterotroph cultures, colonies were collected on sterile foam-tipped applicators (Puritan), and for SRB cultures, 1 mL of each positive culture was collected and centrifuged at 8000 x g for 10 minutes to pellet cells. For a given bentonite sample, biomass from all positive A dilution SRB cultures was collected together, and biomass from all positive B and C dilution SRB cultures was separately collected. Biomass was stored at -20°C until DNA extraction.

#### **4.2.4 DNA extraction from bentonite and culture biomass**

Genomic DNA was extracted from 50 mg of the 105 (5 clay types x 7 temperatures x triplicate) hydrated clay samples and from duplicate aliquots of each of the five as-received clay samples using the DNeasy PowerSoil Pro kit (Qiagen). Genomic DNA was extracted from culture biomass using the DNeasy UltraClean kit (Qiagen). The manufacturer's instructions were followed with the following exceptions. After addition of lysis solution, PowerBead tubes were incubated at 70°C for 10 minutes followed by bead beating for 45 s using a FastPrep-24 Classic Instrument (MP Biomedicals) set to 5.5 m/s. DNA was eluted in 60 µL for PowerSoil extractions and in 50 µL for UltraClean extractions. After extraction, DNA samples were stored at -20°C until further analysis.

#### **4.2.5 Amplicon sequencing of bentonite and culture biomass**

For each genomic DNA sample (hydrated clay, as-received clay, culture biomass), the V4-V5 region of the 16S rRNA gene was amplified in triplicate using universal primers 515F-Y [174] and 926R [157], modified to include a six-base index sequence, Illumina flow cell binding, and sequencing primer binding sites [158]. All PCR mixtures were prepared in a PCR hood (AirClean Systems) with ISO 5-HEPA-filtered air and surfaces wiped with 70% ethanol and UV-treated for 15 minutes before each use. Tubes, water, and BSA used for PCR were also UV-treated on a UV transilluminator (ProteinSimple) using 302 nm wavelength light. Each PCR

consisted of 1x ThermoPol Buffer (New England BioLabs), 0.2  $\mu$ M forward and reverse primer, 200  $\mu$ M dNTPs, 15  $\mu$ g BSA, 0.625 U hot start Taq DNA polymerase (New England BioLabs), and 1  $\mu$ L of template DNA in a final volume of 25  $\mu$ L. The following thermal cycle conditions were used: initial denaturation at 95°C for 5 minutes, followed by 35 (cultures) or 40 (clay) cycles of 95°C denaturation for 30 s, 50°C annealing for 30s, and 68°C extension for 7 min, followed by a final extension step for 7 min at 68°C. No template controls (NTCs) were included in each 96 well plate to test for contamination between wells, and additional NTC and positive controls were included in tubes to test for master mix contamination and appropriate amplification, respectively.

Triplicate PCR products were imaged on a 1% agarose gel stained with ethidium bromide, then triplicates were pooled and quantified on a second 1% agarose gel stained with GelRed (Biotium). Based on gel quantification, equimolar quantities of each sample were pooled. Kit controls and NTCs were added to the pool, regardless of visible amplification, at a volume of 5  $\mu$ L. This pool was electrophoresed on a 1.5% agarose gel stained with ethidium bromide, and the band corresponding to the amplicons was excised and purified using the Wizard SV Gel and PCR Clean-Up System (Promega). The purified pool was quantified using the Qubit dsDNA High Sensitivity Assay Kit (Invitrogen) and with qPCR using P5 and P7 primers (Illumina). The library was denatured and diluted following Illumina guidelines (Document No. 15039740 v01), then spiked with PhiX Sequencing Control v3 (Illumina).

All as-received clay and associated cultures were sequenced on a MiSeq run with a cluster density of 659,000 clusters/mm<sup>2</sup>, average Q30 of 93.0%, and clusters passing filter of 92.5%. All hydrated clay samples were sequenced on second MiSeq run with a cluster density of 774,000 clusters/mm<sup>2</sup>, average Q30 of 90.0%, and clusters passing filter of 91.4%. All culture samples from hydrated clay were sequenced on third MiSeq run with a cluster density of 813,000 clusters/mm<sup>2</sup>, average Q30 of 89.5%, and clusters passing filter of 93.2%.

#### **4.2.6 Microcosm sample sequence analysis**

Sequences were demultiplexed using Local Run Manager version 4. Demultiplexed reads were further analyzed using Quantitative Insights Into Microbial Ecology 2 (QIIME2) [159] including Divisive Amplicon Denoising Algorithm 2 (DADA2) [160]. Paired-end reads were imported into QIIME2, and DADA2 was used to remove low quality reads and primer sequences

and to truncate reads to 250 bases. Next, sequences were denoised, dereplicated, and amplicon sequence variants (ASVs) were generated, again using DADA2 through QIIME2. Taxonomy was assigned to ASVs using a naïve Bayes classifier pre-trained with SILVA databased release 138. The Decontam R package [175] was used to identify potential contaminant sequences. A threshold of 0.5 was selected by examining histograms of Decontam score distribution. For clay samples, several ASVs flagged as contaminants were associated with thousands of reads in culture samples and only a few reads in one or a few controls. As such, any ASV flagged as a contaminant that was associated with >1000 reads in at least one culture and <50 reads in any control was removed from the list of contaminants. Contaminant ASVs were removed from the ASV table prior to subsequent analysis.

All sequence data were deposited in the European Nucleotide Archive with accession number PRJEB79490.

### 4.3 Results

#### 4.3.1 Mineral characterization of as-received bentonite

The mineral composition of the five as-received bentonite samples used throughout were measured. Regardless of source, the mineral composition of the five bentonite samples were similar (Table 4.2).

**Table 4.2** Mineral composition (%) of the five bentonite clays used. When the mineral was not detected, it is reported as “ND,” and when detected in amounts too low to quantify is reported as “trace.”

| Mineral | Montmorillonite<br>( $(\text{OH})_2\text{Si}_2\text{Al}_2\text{O}_{10}\cdot n\text{H}_2\text{O}$ ) | Quartz<br>( $\text{SiO}_2$ ) | Cristobalite<br>( $\text{SiO}_2$ ) | Plagioclase<br>( $\text{NaAlSi}_3\text{O}_8$ -<br>$\text{CaAl}_2\text{Si}_2\text{O}_8$ ) | K feldspar<br>( $\text{KAlSi}_3\text{O}_8$ ) | Mica ( $\text{XY}_2$ -<br>$3\text{Z}_4\text{O}_{10}(\text{OH})_2$ ) | Clinoptilolite<br>( $(\text{Na}_2,\text{K}_2,\text{Ca})_3$<br>$\text{Al}_6\text{Si}_{10}\text{O}_{72}\cdot 24$<br>$\text{H}_2\text{O}$ ) | Kaolinite<br>( $\text{Al}_2\text{Si}_2\text{O}_5(\text{OH})_4$ ) | Calcite<br>( $\text{CaCO}_3$ ) | Gypsum<br>( $\text{CaSO}_4\cdot 2$<br>$\text{H}_2\text{O}$ ) | Goethite<br>( $\text{FeO}(\text{OH})$ ) |
|---------|----------------------------------------------------------------------------------------------------|------------------------------|------------------------------------|------------------------------------------------------------------------------------------|----------------------------------------------|---------------------------------------------------------------------|------------------------------------------------------------------------------------------------------------------------------------------|------------------------------------------------------------------|--------------------------------|--------------------------------------------------------------|-----------------------------------------|
| Asha    | 84.4                                                                                               | 1.4                          | ND                                 | 4.8                                                                                      | ND                                           | ND                                                                  | ND                                                                                                                                       | 3                                                                | 3.1                            | 1.4                                                          | 1.9                                     |
| MX6     | 77.3                                                                                               | 3.9                          | 1.4                                | 9.5                                                                                      | 4.1                                          | 2.2                                                                 | ND                                                                                                                                       | ND                                                               | 1.6                            | trace                                                        | ND                                      |
| MX7     | 77.5                                                                                               | 3.7                          | 1.5                                | 11.8                                                                                     | 3.1                                          | 1.7                                                                 | ND                                                                                                                                       | ND                                                               | 0.7                            | trace                                                        | ND                                      |
| MX9     | 88.4                                                                                               | 1.3                          | ND                                 | 4.6                                                                                      | 2.8                                          | 1.1                                                                 | ND                                                                                                                                       | ND                                                               | 1.8                            | trace                                                        | ND                                      |
| GFM     | 73.7                                                                                               | 1.6                          | 2.7                                | 10.4                                                                                     | 2.9                                          | 2                                                                   | 6.7                                                                                                                                      | ND                                                               | trace                          | trace                                                        | ND                                      |

#### 4.3.2 Cultivation of bentonite from microcosms

Culturable aerobic heterotrophs were detected in each of the clays, dry or hydrated, when cultures were incubated at 15, 30, or 45°C (Figure 4.2). Culturable aerobic heterotrophs grew at

60°C from Asha, MX9, and GFM as-received clays, and from all but MX6 when the clay was first hydrated and incubated at 60°C for a week (Figure 4.2). The average abundance of culturable aerobic heterotrophs in as-received clays at 15, 30, 45, and 60°C was  $5.1 \times 10^2$ ,  $1.8 \times 10^4$ ,  $2.1 \times 10^3$ , and  $3.4 \times 10^1$  CFU/g, respectively. After hydration, the average abundance of aerobic heterotrophs at 15, 30, 45, and 60°C was  $2.8 \times 10^4$ ,  $3.2 \times 10^6$ ,  $3.2 \times 10^6$ , and  $1.3 \times 10^3$  CFU/g, respectively. At 30°C, there were more aerobic heterotrophs cultured from hydrated clay compared to as-received clay for MX6 and MX7, and at 45°C for all clays other than GFM (Figure 4.2 stars; Tukey's HSD,  $p < 0.05$ ). For aerobic heterotroph cultures grown at 15 or 60°C, the abundance before and after hydration was not significantly different for any bentonite type (Figure 4.2; Tukey's HSD,  $p < 0.05$ ). For each hydrated clay, the highest abundance of culturable aerobic heterotrophs was either at 30 or 45°C (Figure 4.2). The value of culturable aerobic heterotrophs at 30 and 45°C was statistically the same for all clays other than GFM, for which the abundance at 30°C was greater than that at 45°C, and was often also the same as abundances at 15°C (Figure 4.2 letters; Tukey's HSD,  $p < 0.05$ ). The abundance of culturable aerobic heterotrophs at 60°C for each hydrated clay was significantly lower than for the same hydrated clay incubated at 30°C. For all as-received clays, there was no statistical difference between the abundance of aerobic heterotrophs cultured at any of the four temperatures (Tukey's HSD,  $p < 0.05$ ).

Culturable anaerobic heterotrophs were detectable in many fewer samples than aerobic heterotrophs (Figure 4.2). Anaerobic heterotrophs were cultured from all as-received bentonites at 45°C, all but MX9 at 30°C, and all but MX6 and GFM at 15°C (Figure 4.2). At 60°C, the only as-received clay from which anaerobic heterotrophs were culturable was GFM (Figure 4.2). The abundance of culturable anaerobic heterotrophs in as-received clay was at most 127 CFU/g (45°C). Once hydrated and incubated for a week, most clays did not have culturable anaerobic heterotrophs regardless of if they did initially in the as-received clay (Figure 4.2). At each temperature, a maximum of one hydrated clay had culturable anaerobic heterotrophs, and no clay had culturable heterotrophs at more than one temperature. At 15°C, MX7 had culturable heterotrophs, at 30°C Asha, at 45°C MX6, and at 60°C, no hydrated clay had culturable anaerobic heterotrophs (Figure 4.2).

SRB were cultured from as-received clay at all four tested temperatures for all bentonite types, at a maximum abundance of 144 MPN/g (30°C; Figure 4.2). They were culturable from all hydrated clays incubated at 15 or 30°C, at similarly low abundances, but were often not detectable in clays hydrated and incubated at 45 or 60°C (Figure 4.2). At 60°C, SRB were only culturable from Asha and GFM, and at 45°C from all but MX7, though for MX6 and MX9 only one tube positive for SRB growth was observed, each for only one of three biological replicate microcosms (Figure 4.2).

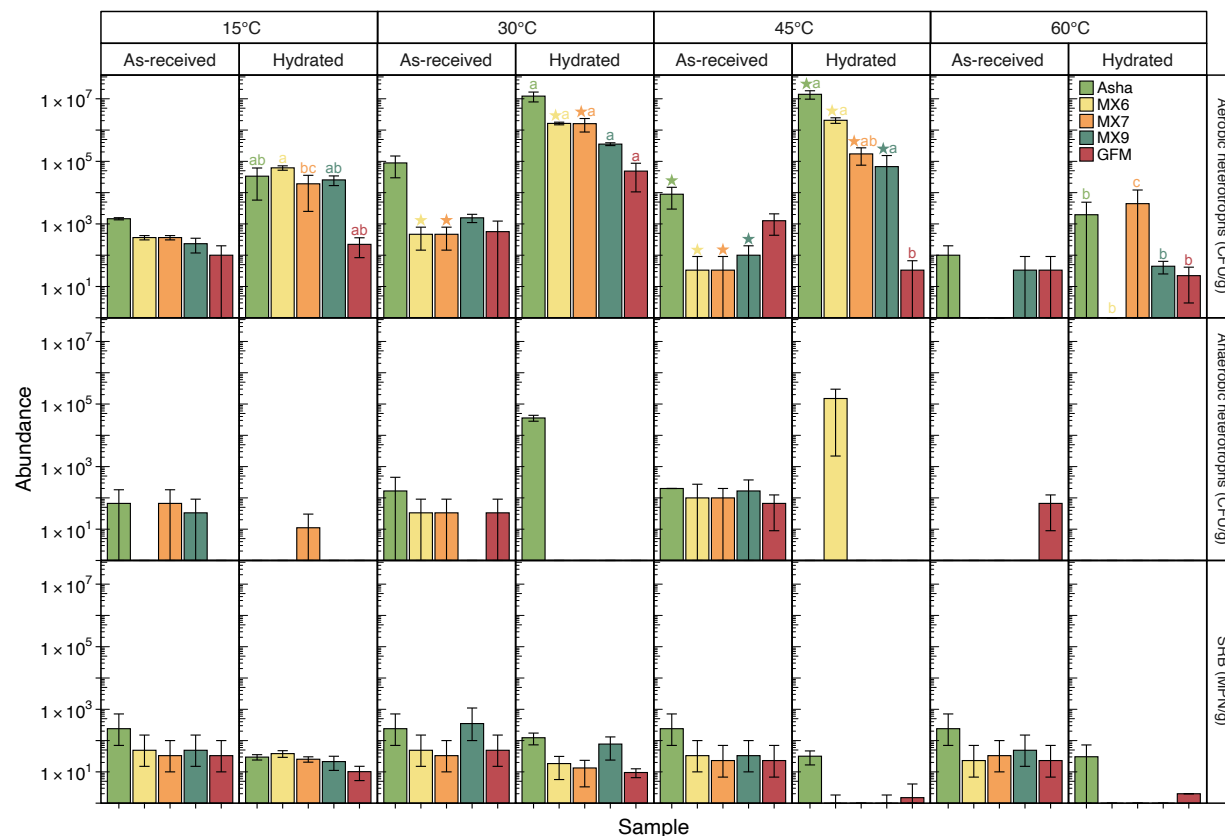


**Figure 4.2** Bar plot showing the abundance of culturable aerobic heterotrophs, anaerobic heterotrophs, and sulfate-reducing bacteria (SRB) grown at 15, 30, 45, or 60°C from as-received or hydrated Asha, MX6, MX7, MX9, or GFM bentonite. For cultures set up from as-received clay, error bars represent the standard deviation between technical triplicates (heterotrophs) or the 95% confidence interval provided by the MPN table (SRB). For cultures set up from hydrated clays, error bars represent the standard deviation between biological triplicate microcosms. Stars in the aerobic heterotrophs panel represent clays for which there was a significant difference between the value of CFU/g before and after hydration at the given temperature (Tukey's HSD test,  $p < 0.05$ ). Small letters in the aerobic heterotroph panel show statistically generated groups for the four temperatures of a given clay (Tukey's HSD test,  $p < 0.05$ ). For dry clays, no letters are included as all incubation temperatures led to the same abundance of culturable heterotrophs for each clay (Tukey's HSD,  $p < 0.05$ ).

#### 4.3.3 16S rRNA gene analysis of as-received bentonite and associated cultures

Although there were differences in 16S rRNA gene profiles between the five different as-received bentonite types, as-received bentonite profiles were generally dominated by sequences associated with *Actinobacteriota* and *Proteobacteria* (Figure 4.3). The most abundant genera among as-received clay samples were *Amycolaptosis*, *Arthrobacter*, *Nocardiopsis*, *Streptomyces*, *Bacillus*, *Paracoccus*, *Xanthomonas*, and family *Micrococcaceae* (Figure 4.3).

Whereas as-received clays were dominated by sequences associated with phyla *Actinobacteriota* and *Proteobacteria*, most cultures from as-received clays were dominated by sequences associated with *Firmicutes*, regardless of clay, culture type, or incubation temperature (Figure 4.3). In aerobic heterotroph cultures incubated at 15 or 30°C, all 16S rRNA gene profiles were dominated (i.e., 68.5-100.0%) by sequences associated with *Bacillus*. At 30°C, sequences associated with *Paenibacillus* also had relatively high abundances in Asha, MX6, and MX7 profiles, and *Sphingomonas* and *Staphylococcus* in MX9 and GFM, respectively (Figure 4.3). Aerobic heterotroph cultures incubated at 45°C were either dominated by sequences associated with *Bacillus* (Asha and GFM), *Streptomyces* (MX6), or *Paenibacillus* (MX9). The aerobic heterotrophs cultures incubated at 60°C had no overlap of dominant genera for the three clays from which aerobic heterotrophs were culturable (Figure 4.3). That said, biomass extracted from MX9 and GFM cultures incubated at 60°C had very low numbers of sequencing reads (i.e., 8 and 44 respectively). Asha was dominated by sequences associated with *Geobacillus*, MX9 by sequences associated with *Comamonadaceae*, and GFM by sequences associated with *Allivibrio*, *Enterobacteriaceae*, and *Fervidobacterium* (Figure 4.3).

Similar to aerobic heterotroph cultures, anaerobic heterotroph cultures incubated at both 15 and 30°C also often had relatively high abundances of sequences associated with *Bacillus* in all but the GFM 30°C cultures, which were instead dominated by sequences affiliated with *Clostridium sensu stricto 1* (Figure 4.3). Genus *Paenibacillus* was also represented by relatively abundant reads in Asha cultures incubated at both 15 and 30°C (Figure 4.3).

The dominant putative SRB represented in the 16S rRNA gene profiles of SRB cultures was associated with the *Desulfosporosinus* genus, although *Desulfotomaculum* sequences dominated Asha cultures incubated at 15 or 30°C, and was additionally detected in lower relative abundance in cultures from GFM and MX6 incubated at 45 and 60°C, respectively (Figure 4.3). Sequences associated with *Desulfosporosinus* and/or *Desulfotomaculum* were detected in all SRB cultures from as-received clay except for SRB cultures from Asha incubated at 15°C, from MX7 and MX9 incubated at 45°C, and from GFM incubated at 60°C, which were instead dominated by *Caldibacillus* (GFM at 60°C) or *Bacillus* (Asha, MX6, MX7, MX9; Figure 4.3).

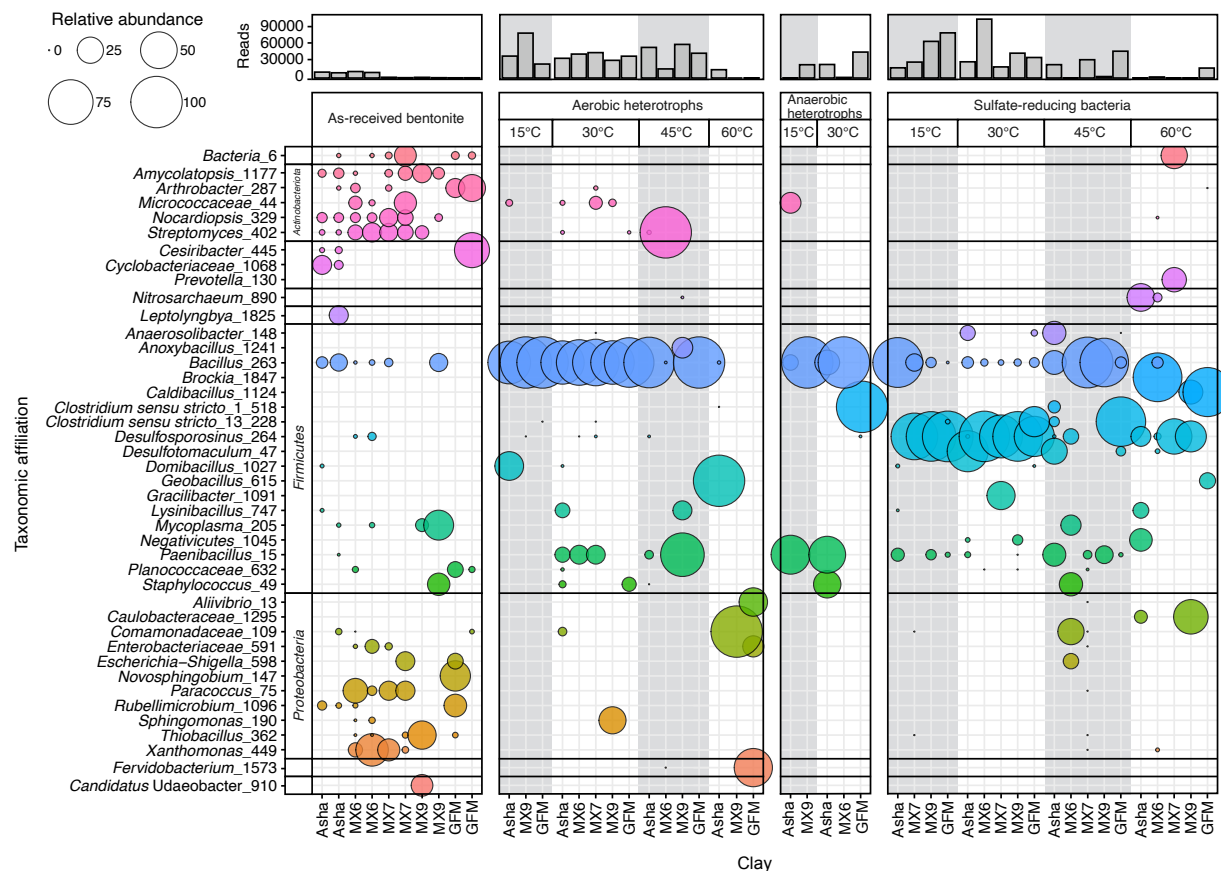


**Figure 4.3** Bubble plot of 16S rRNA gene profiles of as-received bentonite samples and cultures set up from as-received bentonite samples and incubated at 15, 30, 45, or 60°C. Only ASVs with a minimum relative abundance of 5% in at least one sample are displayed. The bar plot displays the number of sequence reads obtained for each sample.

#### 4.3.4 16S rRNA gene analysis of hydrated bentonite and associated cultures

The 16S rRNA gene profiles of hydrated clay samples were often dominated by similar sequences when the bentonite was incubated at 15, 30, or 45°C (Figure 4.4). The three Wyoming MX-80 bentonites (MX6, MX7, and MX9) had profiles dominated by sequences associated with *Streptomyces*. Sequence associated with *Micrococcaceae* were also relatively abundant when the bentonite was incubated at 15 or 30°C, and sequences associated with *Nocardioptosis* were abundant when bentonite was incubated at 30 or especially 45°C (Figure 4.4). Hydrated Asha and GFM replicates had especially low sequence read abundances, likely contributing to replicate profiles with little overlap. In ordination space, all hydrated clays incubated at 15 or 30°C grouped together, both when weighted UniFrac and Bray Curtis distances were used, and separately from the un-hydrated as-received bentonite (Figure 4.5). The bentonite incubated at 45°C had overlap with 15 and 45°C bentonite samples, but also had comparatively higher

relative abundances of sequences associated with *Nocardiopsis*, which was prevalent and relatively abundant in as-received bentonite samples (Figure 4.4). In ordination space, these 45°C samples grouped with the 15 and 30°C samples when weighted UniFrac distances were used, but more closely with starting material when Bray Curtis distances were used (Figure 4.5).

For bentonite hydrated and incubated at 60°C, all replicates of MX6 and MX7 had higher abundances of sequence reads than the remaining three bentonite samples (Figure 4.4). The profiles of these samples were dominated (99-100%) by sequences associated with family *Thermoactinomycetaceae* (Figure 4.4). In ordination space, these *Thermoactinomycetaceae*-dominated samples grouped together and separately from all other samples (Figure 4.5). Overall, temperature was significantly correlated ( $p=1 \times 10^{-4}$ ) to both weighted-UniFrac and Bray Curtis-based ordinations ( $R^2=0.54$  and  $R^2=0.58$ , respectively; Figure 4.5). The family *Thermoactinomycetaceae* was also present, though less abundant, in MX6 and MX7 incubated at 45°C (Figure 4.4). It was not present in MX6 or MX7 incubated at 75, 90, or 105°C, though it was present in one replicate of GFM incubated at 90 and at 105°C (Figure 4.4). Sequences associated with *Thermoactinomycetaceae* were only detected in one as-received clay sample (one replicate of Asha) at 1.5% relative abundance. The 16S rRNA gene profiles of all samples incubated at 75, 90, or 105°C had very little overlap, likely due to the very low sequencing depth for most of these samples (Figure 4.4). In general, bentonite incubated at temperatures of 60°C or higher either had very few (average 241) sequencing reads, perhaps indicative of no or little microbial growth in the bentonite microcosm, or had relatively high sequencing depth and profiles dominated by sequences associated with family *Thermoactinomycetaceae* (Figure 4.6).

Sequences associated with *Desulfosporosinus* and *Desulfovibrionaceae*, both genera of known SRB, were detected in the hydrated clay profiles in samples with very low read counts, perhaps resulting from contamination of these samples from SRB culture samples with high abundances of these sequences. Sequences associated with *Desulfosporosinus* were detected in one MX9 replicate incubated at 75°C, which had a total of two reads, and sequences associated with *Desulfovibrionaceae* were detected in an MX6 replicate incubated at 105°C that had 91 reads total, and in an Asha replicate incubated at 30°C that had 158 reads total.

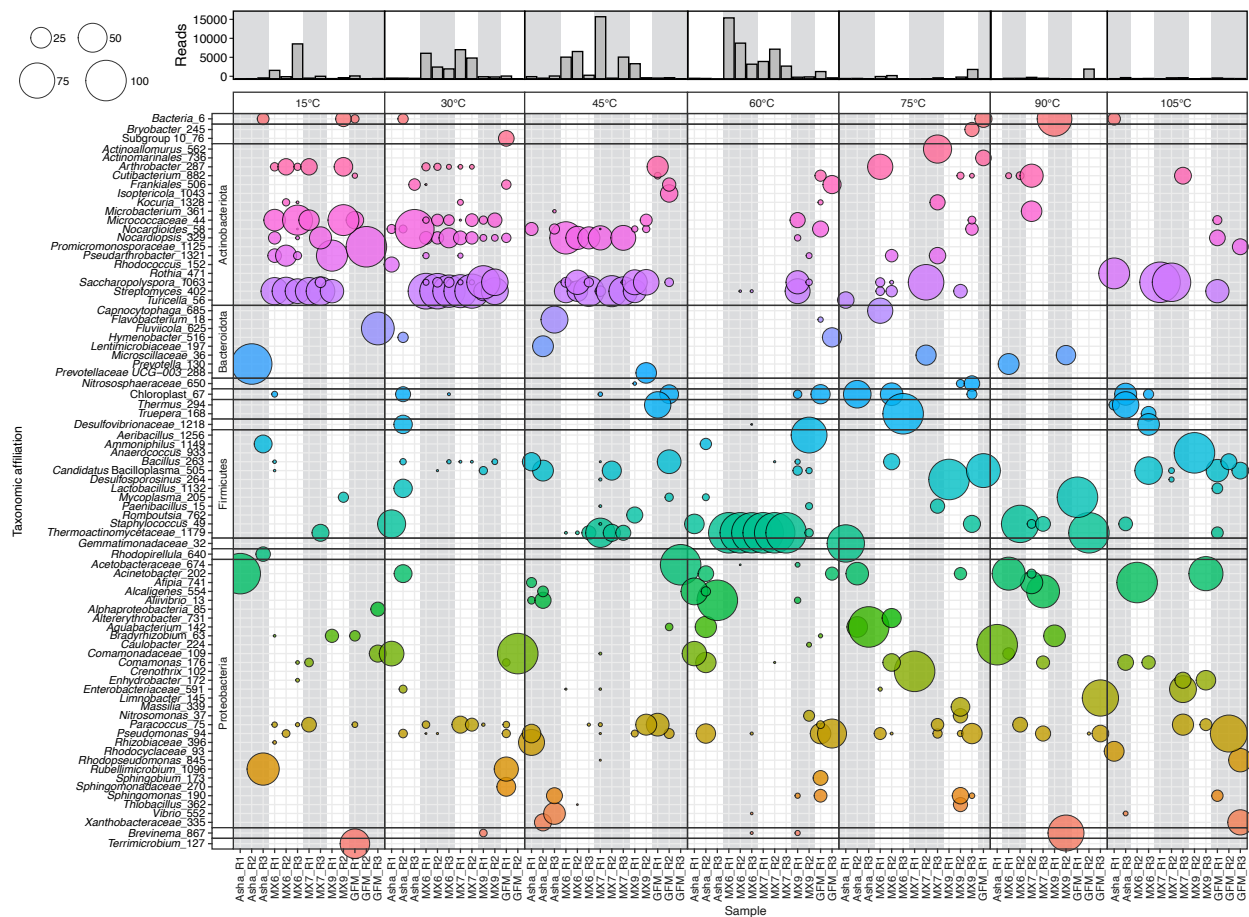


**Figure 4.4** Bubble plot showing the 16S rRNA gene profiles of five bentonite samples (three biological replicates each) hydrated with Type I water and incubated for 7 days at 15, 30, 45, 60, 75, 90, or 105°C. Only ASVs with a minimum relative abundance of 10% in at least one sample are shown. The bar plot displays the total number of sequence reads for each sample.

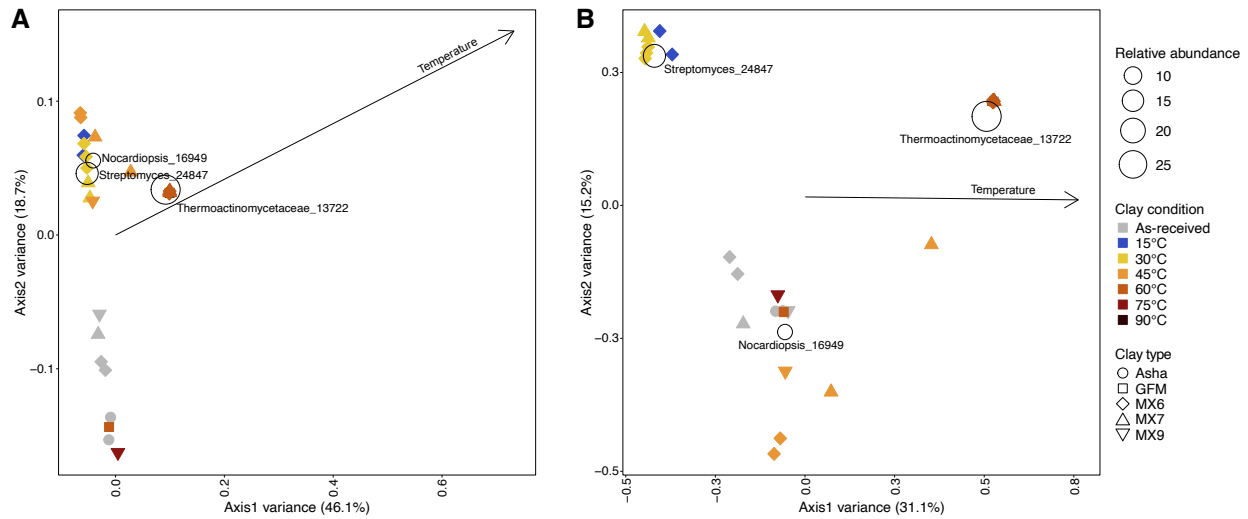


**Figure 4.5** Triplots based on weighted UniFrac (A) and Bray Curtis (B) distances between 16S rRNA gene profiles of as-received bentonite and hydrated bentonite incubated at 15, 30, 45, 60, 75 or 90°C. The vector represents the correlation of temperature with the ordination ( $R^2=0.54$  and  $p=1 \times 10^{-4}$  for weighted UniFrac,  $R^2=0.58$  and  $p=1 \times 10^{-4}$  for Bray Curtis). Taxa bubbles represent taxa that have a minimum relative abundance of 5% across the dataset. The ASV table was normalized to 1000 reads using scaling with ranked subsampling (SRS) prior to computing distances.

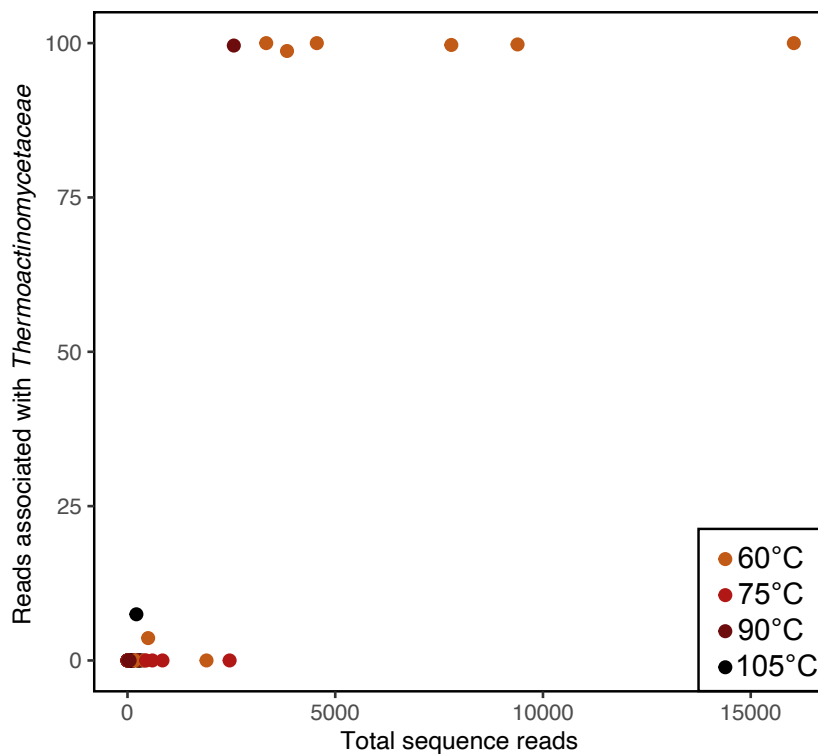


**Figure 4.6** Scatterplot showing the total number of sequence reads and the total number of reads associated with family *Thermoactinomycetaceae* in all hydrated clay samples incubated at 60, 75, 90, or 105°C.

The heterotroph cultures inoculated with hydrated bentonite and incubated at 15, 30, or 45°C had 16S rRNA gene profiles dominated by sequences associated with similar taxa to those that dominated the hydrated clay profiles (Figure 4.4, Figure 4.7). For most aerobic heterotroph cultures incubated at 15 or 30°C, 16S rRNA gene profiles were dominated by sequences associated with *Micrococcaceae*. Exceptions to this were GFM replicates incubated at 15°C, which were dominated by sequences associated with *Bacillus*, and Asha replicates incubated at 30°C, which had a slightly higher relative abundance of *Sinomonas* (Figure 4.7). When grown under anoxic conditions at 30°C, profiles were instead dominated by sequences associated with *Bacillus* (Figure 4.7). With or without oxygen, heterotroph cultures incubated at 45°C were dominated by sequences associated with *Streptomyces* (Figure 4.7). There were three heterotroph samples with very high relative abundances of sequences associated with *Desulfosporosinus* (Figure 4.7). These samples each had a maximum of 16 sequence reads total, and thus this ASV likely represents contamination from a concentrated SRB culture sample. The dominant taxa from high temperature hydrated clay profiles, *Thermoactinomyces*, was only detected in one aerobic heterotroph sample (Asha-R1 incubated at 60°C), where it made up 95% of the 37,889 sequencing reads (Figure 4.7).

Like SRB cultures inoculated with as-received clay, the dominant genus of SRB in SRB cultures inoculated with hydrated clay was *Desulfosporosinus*, except for replicates of Asha bentonite incubated at 30 or 45°C, which were instead dominated by *Desulfotomaculum* (Figure 4.7). In SRB cultures incubated at 60°C, sequences associated with known SRB were only detected in one Asha replicate. Other SRB cultures at this temperature had poorly overlapping 16S rRNA gene profiles with dominant taxa including *Actinomadura*, *Micrococcaceae*, *Aeribacillus*, and *Bacillus* (Figure 4.7).

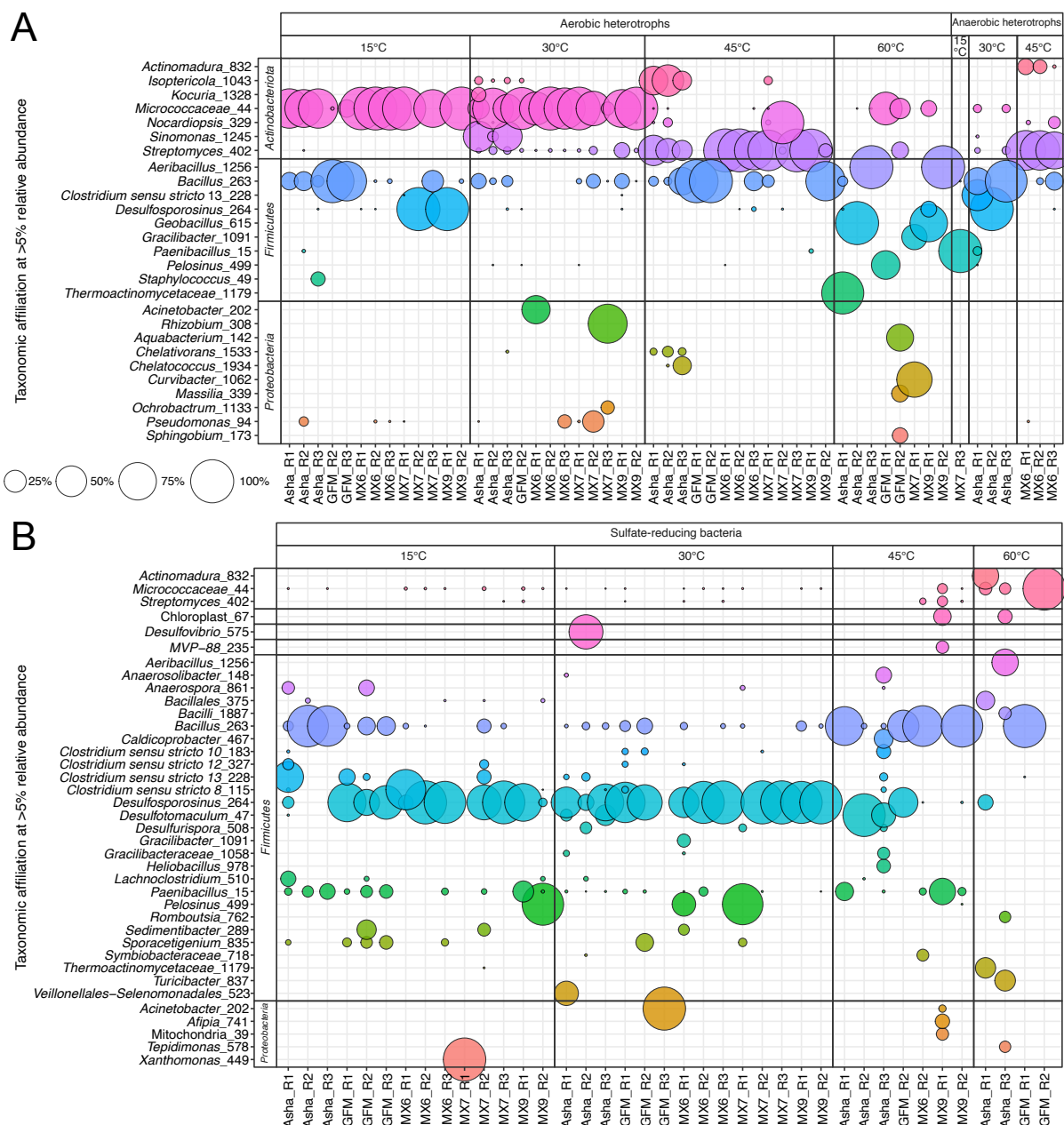


**Figure 4.7** Bubble plot showing the 16S rRNA gene profiles of aerobic and anaerobic heterotrophs (A) and SRB cultures (B) inoculated with hydrated clay, and incubated at either 15, 30, 45, or 60°C. Only ASVs with a minimum relative abundance of 5% in at least one sample are shown.

#### 4.4 Discussion

Studies exploring the microbiology of bentonite clay in the context of long-term nuclear waste storage usually use bentonite incubated at 30°C, room temperature, or even cooler temperatures associated with the subsurface at or above planned repository depth, and microbial cultures from bentonite are most commonly incubated at 30°C, or occasionally slightly cooler

temperatures (e.g., 15 or 20°C) [10, 123–128, 151, 173]. One major limitation of this approach is that, after a relatively short period (e.g., less than a year) with moderate temperatures appropriate for mesophilic microbial growth, a DGR is expected to experience elevated temperatures (as high as 93°C in the models of the Canadian design) for thousands of years [10]. As such, one goal of this study was to determine if DGR-relevant as-received bentonite clay contains microorganisms capable of growing at lower and higher temperatures than 30°C. The cultivation results demonstrate that microorganisms are culturable from a variety of as-received bentonite clays at a temperature of 30°C as has previously been shown [10], but also at temperatures of 15, 45, and 60°C (Figure 4.2). The abundance of culturable microorganisms from as-received clay was similar regardless of incubation temperature. That said, at the highest tested temperature of 60°C, aerobic heterotrophs were not culturable from two bentonite samples and anaerobic heterotrophs were only culturable from one bentonite sample, suggesting that some as-received bentonite types may have microorganisms better adapted to withstanding high temperature than others (Figure 4.2).

Previous experiments showed that, when water is added to bentonite, there is often an increase in abundance of culturable heterotrophs provided that swelling pressure is sufficiently low (either from incubating hydrated bentonite in an unconfined space or from incomplete saturation of a system that will eventually experience elevated swelling pressure) [125, 173]; Chapter 2, Chapter 3]. Similar to these previous experiments conducted at 30°C, in the present study there was also an increase in the abundance of culturable aerobic heterotrophs after bentonite hydration and incubation at 30°C, and also often 15 and 45°C, but never 60°C (Figure 4.2). SRB were always detected in low abundance, and in some clays remained culturable at all tested incubation temperatures, suggesting the presence of thermotolerant but likely not thermophilic SRB (Figure 4.2). The decreased number of hydrated clays, compared to as-received clays, with detectable anaerobic heterotrophs could possibly be because they were outcompeted by aerobic heterotrophs which often saw significant increases in abundance from as-received starting material to hydrated clay (Figure 4.2). These results agree with a previous study that showed that the abundance of culturable microorganisms in highly compacted bentonite (i.e., 1.7 g/cm<sup>3</sup>) incubated at 60°C did not increase [129]. In a separate study of clay slurries, no microbial activity was measured in 60°C incubations [130]. However, microorganisms remained alive throughout incubation at elevated temperature and were still

culturable after incubation [129, 130]. Combined with the results of the current study, cultivation results suggest that the microbial communities within bentonite clay may prefer more moderate temperatures (i.e., 15–45°C) but that members of the community are able to survive incubation at elevated temperature.

Decreased abundance of culturable aerobic heterotrophs incubated at 60°C, compared to lower temperature incubations, does not necessarily mean that thermophiles did not grow in these hydrated bentonite microcosms, and instead could reflect unsuitable cultivation conditions. A DNA-based analysis was included to examine shifts in bentonite community composition before and after hydration and incubation at elevated temperature as a second tool to identify microbial growth at elevated temperature. In agreement with a previous experiment conducted at 30°C using a similar setup, ASVs associated with *Streptomyces* dominated the microbial profiles of hydrated bentonite incubated at 30°C, and also 15 and 45°C [173]. At 15 and 30°C, sequences associated with *Micrococcaceae* were also relatively abundant, as were sequences associated with *Nocardiopsis* in bentonite incubated at 30 or 45°C. *Streptomyces*, *Micrococcaceae*, and *Nocardiopsis* are all members of the phylum *Actinobacteriota*, and are aerobic heterotrophs best known for their production of biotechnologically and medically relevant secondary metabolites [176–178], with some members of family *Micrococcaceae* being cold-adapted [179]. The fact that these taxa increased in relative abundance before and after bentonite hydration and incubation suggests that microorganisms within these taxa grew in the clay. This is further supported by 16S rRNA gene profiles of hydrated clay culture biomass that were dominated by sequences associated with these same taxa, and an increase in culturable aerobic heterotrophs observed for these samples after hydration.

When sequencing depth was sufficiently high in bentonite incubated at temperatures of 60°C or higher, 16S rRNA gene profiles were dominated by sequences associated with family *Thermoactinomycetaceae* (Figure 4.4, Figure 4.6). This was most apparent in the hydrated MX6 and MX7 replicates incubated at 60°C, where sequences associated with this family made up 99–100% of the sequencing reads (Figure 4.4, Figure 4.6). Family *Thermoactinomycetaceae* consists of thermophilic aerobic heterotrophs, often capable of producing endospores [180]. The fact that sequences associated with this family were not detected in as-received MX6 or MX7 replicates, coupled to the high relative abundance of sequences associated with this family in hydrated clays

with high sequence read counts, suggests that microorganisms belonging to family *Thermoactinomycetaceae* grew (i.e., increased in abundance) in several high temperature (e.g., MX6 and MX7, 60°C) microcosms. A similar observation was reported in a study of Bavarian bentonite in a slurry with Opalinus clay porewater, where no microbial activity was measured in 60°C microcosms, but DNA sequencing revealed a shift in microbial community composition from starting material to after hydration and incubation. In this study, after incubation, microbial profiles were dominated by the thermophilic species *Caldinitratiruptor microaerophilus* [130], which is a facultatively microaerophilic, heterotrophic, nitrate-reducing, non-spore-forming bacterium with an optimal growth temperature of 65°C [181]. Together, these results suggest that viable thermophilic microorganisms were present in several bentonite samples, potentially surviving as spores, and can proliferate when provided with suitable conditions (e.g., water and an elevated growth temperature). These results also suggest that using cultivation to demonstrate decreased abundance of viable microorganisms at elevated temperature may not be appropriate. Based on the DNA sequence results of the present study, the cultivation-based abundance results are likely an underestimate of the abundance of viable thermophiles in bentonite. Sequences associated with *Thermoactinomycetaceae* were rarely detected in cultures from hydrated clays, further suggesting that the cultivation conditions used here, and commonly elsewhere, were not ideal for these microorganisms.

In both as-received and hydrated bentonite, sequences associated with known SRB were rarely detected and never at high relative abundance (Figure 4.3, Figure 4.4). The SRB cultures inoculated with both as-received and hydrated clay were usually dominated by sequences associated with *Desulfosporosinus*, which is a commonly detected genus of SRB in bentonite clay [10, 127, 151, 173]; Chapter 2, Chapter 3]. Coupled with the consistently low abundances of culturable SRB in both as-received and hydrated clay, the results do not suggest any growth of SRB in bentonite under any tested conditions. That said, SRB are known to be obligate anaerobes, and the experiments presented here were conducted under oxic conditions.

Overall, the results suggest a temperature-dependent shift in microbial community composition when water is added to bentonite clay. At moderate (i.e., 15-45°C) incubation temperatures, several taxa that were detectable in as-received bentonite increased in abundance, and were confirmed to be viable because the same taxa were detected in 16S rRNA gene profiles

of culture biomass from hydrated clay cultures. At high incubation temperatures (i.e., 60-105°C), thermophilic taxa proliferated that were not detected in as-received clay. These results suggest that as-received bentonite likely contains numerous microbial cells at low relative abundance that can proliferate under specific incubation temperature conditions.

#### 4.5 Conclusion

Temperature is expected to vary substantially within the timeframe of a DGR. Temperature-related design specifications have been established to ensure the stability of copper and bentonite at elevated temperature, but the impact of temperature on microbial communities in bentonite has rarely been studied. The results of this study demonstrate that temperature does influence which microorganisms grow in bentonite when water is added, and that thermophilic microorganisms may exist in as-received bentonite at very low abundance. Cultivation results showed that aerobic heterotrophs, anaerobic heterotrophs, and SRB were culturable from at least some of the five tested bentonites at 15, 30, 45, and 60°C, suggesting that the as-received bentonite microbial community includes members adapted to a range of different temperatures. Adding water to as-received bentonite resulted in an increase in the abundance of culturable aerobic heterotrophs for several bentonite samples incubated at 30 or 45°C, and significantly fewer aerobic heterotrophs were cultured from hydrated bentonite incubated at 60°C compared to at 30°C for all bentonite samples. However, sequence data show a shift in microbial community composition for all MX6 and MX7 60°C microcosms from an initial community in the as-received clay including several taxa mostly from phyla *Actinobacteriota*, *Proteobacteria*, and *Firmicutes*, to profiles dominated (>99%) by sequences associated with the thermophilic family *Thermoactinomycetaceae*, which was not detected in the as-received MX6 or MX7 samples. For one of these bentonite samples (MX6), no culturable aerobic heterotrophs, anaerobic heterotrophs, or SRB were detected in any replicate. For the other, culturable aerobic heterotrophs were detected in a single replicate, and 16S rRNA gene profiles of biomass from this culture did not include sequences associated with *Thermoactinomycetaceae*. Together, these results suggest that thermophilic microorganisms exist within at least some as-received bentonite, though may not be detectable using a direct DNA-sequencing from as-received bentonite approach, and can grow in bentonite given sufficient water and an elevated temperature. These results further suggest that cultivation may underestimate the abundance of

thermophilic microorganisms in bentonite. This highlights the importance of a multifaceted approach to studying community composition of low biomass bentonite clays.

SRB are important targets for microbiology research of bentonite clay given the detrimental role they could play in microbiologically influenced corrosion within a DGR. The results of this study indicate that SRB did not increase in abundance for any of the hydrated bentonite microcosms, though they did remain culturable at low abundances up to 60°C. These results suggest that, at least while the DGR remains oxic, SRB are not likely to grow in bentonite at temperatures between 15 and 60°C, but they may persist in a viable state in bentonite even when exposed to elevated temperatures. The current experiments were conducted under oxic conditions with no compaction, which is a condition that may arise locally in and around a DGR, but generally bentonite is expected to be compacted, water less available, and oxygen consumed relatively quickly. As such, future studies should continue to incorporate different temperatures into their experimental design to test the effect of temperature combined with other parameters that are expected to vary within a DGR spatially and temporally (e.g., swelling pressure, water activity, and oxygen) and others that are inherent to the selected host rock (e.g., groundwater composition). This will allow for a more detailed understanding of the specific microorganisms that may proliferate in a DGR when swelling pressure is sufficiently low (e.g., during periods of incomplete saturation) to not suppress microbial growth altogether.

## **Chapter 5: Microbial analysis of bentonite cores from the Tsukinuno mine: a natural analogue to a deep geological repository for long-term storage of used nuclear fuel**

### **5.1 Introduction**

Bentonite clay is a key component of many proposed deep geological repository (DGR) designs for long-term storage of used nuclear fuel, including the Canadian design, where it acts as a barrier and sealant between used fuel containers (UFCs) and the host rock. As such, bentonite clay has been under study in several different fields. In the context of microbiology, it is critical to understand the composition and abundance of microbial communities within bentonite clay under various DGR-relevant conditions to understand if and how they may alter a DGR environment. Of particular interest are sulfate-reducing bacteria (SRB) because their activity can contribute to microbiologically influenced corrosion in anoxic environments, which may lead to the corrosion of metal used fuel containers in a poorly designed system [12]. Additional microbial metabolisms can lead to the production of gasses, which in high enough quantities could produce permeability fissures in the clay, thus leading to increased groundwater access to UFCs and potentially transporting corrosive compounds [71]. Because microorganisms generally do not live in isolation in an environment, understanding total microbial community responses are beneficial for DGR simulation studies.

Although laboratory and *in situ* experiments provide an ideal opportunity to test the effect of various parameters that are expected to fluctuate over the lifetime of a DGR (e.g., temperature, saturation and associated swelling pressure, moisture content, and water activity) and other parameters that are inherent to the design of the DGR (e.g., dry density, type of bentonite), one factor that cannot be tested effectively on a relevant scale is time. The long-lived fission products of used nuclear fuel have half-lives upwards of 100,000 years, which is thus the planned lifetime of a DGR [138]. To overcome this limitation, lab-scale and *in situ* studies can be applied to the study of natural analogue sites, which mimic aspects of a DGR and have existed in nature for DGR-relevant timeframes. Thus, natural analogues can serve as a “snapshot” of microbial community composition and abundance that could be expected in a DGR once conditions stabilize. The study of natural analogues comes with its own set of limitations, one major one being that it is difficult, if not impossible, to find a site that closely mimics all DGR conditions (e.g., montmorillonite content and related swelling capacity, dry density, saturation,

temperature, and water activity). Further, natural analogues cannot predict the behaviour of microbial communities in the early stages of a DGR when conditions are expected to fluctuate, which could be an important period of time for microbial activity. Nevertheless, coupling the study of natural analogue sites to detailed observations of microbial community response to various conditions through *in vitro* or *in situ* experiments can provide a more holistic prediction for the range of microbial population sizes, community compositions, and activity levels expected to occur in a DGR.

In the context of bentonite and microbiology, natural analogue sites have been identified and studied in three countries: Japan, Spain, and Canada. In Japan, the Kawasaki (Miyagi prefecture) and Tsukinuno (Yamagata prefecture) mines are located on calcium and sodium-type bentonite deposits respectively, and both have bentonites with relatively high montmorillonite contents, making them relevant to DGR bentonite [131]. One study looking at both sites observed higher counts of culturable heterotrophs nearest the drilling mouth, with abundances consistently decreasing to less than  $10^2$  cells/gdw by 1 m into the core [131]. The authors attempted to compare culture counts to several geochemical parameters and found no correlation for any parameter except moisture content and dry density, which were both weakly correlated (moisture content positively and dry density negatively) to abundance of culturable aerobic and anaerobic heterotrophs [131]. A more recent study at the Tsukinuno site incorporated cultivation of SRB and DNA-based techniques and measured low or zero culturable SRB, relatively low counts of culturable heterotrophs, and 16S rRNA gene profiles dominated by sequences associated with *Proteobacteria*, *Actinobacteria*, and *Euryarchaeota* [133]. Sequences associated with *Proteobacteria* and *Actinobacteria*, as well as *Bacteroidetes*, dominated 16S rRNA gene profiles of bentonite from El Cortijo de Archidona, El Toril, and Los Trancos (Spain) [135]. Similar to experiments on Japanese bentonites, the authors found no correlation between measured geochemical parameters and microbial community composition [135]. Although not specifically bentonite, an additional DGR-relevant natural analogue site in Canada, Cigar Lake, is a uranium deposit located approximately 450 m below ground and surrounded by a clay, such that no radioactivity is detectable at the surface [136]. Ground water associated with clay from this site was shown to have relatively low abundances of heterotrophs (i.e.,  $10^3$  to  $10^5$  cells/mL) and occasional detection of SRB, also at low abundance [136].

Although the study of DGR-relevant natural analogue sites focusing on microbiology began more than 30 years ago, natural analogue research remains relatively limited, and often predates the era of high-throughput DNA sequencing. To date, the study of natural analogues has revealed low abundances of culturable microorganisms, with SRB either not detected or detected in very low abundance, and attempts to correlate geochemical and physical parameters to microbial community composition or abundance has so far mostly revealed no correlations [131, 135]. This current study expands on a previous study of Tsukinuno bentonite grab samples [133]. The purpose was to assess the abundance and composition of microbial communities in 4-6 m bentonite cores, using both cultivation and DNA-based techniques, and to compare these to measured geochemical variables. Using a combination of cultivation and DNA-based techniques allowed for the study of both (a portion of) the viable community and the total DNA-based community, which previous studies have indicated could be in large part made up of relic DNA [133].

## **5.2 Materials and methods**

### **5.2.1 Sample site and bentonite sampling**

Four cores were drilled from the Tsukinuno mine. The Tsukinuno mine is situated in the Yamagata prefecture in Japan and consists of 35 layers of Na-type bentonite within the mining area, up to 7 m thick and located between parallel strata of shale [132]. The deposit formed in the middle Miocene epoch, between 12 and 16 million years ago, from diagenic alteration of volcanic ash deposited on the sedimentary basin during a period of high volcanic activity [132].

Three cores (D4A, D4B, and D4C) were drilled from bentonite bed 31 (Figure 5.1A), and one core (D6) was drilled from bentonite bed 29 (Figure 5.1B). Core D4A was drilled horizontally through and perpendicular to the tunnel wall (4.35 m total), and D4B and D4C were drilled from the same wall at an incline (3.55 and 4.75 m total). Core D6 was drilled vertically (5.95 m total). Cores were drilled in 1 m sections using a quadruple barrel corer. When cores were removed from the two outer steel layers, a flexible plastic (directly in contact with the bentonite) and acrylic layer remained around the bentonite throughout shipment (Figure 5.1C). Corer caps were screwed to the acrylic tube to seal the bentonite within.



**Figure 5.1** Photo of the drilling setup at the site where the D4 (A) and D6 (B) cores were retrieved, an example of a core in its acrylic casing (C) and once removed from the acrylic casing (D).

Three types of water were collected throughout bentonite sampling: tap water, which was used for drilling, drilling water at the end of drilling, and groundwater. For tap water and groundwater, 1 L was filtered first through a 0.22  $\mu\text{m}$  Stericup Vacuum Filter (Millipore), then the flow through was subsequently filtered through a 0.10  $\mu\text{m}$  Stericup Vacuum Filter (Millipore). This process was repeated with duplicate aliquots of each type of water. The drilling water had a large amount of suspended clay material and immediately clogged the filter. As such, water was collected and DNA subsequently extracted directly from the water with no filtration. All cores and filters were stored at 4°C from right after sampling, throughout shipment to the University of Waterloo, and upon arrival at the University of Waterloo.

### 5.2.2 Core processing

Cores were removed from 4°C storage one at a time for subsampling. For each of the four cores, 30 cm subsamples were collected at each meter mark, except for the end of the core nearest the tunnel opening, where the outside few mm of material was collected (“0 m” for the D4 cores and “5.2 m” for the D6 core) separately from the full 30 cm subsection (“0.1 m” for the D4 cores and “5.3 m for the D6 core; Figure 5.2). Cores are referred to throughout using the

name of the core (D4A, D4B, D4C, or D6) followed by the meter marker after a dash (e.g., D4A-1 refers to the 1 m subsample of the D4A core). The outside of the acrylic casing was wiped with 70% ethanol and a reciprocating saw with an ethanol-sterilized blade was used to cut the casing open on either side of the 30 cm section. Next to a flame, the inner plastic layer was removed with a sterile scalpel blade and the 30 cm section of core was collected on a sterile sheet of aluminum foil using a hammer and sterile chisel to separate the subsection from the rest of the core. A small amount of clay was collected from the ends of the subsample for immediate water activity and moisture content measurements in triplicate. The subsection was then wrapped in sterile foil and transported into an anaerobic chamber (Coy) for further processing to minimize oxygen exposure (Figure 5.1D).

Inside the chamber, the outside of the core was shaved using a sterile scalpel to remove any potential contamination introduced during the sampling process. The inner layer was then split as follows: 2 g was collected in triplicate for cultivation, 5 g was collected in triplicate for DNA extraction, 10 g was collected for XRD analysis, and 250 g was collected for geochemical analyses. For the two 0 m samples and the one 5.2 m sample, only a small amount of outer layer material was available, so XRD and geochemical analyses were excluded. For each analysis, bentonite from different parts of the 30 cm subsection were mixed. Bentonite intended for cultivation, XRD, and geochemical analyses was stored at 4°C prior to processing, and bentonite intended for DNA extraction was stored at -20°C prior to extraction.



**Figure 5.2** Schematic of the ~1 m core segments retrieved and the subsamples that were collected for analysis (purple and blue). Light grey cores represent depths that were not analyzed for microbiology.

### 5.2.3 Water activity and moisture content measurement of core samples

An Aqualab 4TE water activity meter was used to analyze triplicate aliquots of clay from each core subsample. After water activity measurement, the same aliquot of clay was transferred to a pre-weighed aluminum foil container, weighed, and dried in an oven at 110°C for at least 24

hours. After drying, samples were weighed again and the difference in mass before and after drying was used to calculate the moisture content (equation 2.1).

#### **5.2.4 XRD and geochemical analysis of bentonite core samples**

Quantitative X-ray diffraction (XRD) analysis and clay speciation analysis was completed by Activation Laboratories Ltd. Briefly, the XRD analysis was performed in a Bruker D8 Endeavour diffractometer equipped with a Cu X-ray source, at 40 kV and 40 mA over a  $2\theta$  range of  $4\text{--}70^\circ$ , with a step size of  $0.02^\circ$  and a time per step of 0.5 seconds, a fixed divergence slit at  $0.30^\circ$ , and sample rotation at 15 rpm. For clay speciation, a portion of each sample was dispersed in distilled water and the  $<25\text{ }\mu\text{m}$  size fraction was separated by gravity settling. The PDF4 minerals database (International Centre for Diffraction Data) was used for mineral quantification and the Rietveld method [152] was used to determine the quantity of each crystalline mineral. The proportion of clay minerals was calculated using the ratios of their basal-peak areas.

Geochemical analyses were performed by the Agriculture and Food Laboratory at the University of Guelph. The pH and cation exchange capacity of each sample was measured using the barium chloride method [182]. Total, organic, and inorganic carbon were measured using the SC-444 combustion method (Leco Corp). For measurement of total sulfur, homogenized samples were closed-vessel microwave digested with nitric and hydrochloric acids, and total sulfur was quantified using inductively coupled-plasma-optical emission spectroscopy (ICP-OES) with a pneumatic nebulizer [183]. Concentrations of arsenic, cadmium, chromium, cobalt, copper, molybdenum, nickel, mercury, lead, zinc, and selenium were measured using closed vessel, mixed acid, microwave digestion followed by inductively coupled plasma mass spectrometry (ICP-MS). Concentrations of calcium, magnesium, potassium, and sodium were measured by ICP-OES after extraction with ammonium acetate [184], iron by ICP-OES after extraction with DPTA [185], and phosphorus colourimetrically using a Seal AA3 after extraction with sodium bicarbonate [186]. Ammonium was measured using a spectrophotometer at 650-660 nm after a reaction with alkaline phenol and hypochlorite to form indophenol blue. Nitrate was quantified using a spectrophotometer at 520 nm after reduction by copperized cadmium to form nitrite, which has a reddish-purple dye in dilute phosphoric acid and N-(1-naphthyl)-ethylenediamine dihydrochloride.

### 5.2.5 Cultivation of bentonite core samples

All cultivation was completed in an anaerobic chamber. Prior to cultivation, liquid SRB medium (M803; HiMedia Laboratories) and phosphate buffered saline solution (PBS; 8 g NaCl, 0.2 g KCl, 1.44 g Na<sub>2</sub>HPO<sub>4</sub>, and 0.24 g KH<sub>2</sub>PO<sub>4</sub> in a final volume of 1 L Type I water, with pH adjusted to 7.4 using HCl), were both flushed with N<sub>2</sub> gas using sterile tubing downstream of a 0.22 µm PES filter (Sarstedt) and then shuttled into the anaerobic chamber. Lids were briefly opened to allow any oxygen trapped in the headspace to escape. Each 1-L bottle of PBS was flushed for 30 minutes, and 1-L bottles of SRB medium for 20 minutes plus an additional 15 minutes after the addition of parts B and C. Reasoner's 2A (R2A; M1687; HiMedia Laboratories) agar was not flushed, but agar plates were transported into the anaerobic chamber at least 24 hours before being used. Resazurin was added to all SRB medium and agar to a final concentration of 0.5 mg/mL to detect visibly if oxygen was present in the medium.

The 2 g triplicate aliquots of bentonite clay for each core, previously weighed into 25 mL tubes, were removed from 4°C storage a few at a time to minimize time at room temperature. To each 25 mL tube, 18 mL of PBS was added, and tubes were vortexed horizontally for 30 minutes at low speed to suspend the clay in the PBS. This clay suspension is referred to as dilution A throughout. From dilution A, two 10-fold serial dilutions were prepared in PBS with a final volume of 10 mL and are referred to as dilution B and C.

Dilution A (100 µL) was added to twelve R2A agar plates, dilution B (100 µL) to six R2A agar plates, and dilution C (100 µL) to three R2A agar plates. Clay suspension was spread with sterile 2 mm glass beads until dry. Three dilution A, three dilution B, and three dilution C R2A plates were incubated at 30°C for 5 days under oxic conditions, and three dilution A and three dilution B plates were incubated at 30°C under anoxic conditions for 28 days. A second set of cultures was incubated at 15°C to more closely mimic in situ conditions: three dilution A plates were incubated under oxic conditions, and three under anoxic conditions. All plates incubated under anoxic conditions were placed in airtight heat-sealed bags containing a GasPak EZ anaerobe sachet (BD) to help remove any residual oxygen, and an anaerobic indicator strip (BD) to verify anoxic conditions throughout incubation. After incubation, the colonies on each plate were counted and a value of CFU/g was calculated for each sample. Culture biomass was collected on sterile foam-tipped applicators (Puritan) and stored at -20°C until DNA extraction.

For each sample, two sets of SRB cultures were additionally created, one incubated at 15°C and one at 30°C. A total of 15 test tubes each containing 9 mL of SRB medium were inoculated with 1 mL of dilution A (5 tubes), B (5 tubes), or C (5 tubes) for each temperature. Test tubes were placed in vacuum chambers (BVV3GSSW, BVV) containing a GasPak EZ anaerobe sachet (BD) and anaerobic indicator strip (BD), then flushed five times with a 90% N<sub>2</sub> and 10% CO<sub>2</sub> gas mixture and incubated at either 15°C or 30°C for 28 days. After incubation, each test tube was assessed for evidence of SRB growth by observing presence of black ferrous sulfide precipitate produced by a reaction between SRB-derived sulfide and the ferric ammonium sulfate present in the medium. The number of positive tubes at each dilution was used to estimate the most probable number of cells per g of clay by comparison to an MPN table [153]. One mL of each positive SRB culture was pooled for each sample, and these pooled tubes were centrifuged at 8000 x g for 10 minutes to pellet cells. The supernatants were discarded and the cell pellets were stored at -20°C until DNA extraction.

Values of CFU or MPN per gram were converted to values of CFU and MPN per gram dry weight (gdw) using the measured moisture content values and equation 2.2.

### **5.2.6 DNA extraction from bentonite core samples**

Genomic DNA was extracted from the 66 (22 core samples x triplicate) previously frozen 5 g bentonite samples using the DNeasy PowerMax soil kit, from duplicate drilling water samples and from duplicate 0.22 µm and 0.10 µm filters of groundwater and of tap water using the DNeasy PowerSoil Kit (Qiagen), and from culture biomass using the DNeasy UltraClean Kit (Qiagen). The manufacturer's instructions were followed except for the cell lysis and elution steps. After addition of lysis solution, PowerBead tubes were incubated at 65°C for 30 minutes for PowerMax extractions, and at 70°C for 10 minutes for PowerSoil and UltraClean extractions. This was followed by bead beating for 10 minutes at 30 Hz using an MM 400 Mixer Mill (Retsch) for PowerMax extractions, and for 45 seconds at 5.5 m/s using a FastPrep-24 Classic Instrument (MP) for PowerSoil and UltraClean extractions. Final elution volumes were reduced to concentrate DNA as yield was expected to be low, especially for clay and water samples. For PowerMax, PowerSoil, and UltraClean extractions, 2 mL, 60 µL, and 50 µL were used, respectively. Genomic DNA extracts were stored at -20°C until further analysis. The method

used for DNA extraction from bentonite clay samples has been previously validated for bentonite [154].

### 5.2.7 Quantitative PCR of bentonite core samples

For each bentonite sample, bacterial 16S rRNA genes and fungal 18S rRNA genes were quantified in duplicate using a CFX Opus Real-Time PCR system (Bio-Rad). The total volume of each reaction was 15  $\mu$ L, which included 1x Sso Advanced SYBR Green Supermix (Bio-Rad), 0.3  $\mu$ M each of forward and reverse primer, 7.5  $\mu$ g of bovine serum albumin (BSA), and 2  $\mu$ L of template DNA. For 16S rRNA gene qPCR, primers 341F and 518R were used [188], and for 18S rRNA gene qPCR, FungiQuant-F and FungiQuant-R primers were used [189]. For both 16S and 18S rRNA gene qPCR, the following thermal cycling conditions were used: initial denaturation at 98°C for 3 minutes, followed by 40 cycles of 98°C for 15 s and 55°C for 30 s. For the 16S rRNA gene standard, M13 forward and reverse primers amplified a region of a previously constructed plasmid containing the full length of the 16S rRNA gene from *Thermus thermophilus* (Figure 3.1). For the 18S rRNA gene qPCR standard, primers EMP515F [174] and Uni1392R [190] generated an 18S rRNA gene amplicon from *Saccharomyces cerevisiae* DNA. A fresh aliquot of standard DNA was thawed and quantified using the Qubit dsDNA High Sensitivity Assay Kit (Invitrogen) with 4  $\mu$ L of sample immediately prior to preparing the qPCR standard curve. Samples were split between two 96 well plates for each qPCR primer set and were processed on the same day using a single tube of master mix and set of standard dilutions.

Bacterial 16S rRNA gene and fungal 18S rRNA gene copy numbers were calculated using linear regression of the standard curve based on the Qubit quantification of the standards. DNA extraction kit controls were included in the qPCR, and the average SQ was calculated for all kit controls that came from kits with the same lot number. This SQ value was subtracted from the SQ value of each clay sample extracted using a kit with the same lot number. Adjusted SQ values were converted to 16S or 18S rRNA gene copies/gdw (equation 5.1).

#### 5.1 16S or 18S rRNA gene copies/gdw<sup>-1</sup>

$$= \frac{(\text{average SQ sample} - \text{average SQ of kit controls}) \times 2000 \mu\text{L elution in DNA extraction}}{2 \mu\text{L template in qPCR} \times 5 \text{ g clay in DNA extraction}}$$

### **5.2.8 Amplicon sequencing of bentonite core, culture biomass, and water filter samples**

To help ensure sufficient DNA for sequencing from bentonite samples, a 1 mL aliquot of genomic DNA from each bentonite sample was concentrated using the NucleoSpin gDNA Clean-up XS kit (MN). The manufacturer's instructions were followed, and DNA was eluted in two steps using 12 followed by 8  $\mu$ L of elution buffer.

For each genomic DNA sample (clay, water, filter, or culture biomass), the V4-V5 region of the 16S rRNA gene was amplified in triplicate using universal primers 515F-Y [174] and 926R [157], modified to include a six-base index sequence, flow cell binding, and sequencing primer binding sites [158]. The PCR was set up in a PCR hood (AirClean Systems) with ISO 5-HEPA-filtered air. Surfaces of the PCR hood were washed with 70% ethanol and UV-treated for 15 minutes before each use. All tubes, water, and BSA were also UV-treated on a UV transilluminator (ProteinSimple) using 302 nm wavelength light. The final volume of each PCR was 25  $\mu$ L and included 1x ThermoPol Buffer (New England BioLabs), 0.2  $\mu$ M forward and reverse primer, 200  $\mu$ M dNTPs, 15  $\mu$ g BSA, 0.625 U hot start Taq DNA polymerase (New England BioLabs), and 1  $\mu$ L of template DNA. The following thermal cycling conditions were used: initial denaturation at 95°C for 5 minutes, followed by 35 (cultures) or 40 (clay, water, filters) cycles of 95°C denaturation for 30 s, 50°C annealing for 30s, and 68°C extension for 7 min, followed by a final extension step for 7 min at 68°C. No template controls (NTCs) were included in each 96 well PCR plate to test for contamination between wells, and additional no template controls and positive controls were included separately in tubes to ensure the master mix was prepared properly.

Triplicate PCR products were visualized on a 1% agarose gel stained with ethidium bromide, then triplicates were pooled and quantified on a second 1% agarose gel stained with gel GelRed (Biotium). Based on relative gel quantification, equimolar amounts of each amplicon were pooled. For samples with no visible amplification on the agarose gel, a maximum of 20  $\mu$ L of PCR product was included. Kit controls and NTCs were also added to the pool, even if they did not have visible amplification, at a volume of 5  $\mu$ L. This pool was excised from a 1.5% agarose gel stained with ethidium bromide to remove primer dimers and other PCR components, and the excised band was purified using the Wizard SV Gel and PCR Clean-Up System

(Promega). The purified pool was quantified using the Qubit dsDNA High Sensitivity Assay Kit (Invitrogen) as well as qPCR using P5 and P7 primers (Illumina). The library was denatured and diluted (9.0 pM for the MiSeq run including clay and culture samples and 12.0 pM for the MiSeq run including water and filter samples) following Illumina guidelines (Document No. 15039740 v01), then spiked with PhiX Sequencing Control v3 (Illumina) to a final 20% concentration.

All clay and culture samples were sequenced on a single MiSeq run with a cluster density of 656,000 clusters/mm<sup>2</sup>, average Q30 of 92.5%, and clusters passing filter of 88.8%. Water and filter samples were sequenced on a second MiSeq run with a cluster density of 774,000 clusters/mm<sup>2</sup>, average Q30 of 90.0%, and clusters passing filter of 91.4%.

### **5.2.9 Sequence analysis of bentonite core, culture biomass, and water filter samples**

Sequences were demultiplexed using Local Run Manager version 4 and further analyzed using Quantitative Insights Into Microbial Ecology 2 (QIIME2) [159] including Divisive Amplicon Denoising Algorithm 2 (DADA2) [160]. Paired-end reads were imported into QIIME2, where DADA2 was used to remove low quality reads and primer sequences and to truncate forward and reverse reads to 250 bases. Denoising, dereplicating, and generation of amplicon sequence variants (ASVs) was additionally accomplished using DADA2 through QIIME2. Taxonomy was assigned to ASVs using a naïve Bayes classifier pre-trained with SILVA database release 138. Potential contaminant ASVs were identified using the Decontam R package [175] and a threshold value of 0.5 (selected by examining histograms of Decontam scores). All ASVs flagged as contaminants were removed from the ASV table for subsequent analysis.

All sequence data were deposited in the European Nucleotide Archive with accession number PRJEB79453.

## **5.3 Results**

### **5.3.1 Mineral and chemical characterization of Tsukinuno bentonite**

The bentonite cores had variable amounts of clay content, ranging from 15-80%, as compared to 87%, 83%, and 74% for the Wyoming MX-80 powdered and two gapfill bentonite samples. The clay fraction of the Tsukinuno bentonite cores were predominantly associated with montmorillonite, the primary clay mineral in bentonite, but several core samples also had large

proportions of interstratified illite and smectite (a group of clay minerals, of which montmorillonite is the most common; Figure 5.3). Dominant non-clay minerals were quartz, which made up the majority of some samples, and plagioclase, which was especially abundant in D6-10 and 11 (Figure 5.3). A relatively high proportion (27%) of sample D6-5.3 was comprised of calcite, which was no more than 5% abundant in any other sample and often was not detected at all (Figure 5.3). Although mineral abundances did vary between samples, there was no obvious trend with distance from tunnel wall/floor.

The clay samples exposed to the tunnel wall (D4A and B 0 m samples) had highest average moisture content values of 53.4% and 41.5%, respectively, after which there was no obvious trend with depth. They also had the highest water activities of 1.00 and 0.998, respectively, after which, once again, there was no obvious trend with depth. Of the inner layer samples, the moisture content ranged from 7.6% (D4B-1) to 31.9% (D4A-2), with an average moisture content of 18.9%. Water activity ranged from 0.935 (D4B-1) to 0.998 (D4A-2), with an average of 0.986. The D4B-1 samples were the only samples with water activities less than 0.96. Most core samples had pH values between 9.0 and 9.5. Exceptions to this were D4B-3, which had a pH of 7.6, and D6-11, which had a pH of 6.3 (Figure 5.4). These were also the samples with the lowest clay content. The clay cores additionally had low carbon content, with inorganic carbon making up a maximum of 1.31% (D6-10) and organic carbon concentrations less than 0.2% in all cores other than D4B-3 (1.04%). Three clay cations were measured: sodium and calcium, which are the exchangeable cations associated with montmorillonite, and magnesium, which can substitute the central aluminum atoms. Of the three, sodium was consistently most abundant, which was expected because the Tsukinuno deposit is characterized by a sodium-type bentonite (Figure 5.4). The cores overall had high concentrations of ammonium (average of 260 mg/kg dry clay) and low concentrations of nitrate (average 0.91 mg/kg dry clay). Iron, phosphorus, and potassium were all detectable in all clay samples other than D6-11 for phosphorus, at average concentrations of 19.6, 1.3, and 353 mg/kg dry clay, respectively. Of the heavy metals measured, zinc was consistently the most abundant, especially in the D4C core (Figure 5.4).

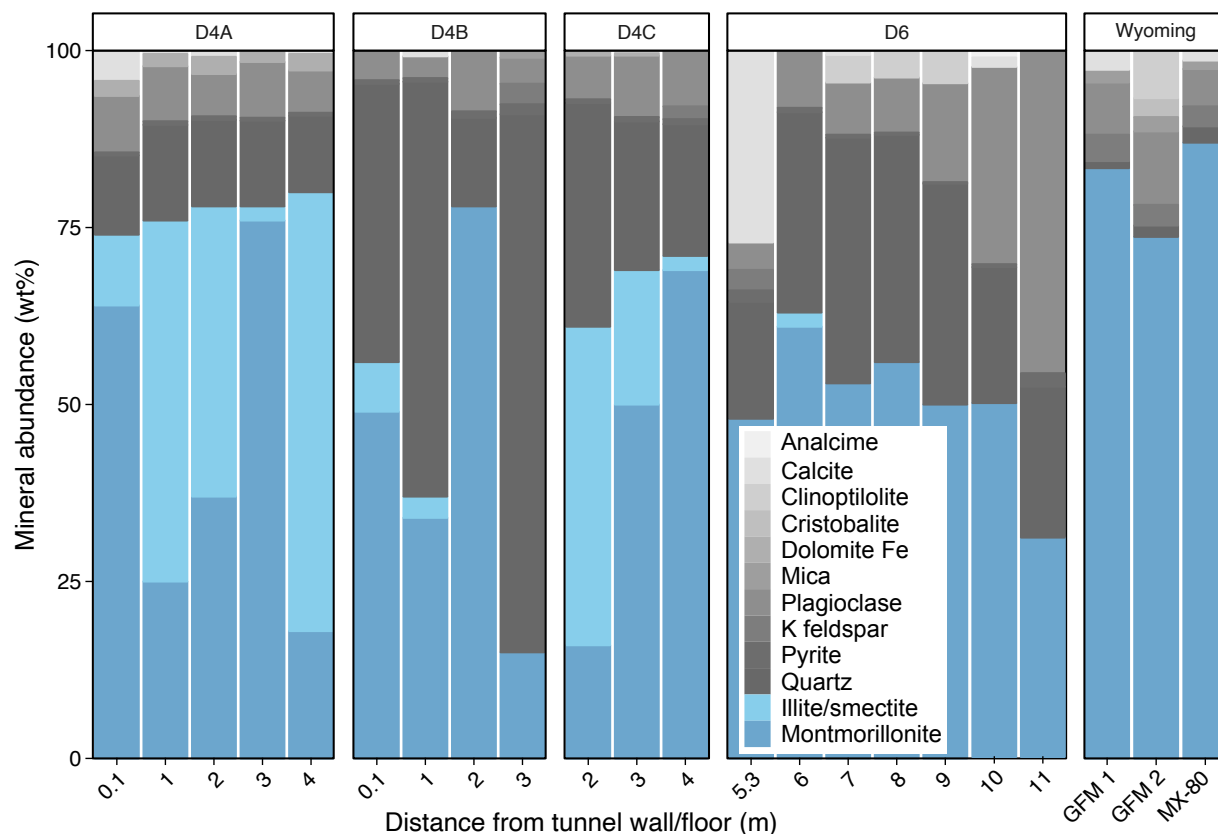


**Figure 5.3** Stacked bar plot showing the mineral composition of the 19 Tsukinuno core samples based on XRD analysis. One Wyoming MX-80 powdered bentonite and two gapfill bentonite samples were included for comparison. Clay minerals are shown in blue and non-clay minerals in grey.

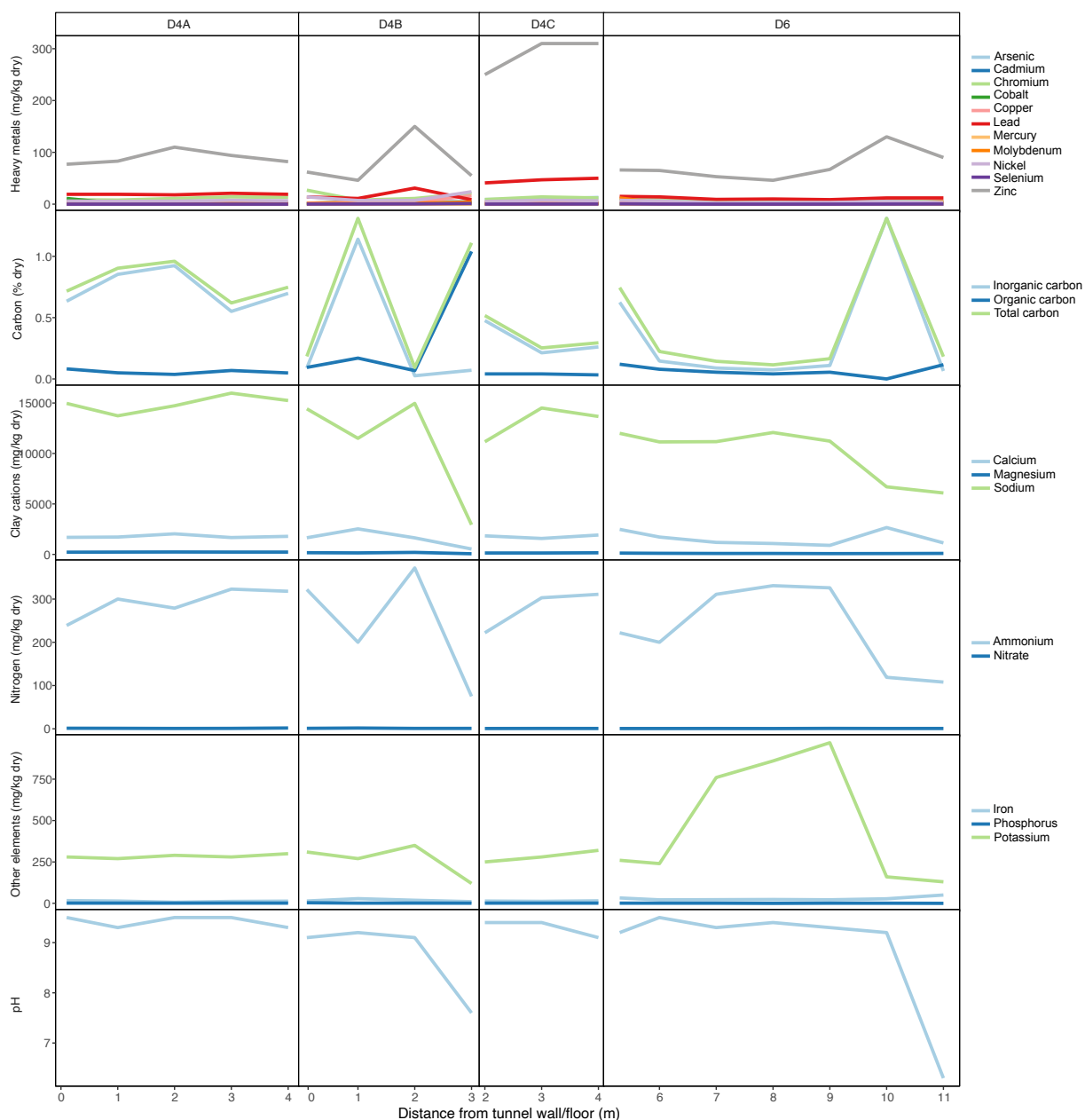


**Figure 5.4** Measured concentrations of chemical parameters in 19 Tsukinuno core samples.

### 5.3.2 Cultivation and qPCR

Aerobic heterotrophs were detected via cultivation, at both 15°C and 30°C, in most bentonite samples. The highest abundance of culturable aerobic heterotrophs grown at 30°C was  $2.4 \times 10^6$  in D4A-0, and this same sample had the highest abundance of culturable aerobic heterotrophs grown at 15°C of  $3.5 \times 10^5$  CFU/gdw (Figure 5.5). The average abundance of culturable aerobic heterotrophs at 15°C and 30°C were  $2.5 \times 10^5$  and  $3.8 \times 10^4$  CFU/gdw,

respectively. The cultivation results for anaerobic heterotrophs closely mimicked those of aerobic heterotrophs: the highest abundance of culturable anaerobic heterotrophs at both 15°C ( $8.7 \times 10^3$  CFU/gdw) and 30°C ( $1.6 \times 10^4$  CFU/gdw) was also in the D4A-0 sample, and when culturable aerobic heterotrophs were not detected in a sample, neither were culturable anaerobic heterotrophs (Figure 5.5). The abundance of culturable aerobic and anaerobic heterotrophs had a high Spearman correlation of 0.90 (Figure 5.6). The average abundances of culturable anaerobic heterotrophs at 15°C and 30°C were  $8.7 \times 10^3$  CFU/gdw and  $1.9 \times 10^3$  CFU/gdw, respectively. Culturable SRB were often not detected: at 15°C, SRB were detected in 12 of the 22 core samples, and at 30°C in 9 of the 22 core samples (Figure 5.5). When they were detected, their abundance was very low. At 15 and 30°C, the maximum abundances of culturable SRB were 5.9 and 5.8 MPN/gdw, respectively, and both maxima occurred in the D4A-0 sample. The abundance of culturable SRB was not significantly correlated with the abundance of culturable aerobic or anaerobic heterotrophs (Figure 5.6).

The abundances of culturable aerobic and anaerobic heterotrophs were also negatively correlated with the concentration of montmorillonite ( $r_s = -0.62$  for aerobic heterotrophs and  $-0.49$  for anaerobic heterotrophs; Figure 5.6). The abundance of culturable aerobic heterotrophs was additionally significantly correlated negatively to the concentration of plagioclase ( $r_s = -0.48$ ) and positively to the concentration of copper ( $r_s = 0.46$ ; Figure 5.6). The abundance of culturable anaerobic heterotrophs was positively correlated with the concentration of nitrate ( $r_s = 0.59$ ; Figure 5.6). The abundance of culturable sulfate-reducing bacteria was significantly positively correlated with the concentrations of phosphorus ( $r_s = 0.47$ ), sodium ( $r_s = 0.54$ ), magnesium ( $r_s = 0.63$ ), inorganic carbon ( $r_s = 0.53$ ), and calcium ( $r_s = 0.49$ ), as well as to moisture content ( $r_s = 0.54$ ) and cation exchange capacity ( $r_s = 0.63$ ), and was negatively correlated with the concentrations of plagioclase ( $r_s = -0.46$ ), arsenic ( $-0.49$ ), and mercury ( $r_s = -0.53$ ; Figure 5.6).

The qPCR analysis revealed detectable 16S rRNA gene copies in all core samples, and 18S rRNA gene copies in 14 of the 22 core samples (Figure 5.5). The highest abundance of 16S rRNA gene copies was  $5.7 \times 10^6$  copies/gdw (D4B-3) and the average was  $5.7 \times 10^5$  copies/gdw. For 18S rRNA genes, the maximum abundance was  $9.0 \times 10^3$  copies/gdw (D4B-1) and the average was  $8.1 \times 10^2$  copies/gdw. For both 16S and 18S qPCR, there was a high degree of variation between biological replicates of the same core sample (Figure 5.5). The abundance of

16S rRNA genes was significantly correlated with the abundance of culturable aerobic heterotrophs ( $r_s=0.50$ ) but not to anaerobic heterotrophs or SRB, and the abundance of 18S rRNA genes was not significantly correlated with any abundances of cultured microorganisms, though it was correlated with the abundance of 16S rRNA genes ( $r_s=0.56$ ; Figure 5.6).

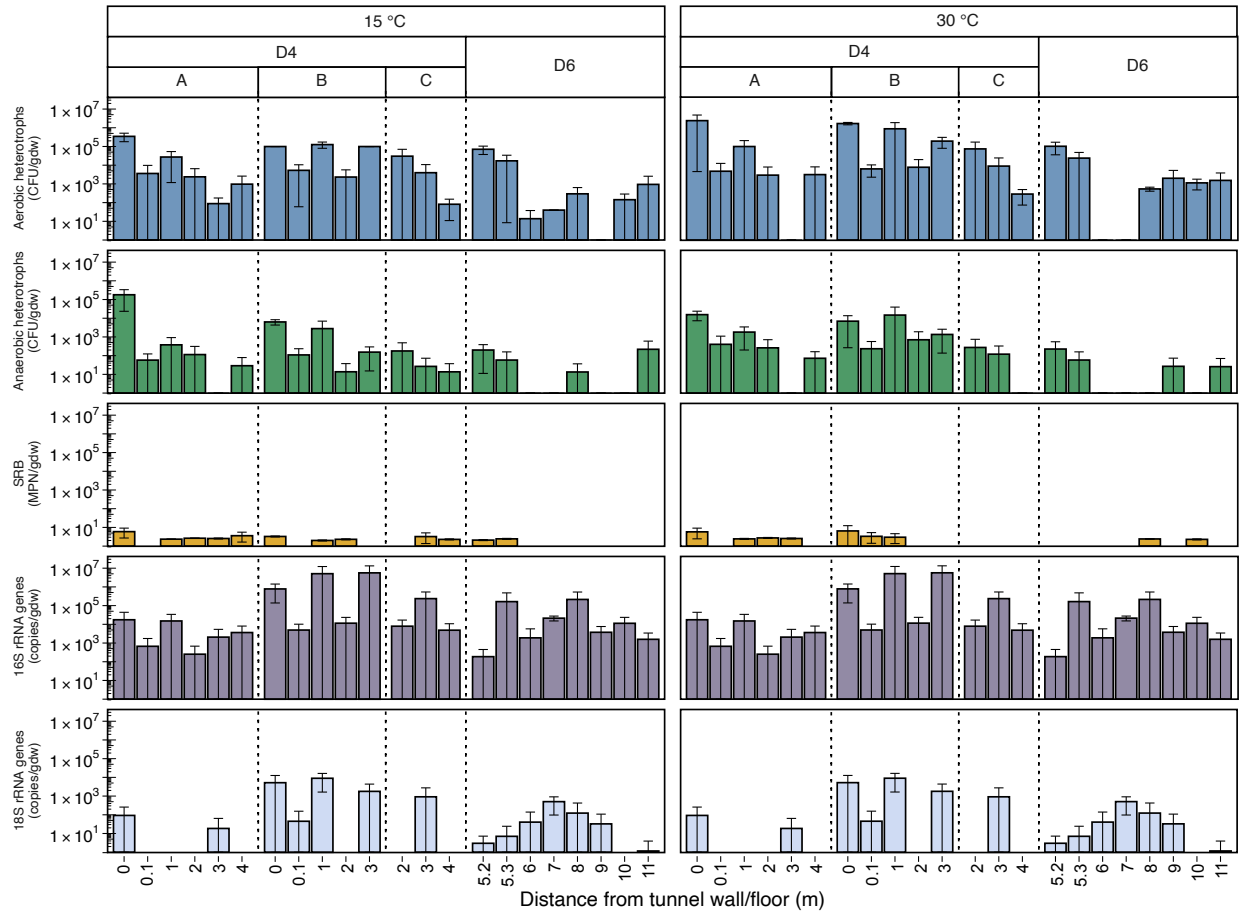


**Figure 5.5** Bar plot showing the abundance of culturable aerobic heterotrophs, anaerobic heterotrophs, and SRB, as well as 16S and 18S rRNA gene copies. For culture counts, error bars are based on the standard deviation between biological triplicate samples. For qPCR measurements, error bars are based on the standard deviation of biological triplicate samples and each of their technical duplicate measurements. For abundances of culturable SRB, bars were removed if no SRB were detected in any replicates even though MPN tables do not produce a value of 0 when no tubes are positive for growth.

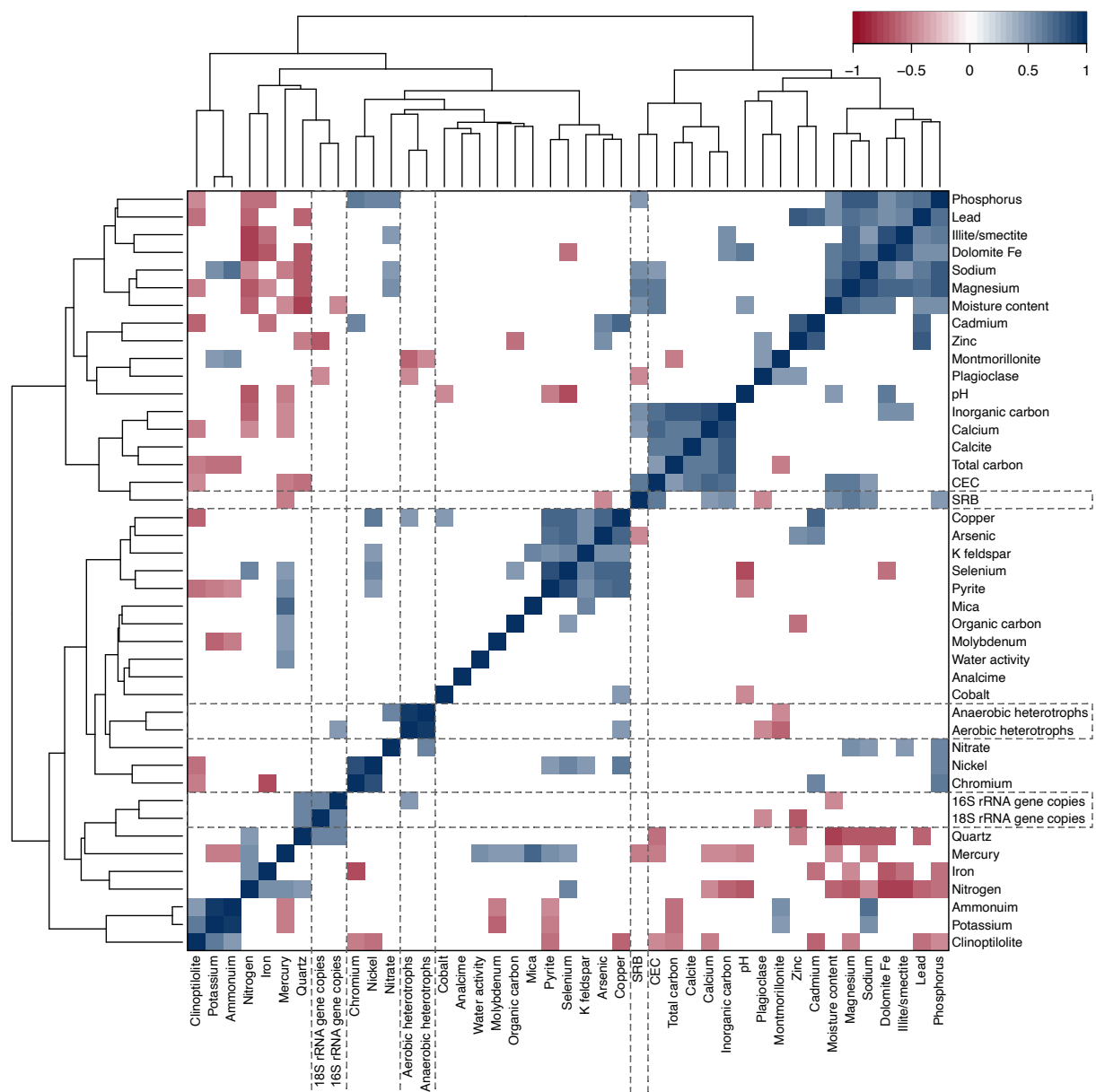


**Figure 5.6** Heat map showing Spearman correlations between measurements of chemicals, minerals, moisture content, water activity, cation exchange capacity, pH, and abundances of 16S and 18S rRNA genes and culturable aerobic heterotrophs, anaerobic heterotrophs, and SRB grown at 30°C. The 0 and 5.2 m samples were excluded as only a subset of analyses were performed for these samples. Samples are ordered based on hierarchical clustering. Negative correlations are indicated in red and positive correlations in blue. Any correlations with insignificant  $p$  values ( $>0.05$ ) are not shown.

### 5.3.3 16S rRNA gene analysis of clay and water samples

The 16S rRNA gene profiles of clay samples were overall quite similar to each other, regardless of core and distance from tunnel wall/floor, and were dominated by sequences associated with phylum *Proteobacteria* (Figure 5.7). Cores D4B and D4C were especially

similar. The most abundant genus detected in these cores was *Thiobacillus*, which was represented by three ASVs together representing an average of 45.1% of reads (Figure 5.7). Sequences associated with *Hydrogenophaga* were also relatively abundant in these samples, comprising a combined average of 22.5% of reads between the five ASVs associated with this genus (Figure 5.7). Samples from the D4A core often had very low sequencing read counts, especially in the 3 m replicates, likely explaining the profiles with poor overlap between replicates (Figure 5.7). The samples with higher read counts generally had 16S rRNA gene profiles similar to the D4B and D4C cores. These samples similarly had several dominant ASVs associated with *Hydrogenophaga* and with family *Comamonadaceae* (Figure 5.7), potentially representing additional more poorly resolved *Hydrogenophaga*-associated ASVs. Compared to D4B and D4C, the D4A samples overall had fewer sequences associated with *Thiobacillus*. Instead, the dominant ASV in the D4A samples was associated with *Pseudomonas stutzeri*, which had an average relative abundance of 9.7%. This same ASV was also detected in all D4B and D4C core samples at lower relative abundance (Figure 5.7).

The D4A (horizontal drill), D4B and D4C (inclined drill), and D6 replicates were plotted separately and geochemical data was overlayed to test if any of these measured variables led to differences in microbial community composition (Figure 5.8). In ordination space, the triplicate samples at a given depth grouped more closely with each other than with any other samples (Figure 5.8). In general, more separation between samples was observed with Bray Curtis distances than when phylogeny was considered with weighted UniFrac distances. The separation of the D4A core samples in one direction with Bray Curtis distances could be explained by increasing concentration of zinc (Figure 5.8 A). The 2 m samples, which had the highest concentration of zinc amongst D4A core samples (Figure 5.4), additionally had elevated relative abundances of *Pseudomonas stutzeri* compared to some other D4A core samples (Figure 5.8A). The separation of samples in the opposite direction could be explained by cation exchange capacity as well as increasing concentrations of inorganic and total carbon, which were all higher in the 1 and 2 m samples than in the 0.1, 3, or 4 m samples (Figure 5.4). The 1 and 2 m samples were also associated with increased relative abundance of *Paracoccus marcusii*, *Dietzia*, and one ASV each of *Hydrogenophaga* and *Comamonadaceae* compared to other D4A samples (Figure 5.8A).

The concentration of nitrogen was significantly correlated with the distribution of D4B and C samples in ordination space with both Bray Curtis and weighted UniFrac distances (Figure 5.8C-D). The concentration of nitrogen was highest in 1 and 3 m samples from the D4B core (Figure 5.4). These samples were associated with higher relative abundance of a specific *Hydrogenophaga* ASV compared to other D4B and C samples. Using Bray Curtis distances, concentrations of nickel, selenium, quartz, and organic carbon were also significantly correlated with the distribution of samples (Figure 5.8C). These variables were also all highest in the D4B 3 m samples (Figure 5.4). In the opposite direction within ordination space, concentrations of lead, zinc, cadmium, dolomite, arsenic, and illite/smectite were significantly correlated with both the Bray Curtis and weighted UniFrac ordinations (Figure 5.8C-D). All but illite/smectite were consistently higher in D4C 2-4 m samples compared to the D4B samples (Figure 5.4). These samples were associated with especially high relative abundances of sequences associated with *Thiobacillus* (Figure 5.8C-D).

Similar to samples from core D4A, samples from core D6 also generally lacked sequences associated with *Thiobacillus* and had higher relative abundances of *Pseudomonas stutzeri* compared to the D4B and D4C cores (Figure 5.7). Making up an average of 23.3% of reads, the dominant ASV in the D6 cores was associated with *Comamonadaceae*. With the exception of the 5.2 and 5.3 m samples, ASVs associated with *Hydrogenophaga* dominated, similar to the other cores, making up an average of 14.1% of reads. Instead, in the 5.3 m samples an ASV associated with *Halothiobacillus* dominated (62.4%) the profiles (Figure 5.7). These 5.3 m samples grouped most distantly from other D6 samples in ordination space and were associated with a high relative abundance of *Halothiobacillus* (62.4%), which was not detected in any other sample. Additionally, they had increased relative abundances of *Pseudomonas stutzeri* compared to other D6 samples, although the same ASV was detected in most D6 samples at lower relative abundance. No measured geochemical variables were significantly correlated with the D6 ordinations at the  $R^2$  threshold of 0.3.

Sequences associated with known SRB were rare in clay samples. Four ASVs were detected, each in a single sample, and with fewer than 20 sequencing reads each. One ASV was associated with *Desulfosporosinus* (D6-10), two with *Candidatus Desulforudis* (both in D6-5.3), and one only resolved to the family *Thermodesulfobrevibrio* (D6-11).

The DNA from tap water, drilling water, and groundwater was extracted and sequenced for comparison to the bentonite cores. Tap water was used to drill the cores, and was included in the analysis to indicate potential core contamination by tap water during drilling. The four filters had very low read counts and the profiles did not overlap with clay samples (Figure 5.7). After drilling, the drilling water had a large amount of suspended clay material and the 16S rRNA gene profiles looked similar to the clay cores (Figure 5.7). Ground water was included in the analysis to determine if the microbial communities detected in the clay were unique to the clay or similar to those associated with groundwater. Predominant ASVs in these samples were not detected in the clay and were instead associated with *Turneriella* (32% in one replicate and not detected in the other three), an uncultured bacterium from order *Xanthomonadales* (6.8%), *Porphyrobacter* (6.4%), and *Nevskia* (6.0%). All other ASVs, including four associated with *Comamonadaceae* and three associated with *Hydrogenophaga* that were common to the clays, were detected at an average relative abundance of less than 5% (Figure 5.7).



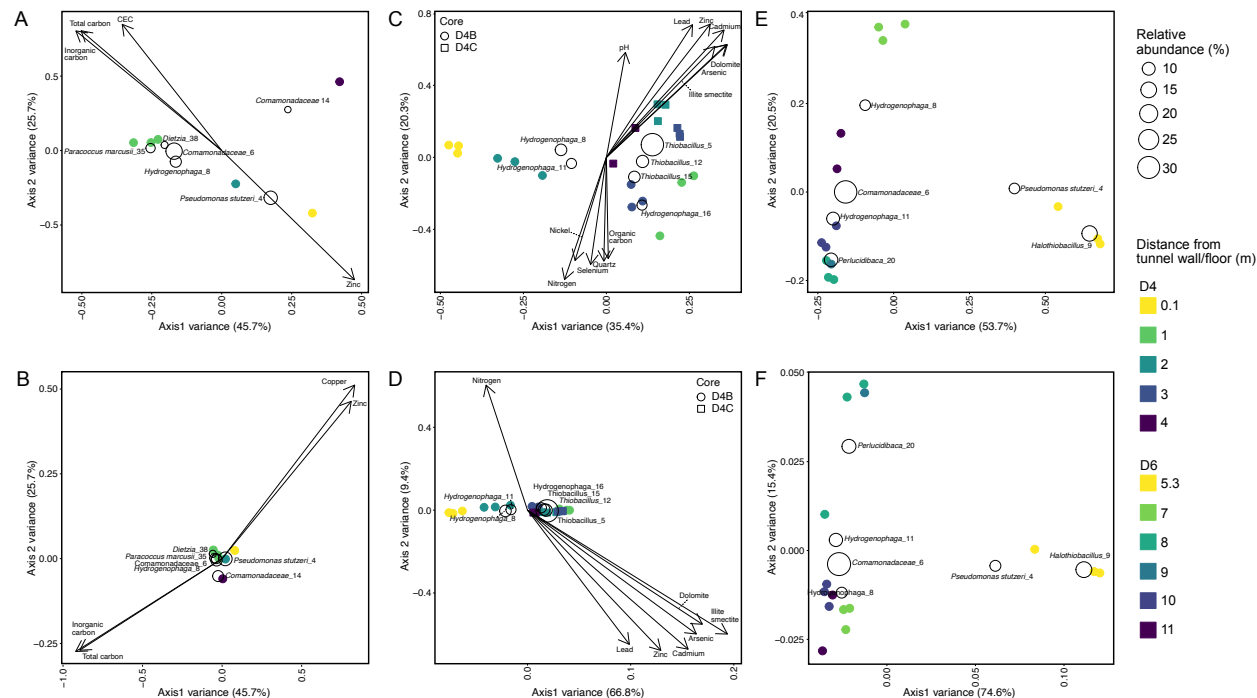


**Figure 5.8** Triplots based on Bray-Curtis (top row) and weighted UniFrac (bottom row) distances between 16S rRNA gene profiles of D4A (A and B), D4B and D4C (C and D), and D6 (E and F) bentonite cores. Geochemical and mineral parameters with a significant ( $p < 0.05$ ) correlation of at least 0.3 are displayed as vectors. Taxa bubbles represent taxa at least 5% abundant across the dataset. The ASV table was normalized to 5000 reads using scaling with ranked subsampling (SRS) prior to computing distances.

### 5.3.4 16S rRNA gene analysis of cultures

Biomass was collected from all cultures where growth was observed. The DNA was extracted from this biomass and the 16S rRNA gene was amplified and sequenced to identify the taxa associated with the cultured microorganisms. Most cultures were dominated by one or a few different genera, and these taxa were quite similar in cultures incubated at 15 and 30°C for a given sample (Figure 5.9). Overall, the dominant genus detected in aerobic heterotrophs cultures at both 15 and 30°C was *Pseudomonas*. Several D4A core samples also had a similarly high relative abundance of sequences associated with *Glutamicibacter*, and the shallower (5.2-6 m) D6 samples had abundant sequences associated with *Micrococcaceae* (Figure 5.9). Although overall less prevalent across the data set, several samples had relatively high abundance of sequences associated with *Microbacterium*, *Rhodococcus*, *Chryseobacterium*, *Bacillus*, *Acinetobacter*, *Brevundimonas*, and *Comamonadaceae* (Figure 5.9).

Similar to the aerobic heterotroph cultures, anaerobic heterotroph cultures were also dominated by sequences associated with *Pseudomonas*. Samples that had fewer *Pseudomonas* sequences tended to have higher relative abundance of sequences associated with members of the *Actinobacteria* phylum, specifically *Microbacterium*, *Cellulomonas*, *Cutibacterium*, *Oersokovia*, and/or *Sanguibacter* (Figure 5.9). Similar taxa were detected in samples grown at 15 and at 30°C. The SRB cultures were also associated with *Pseudomonas* sequences (Figure 5.9). The D4B samples had a lower relative abundance of *Pseudomonas* sequences than other cores, and instead had abundant sequences associated with *Shewanella*, which were not detected in any of the other cores. A total of 19 ASVs associated with known SRB were detected in the cultures. Of these, 14 were associated with genus *Desulfosporosinus* (two with *Desulfosporosinus meridiei*, one with *Desulfosporosinus orientis*, two with *Desulfosporosinus hippie*, and nine only resolved to the genus level), three were associated with *Desulfotomaculum*, one with *Desulfogalotomaculum*, and one resolved only to family *Desulfitobacteriales* (the same family as *Desulfosporosinus*). Genus *Desulfosporosinus* was the most prevalent: 10 of the 35 SRB cultures positive for SRB growth included sequences assigned to *Desulfosporosinus*, whereas only one sample (D4B-0) had sequences associated with other genera.

One reason to sequence biomass from cultures was to determine if the taxa cultured overlapped with those detected in the DNA extracted directly from clay. Although this approach cannot determine if all sequences detected in the clay directly were associated with viable microorganisms, it can provide insight into the viability of taxa that are detected in both the clay and the associated cultures. There were 16 ASVs with an average relative abundance of greater than 1% in clay samples and 11 of these were detected in at least one culture (Figure 5.10). The five ASVs not detected in a culture were associated with *Hydrogenophaga*, *Halothiobacillus*, *Comamonadaceae*, and two with *Thiobacillus*. Of these, all but *Halothiobacillus* were represented in cultures by a different ASV with the same taxonomic affiliation.

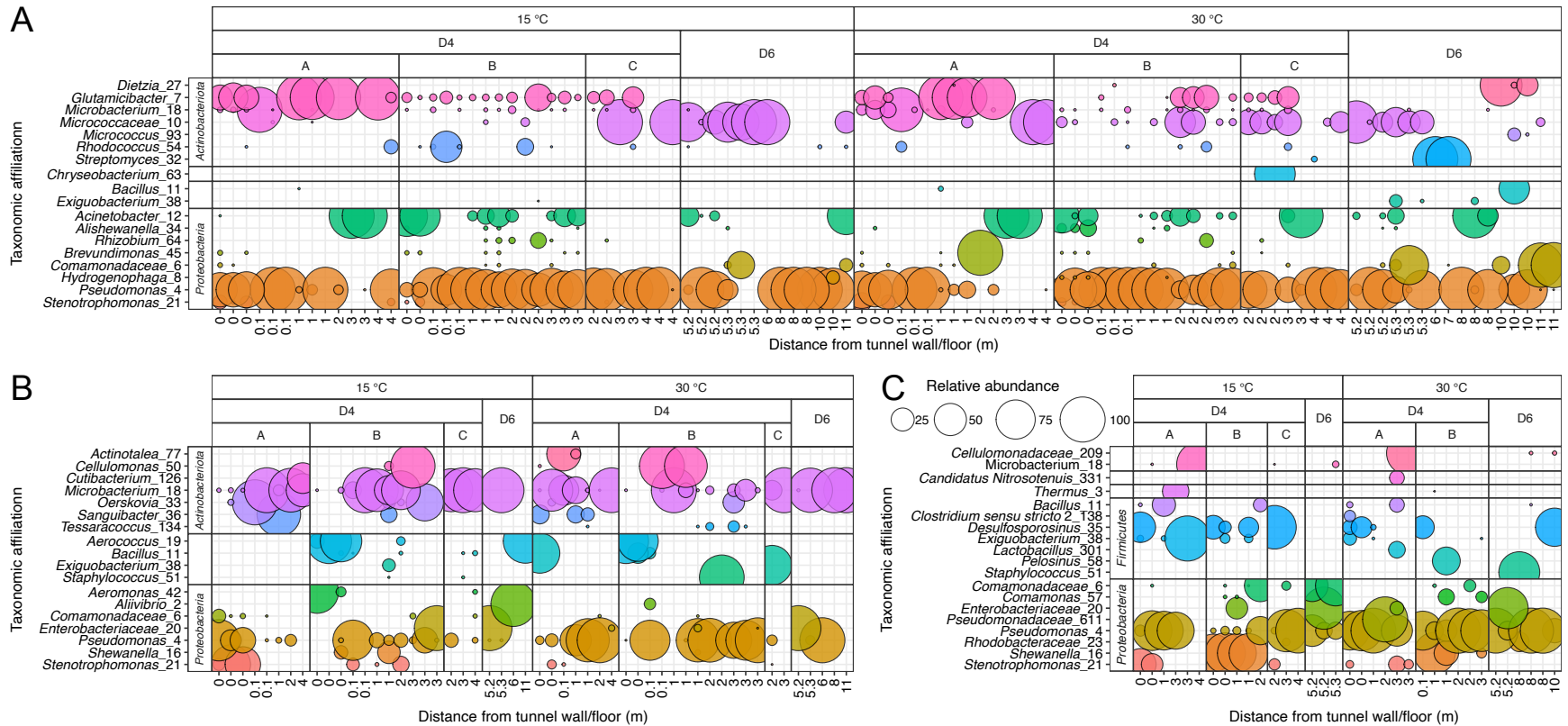


**Figure 5.9** Bubble plot showing the 16S rRNA gene profiles of biomass from cultures of aerobic heterotrophs (A), anaerobic heterotrophs (B), and sulfate-reducing bacteria (C). Only genera with a minimum relative abundance of 5% in at least one sample are displayed.

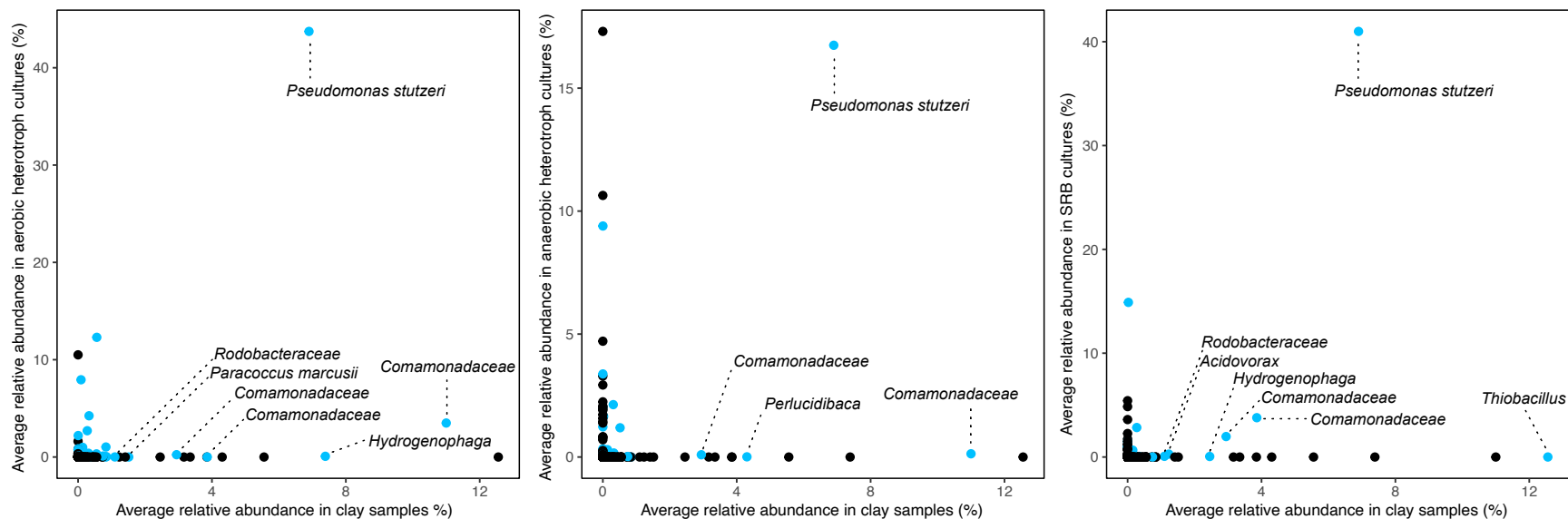


**Figure 5.10** Scatterplot showing the average relative abundance of ASVs in clay samples (x axes) and culture samples (y axes). Each plot includes the 2838 ASVs detected in the clay and/or culture samples. All ASVs that were detected in both the clay and culture samples are blue, whereas ASVs that were either only detected in the clay or only detected in the culture samples are black. All ASVs common to both clay and culture samples with a minimum average relative abundance in the clay of 1% are labelled with their associated taxa.

## **5.4 Discussion**

### **5.4.1 Tsukinuno as a DGR-relevant natural analogue site**

No natural analogue will perfectly mimic all aspects of bentonite in a DGR, but an ideal natural analogue will mimic some key aspects of a DGR. The bentonite proposed for use in some DGR designs, including the Canadian design, is Wyoming MX-80 bentonite. The Canadian design also includes a second type of bentonite, gapfill material, which is created from Wyoming MX-80 through a roll compaction/milling process. For this discussion, DGR-relevance was assessed by comparing properties of Tsukinuno bentonite to those of Wyoming bentonite.

From a microbiology perspective, a key aspect to DGR design is dry density and associated swelling pressure, which is expected to limit or inhibit microbial growth and activity [123, 124, 126, 127]. There are two key factors that contribute to the swelling capacity of bentonite: montmorillonite content, and the primary exchangeable cation associated with the montmorillonite structure. Montmorillonite is the predominant clay mineral in bentonite and confers its swelling property. As such, the higher the content of montmorillonite, the higher the swelling capacity. The Tsukinuno bentonite core samples had variable amounts of clay content, ranging from 15-80% (Figure 5.3). Although the clay fraction of most cores was dominated by montmorillonite, some cores had high proportions of interstratified illite and smectite (the group of clay minerals that montmorillonite belongs to; Figure 5.3). The cores with the highest montmorillonite content, D4A-3 (76%) and D4B-2 (78%) were within the range of measured montmorillonite content in Wyoming MX-80 (87%) and two batches of gapfill (83 and 74%; Figure 5.3). Montmorillonite has an exchangeable cation in the interlayer space of its structure, which can either be sodium or calcium. Sodium-type bentonites have much higher swelling capacities than calcium-type bentonites [191]. Both Wyoming bentonite and Tsukinuno bentonite are sodium-type bentonites, adding to the relevance of Tsukinuno bentonite as a natural analogue [132].

It is challenging to compare other chemical measurements between Tsukinuno bentonite and Wyoming bentonites because these are largely influenced by the water saturating the Wyoming bentonite (in the case of a DGR, the groundwater at the DGR site). In terms of microbial growth, one key factor that is less dependent on groundwater composition is moisture. The moisture content in Tsukinuno bentonite ranged from 7.6-31.9%, with an average value of

18.9%. By comparison, gapfill bentonite compacted to dry densities of 1.25, 1.40, 1.45, 1.50, and 1.60 g/cm<sup>3</sup> had average moisture contents of 29.8, 26.2, 24.3, 23.1, and 20.6%, respectively (data not shown). The D4B and C and D6 core samples generally had moisture contents below the range of compacted MX-80, but all D4A core samples had moisture contents within this range. Although expected to be influenced by the salinity of ground water in a DGR, gapfill bentonite compacted to the same dry densities reported above and saturated with Type I water had water activities between 0.617 and 0.997, putting most of the Tsukinuno bentonite water activities at the high end of this range. As groundwater with a higher salinity than Type I water would be expected to reduce water activity, it is likely that the water activities of Tsukinuno bentonites would be higher than expected water activities in DGR bentonite.

#### **5.4.2 Cultivation and qPCR**

Similar to a previous study using cultivation techniques to study clay cores from the Tsukinuno mine, the highest abundances of culturable aerobic and anaerobic heterotrophs on R2A agar occurred in the bentonite exposed to the tunnel wall (Figure 5.5) [131]. Unlike this previous Tsukinuno study where culturable heterotrophs had a maximum abundance of 10<sup>2</sup> CFU/gdw by a depth of 1 m into the core, in the present study abundances of culturable aerobic heterotrophs in the inner layers of the core were often greater than 10<sup>2</sup> CFU/gdw and occasionally reached 10<sup>6</sup> CFU/gdw (Figure 5.5). Abundances of culturable anaerobic heterotrophs were generally lower than those of aerobic heterotrophs, and in inner layer samples had a maximum of 10<sup>4</sup> CFU/gdw (Figure 5.5). A recent study that analyzed hand samples (i.e., small samples collected from tunnel walls) from the Tsukinuno mine reported abundances of culturable aerobic heterotrophs between 10<sup>1</sup> and 10<sup>4</sup>, on average lower than in the current study, and several samples with undetectable anaerobic heterotrophs, similar to the current study [133]. One commonality between the current study and all previous natural analogue studies is the minimal presence of culturable SRB [133, 135, 136].

The abundance of 16S rRNA genes was significantly and positively correlated with the abundance of culturable aerobic heterotrophs ( $r_s=0.50$ ; Figure 5.6). The abundance of 16S rRNA genes was on average an order of magnitude higher than the abundance of culturable aerobic heterotrophs (5.7 x10<sup>5</sup> 16S rRNA gene copies/gdw compared to 3.8 x10<sup>4</sup> CFU/gdw; Figure 5.5). These values are more similar than in the most recent study of Tsukinuno bentonite, where the

abundances of 16S rRNA genes were up to 4 orders of magnitude more abundant than culturable heterotrophs or SRB [133]. The relative similarity between 16S rRNA gene copies and culturable heterotrophs in the present study could indicate that a higher proportion of the total DNA in the bentonite is associated with viable microorganisms.

No known previous study has analyzed eukaryotic communities within natural analogue samples. Although detected in relatively few samples, several Tsukinuno bentonite samples had measurable abundances of fungal 18S rRNA genes, suggesting that fungi persist within the samples (Figure 5.5). With no sequencing information to accompany these results, it is unknown which fungal taxa are associated with the detected DNA; a next step will be to explore eukaryotic community profiles of these bentonite cores.

Several geochemical parameters were measured for each clay sample to study correlations among these parameters and the abundance and composition of microbial communities. For the initial Tsukinuno bentonite study, a similar approach was taken and only moisture content and dry density were found to be moderately correlated with the abundance of culturable heterotrophs [131]. In the present study, moisture content was only found to be correlated with the abundance of culturable SRB, but not the abundance of culturable heterotrophs (Figure 5.6). However, all but one sample had water activities greater than 0.96, and several were nearing 1.0, so available water was likely not limiting for microorganisms in most samples. Although dry density was not measured, montmorillonite concentration would similarly be expected to have a negative impact on microbial growth due to increased swelling pressure. Indeed, montmorillonite concentration was negatively correlated with the abundance of both aerobic ( $r_s = -0.62$ ) and anaerobic ( $r_s = -0.49$ ) heterotrophs. The abundances of aerobic and anaerobic heterotrophs were also significantly correlated with copper and nitrate concentrations, respectively (Figure 5.6). Copper is an important component of many cellular proteins, and the concentrations of copper measured in the bentonite samples were generally lower than concentrations shown to be inhibitory in water and soil systems [192, 193]. The positive correlation between nitrate and abundance of anaerobic heterotrophs could be explained by the high relative abundance of *Pseudomonas stutzeri*, a known denitrifying bacterium, in anaerobic heterotrophs cultures [194]. The abundance of SRB was correlated positively to several biologically necessary elements (i.e., phosphorus, sodium, magnesium, carbon, and calcium) and

negatively to heavy metals arsenic and mercury (Figure 5.6). However, culturable SRB were in similarly very low abundance, just above the limit of detection, in all samples where they were detected, so these correlations should be interpreted cautiously.

### 5.4.3 16S rRNA gene analysis

A first step in DNA analysis of the clays was to compare 16S rRNA gene profiles of bentonite cores to those of the tap water used for drilling (Figure 5.7). The tap water used for drilling did not include dominant taxa detected in the clay samples, indicating that the samples were not contaminated by the drilling process (Figure 5.7). Many of the dominant ASVs detected in clay were also not found in the associated ground water, although there was some overlap in taxa such as *Hydrogenophaga* and *Thiobacillus* (Figure 5.7).

Although few previous studies on the microbiology of natural analogue sites have implemented DNA-based analyses, *Proteobacteria* and *Actinobacteria* have been identified as dominant phyla [133, 135]. Consistent with previous literature, the majority of sequences detected in the Tsukinuno cores were associated with phylum *Proteobacteria*, as well as a few taxa within phylum *Actinobacteria* at generally lower relative abundances (Figure 5.7). Cores D4B and D4C had very similar 16S rRNA gene profiles overall and were dominated by sequences associated with *Thiobacillus* and *Hydrogenophaga* (Figure 5.7). Species within *Hydrogenophaga* are known for being autotrophic hydrogen oxidizers [195]. Members of the genus *Thiobacillus* are best known for coupling denitrification to sulfur oxidation [196–200], but some species have been shown to instead oxidize arsenic and fix nitrogen [199], and two studies have shown that members of *Thiobacillus* have the genes necessary for dissimilatory sulfate reduction [198, 200]. Although metagenomic sequencing was not conducted and so it is not known what *Thiobacillus* species in the clay cores encode within their genomes, the relative abundance of reads associated with *Thiobacillus* was strongly correlated with the concentration of arsenic ( $r=0.71$ ), but much less so for nitrate ( $r=0.05$ ) or sulfur ( $r=0.15$ ; Figure 5.8). The concentration of arsenic, and of other heavy metals zinc and cadmium, was additionally significantly correlated with the distribution of D4B and C core samples in ordination space, in the direction of samples dominated by *Thiobacillus*-associated sequences (Figure 5.8). This analysis also showed a significant correlation with nitrogen in the direction of samples with a high relative abundance of a specific *Hydrogenophaga*-associated ASV (Figure 5.8).

Samples from cores D4A and D6 also had ASVs associated with *Thiobacillus* and *Hydrogenophaga*, but generally had higher representation of sequences associated with *Pseudomonas stutzeri* and *Comamonadaceae* (Figure 5.7). *Pseudomonas* is a commonly detected genus of bacteria in both bentonite clay and cultures from bentonite clay [10, 127, 151, 201] and is a known denitrifier [194]. *Comamonadaceae* is a diverse family with members capable of different metabolisms such as denitrification and hydrogen oxidation [202, 203]. An ordination of D4A 16S rRNA gene profiles was significantly correlated with four measured variables, which were not always reflected among dominant taxa for these samples (Figure 5.8). The samples with the highest relative abundance of *Pseudomonas stutzeri* also had the highest concentrations of zinc (Figure 5.8). Cation exchange capacity, inorganic carbon, and total carbon were all correlated within ordination space in the direction of samples with increased relative abundance of *Paracoccus marcusii*, *Dietzia*, *Hydrogenophaga*, and *Comamonadaceae* (Figure 5.8). In general, it is challenging to determine how the measured correlations relate to microbial community composition. In some cases, they may reflect a key nutrient or co-factor that a particular group of microorganisms require, and in other cases they may be indirectly related to microbial community composition. For example, they could be correlated with a factor that was not measured that is related to microbial requirements. In this study, very similar taxa were detected across most samples, adding an additional challenge. As more diverse natural analogue samples are studied, it may be easier to draw connections between geochemical parameters and microbial community composition. Incorporating a metagenomic approach may offer insight into the physiological requirements that account for these correlations.

Sequencing DNA extracted from clay directly offers the ability to study the microbial community beyond the portion of the community capable of growing under the selected cultivation conditions, but one limitation is that the DNA detected could be associated with living bacteria or instead could represent relic DNA. To minimize this limitation, DNA sequencing directly from clay was combined with cultivation and subsequent sequencing of DNA from culture biomass. Most dominant ASVs detected using the direct from clay approach were also detected in the biomass of at least one culture, suggesting that the dominant taxa detected in the clay could represent viable microorganisms (Figure 5.10). This interpretation is further supported by the relatively similar (i.e., usually approximately one order of magnitude greater) abundances of 16S rRNA gene copies and culturable aerobic heterotrophs (Figure 5.7,

Figure 5.9). One caution with this interpretation is that, especially for liquid SRB cultures, there is some clay present in the cultures that could contribute to sequencing reads. For the 16 most abundant (>1% relative abundance) ASVs in clay samples, 11 were also detected in at least one culture, but only four (one ASV associated with *Pseudomonas* and three with *Comamonadaceae*) at greater than 1% relative abundance in that culture (Figure 5.10). Although no known natural analogue studies have used this approach, the microbiology of bentonite clay outside of the study of natural analogues has implemented this approach. Compared to a series of manufactured powdered or gapfill bentonite samples, the Tsukinuno samples had a higher degree of overlap between dominant clay ASVs and culture ASVs [10]. However, when similar manufactured dry clays were hydrated with Type I water in pressure vessel experiments, there was increased overlap between dominant clay ASVs and dominant culture ASVs (Chapter 2). To better predict if the DNA sequenced from Tsukinuno cores was associated with living microorganisms, additional techniques should be implemented, such as PMA-treatment or ancient DNA analysis.

Consistent with cultivation results, sequences associated with known SRB were not detected in most samples, and when they were it was at very low relative abundance (Figure 5.7). Of the taxa detected, *Desulfosporosinus* was the only one also detected in cultures (Figure 5.9). That said, the medium used is intended for the cultivation of heterotrophic bacteria like *Desulfosporosinus*, so the remaining genera detected in the clay could be viable but not culturable under the selected conditions (e.g., the pH values of the media were not the same as the bentonite). Low SRB abundance is common to all natural analogue studies that have specifically looked for them [133, 135, 136]. Although the conditions of these analogues differ from bentonite in a DGR in various ways, the natural analogue literature thus far, including the present study, suggests that low SRB abundance could be expected for DGR-associated bentonite.

## 5.5 Conclusion

The study of microorganisms in bentonite clay in the context of long-term nuclear waste storage has largely focused on short-term experiments where manufactured clay is exposed to one or a few DGR-relevant conditions, either in a lab setting or in boreholes. These types of experiments are critical for testing the effect of various DGR conditions (e.g., temperature, dry density, pressure) on microbial community composition and abundance, but have a time-related

limitation. A DGR is being designed to last upwards of one hundred thousand years, which is not a realistic timescale for these types of experiments. Coupling these short-term experiments to the study of natural analogues can help to alleviate the issue of time, given that natural analogues have already existed in nature for DGR-relevant timeframes. The results of this study demonstrate relatively low abundances of culturable heterotrophs in four bentonite cores taken from the Tsukinuno mine. The abundance of culturable heterotrophs was correlated with the concentration of montmorillonite, potentially due to increased swelling pressure characteristic of clays with a high montmorillonite proportions. The abundance of anaerobic heterotrophs was additionally correlated with the concentration of nitrate, perhaps indicative that nitrate stimulates the growth of certain anaerobic heterotrophs in clay such as known denitrifier *Pseudomonas*, which was affiliated with dominant sequences from anaerobic heterotrophs cultures.

Similar to previous bentonite natural analogue studies, the Tsukinuno core samples were dominated by sequences associated with *Proteobacteria* and *Actinobacteria*, and more specifically *Thiobacillus*, *Hydrogenophaga*, *Pseudomonas*, and *Comamonadaceae*. Although ordinations of the 16S rRNA gene profiles overlayed with measured geochemical parameters revealed several significant correlations, in many cases it was challenging to link the correlations to growth requirements of the taxa detected via DNA sequencing. That said, the 16S rRNA gene profiles of all samples were quite similar, and more obvious relationships may exist if a larger diversity of bentonite clay communities were included.

Culturable SRB were consistently detected at very low abundance (maximum 5.9 MPN/gdw) and were not detected at all in approximately half of the bentonite core samples. Sequences associated with known SRB were additionally detected in very few samples and at very low abundances. These results are consistent with previous natural analogue studies, where SRB were similarly either not detected or detected in low abundance [133, 135, 136, 204].

The results of this study, in combination with the few other bentonite analogue studies conducted to date [133, 135, 136, 204], suggest that bentonite associated with a future DGR could host relatively low abundance microbial communities, likely including heterotrophic bacteria like *Pseudomonas*, and potentially hydrogen and sulfur oxidizers. Although Tsukinuno cores also had high representation of ASVs associated with *Thiobacillus*, *Hydrogenophaga*, and

*Comamonadaceae*, it is challenging to say whether these sequences were associated with living bacteria. Detection of many dominant clay ASVs in cultures, coupled with overall 16S rRNA gene copies being relatively similar in abundance to culturable heterotrophs, support that much of the DNA detected could be associated with living organisms. However, additional techniques to differentiate between living microorganisms and relic DNA should continue to be developed and implemented. In addition, conditions of the Tsukinuno bentonite do not fully mimic expected conditions for DGR bentonite. For example, most Tsukinuno samples had lower proportions of montmorillonite compared to Wyoming bentonite. Although SRB are common constituents of deep subsurface microbial communities, current natural analogue studies would not suggest that they would be dominant members of DGR bentonite communities.

## **Chapter 6: Testing conditions for growth of aerobic heterotrophs and SRB in bentonite clay**

### **6.1 Introduction**

Bentonite clay is an integral component of deep geological repository designs for long-term storage of used nuclear fuel. Given the long timeframe a DGR needs to persist (e.g., between one hundred thousand and one million years), and the importance of isolating radioactive used nuclear fuel from the surrounding environment, it is important to study bentonite from several different perspectives, including microbiology [138]. Microorganisms could impact the integrity of a DGR through their participation in microbiologically influenced corrosion (MIC), or through gas production, which in large enough volumes could produce permeability fissures [12, 71]. Under anoxic conditions, which are expected for most of the lifetime of a DGR, sulfate-reducing bacteria (SRB) have been shown to dominate occurrences of MIC in other environments and are thus primary targets of microbiology research on bentonite clay in the context of long-term used nuclear fuel storage [12]. Certain observations in previous studies of bentonite clay microbiology remain unresolved and will be addressed here. The first is whether increases in abundance of culturable aerobic heterotrophs sometimes observed in hydrated bentonite in the absence of compaction or prior to complete saturation is due to a recovery from the VBNC state or instead due to growth of aerobic heterotrophs (Chapter 2 and 3). The second is whether variable abundances of culturable aerobic heterotrophs observed in some hydrated clay microcosms is due to inherent heterogeneity of bentonite or instead due to experimental setup conditions introducing heterogeneity (Chapter 4). The third observation is that SRB have been found to grow in bentonite under some conditions (Chapter 3), but not other similar conditions, and the factors that influence their ability to grow in bentonite are poorly understood.

As-received bentonite (i.e., relatively dry bentonite, “as-received” from the manufacturer) contains viable microorganisms at low abundances [10]. When water is added to as-received bentonite, an increase in abundance of culturable aerobic heterotrophs is commonly observed, provided that the swelling pressure is sufficiently low, either due to low dry density or prior to complete saturation [123, 124, 126, 127]; Chapter 2, Chapter 3]. Although a high dry density (e.g., 1.4-1.6 g/cm<sup>3</sup>) suppresses microbial growth in saturated bentonite (Chapter 2-3), it does not

appear to kill microorganisms [125]. In an experiment with saturated Wyoming MX-80 bentonite compressed to a high dry density of  $1.6 \text{ g/cm}^3$ , no increase was observed for the abundance of culturable aerobic heterotrophs beyond that of the as-received MX-80 bentonite [125]. When lids of the vessels were removed to allow the bentonite to expand beyond the limits of the vessels, effectively decreasing the dry density of the bentonite, the abundance of culturable aerobic heterotrophs increased [125]. The authors proposed that the most likely explanation for the increase in abundance of culturable aerobic heterotrophs was a recovery from the viable but not culturable (VBNC) state rather than actual cell division [125].

The VBNC state was first proposed in 1985 after an observation that many non-culturable cells could become culturable under the same cultivation conditions after a “reversal” event [205]. Though still relatively poorly understood, the VBNC state is characterized by a physiological change that can help cells survive under adverse conditions, and differs from dormancy because cells can remain metabolically active [206–208]. Generally, the VBNC state is induced by harsh conditions, which have been thoroughly summarized in a recent review [208]. Of the reported conditions to cause cells to enter the VBNC state, the most relevant to the conditions of as-received bentonite include desiccation and potentially starvation, and additionally high pressure when bentonite is saturated in a confined space [208]. That said, other conditions have also been reported to induce the VBNC state in certain microorganisms, including low or high temperature, pulsed electric fields, low or high pH, altered osmotic pressure, UV, lack of carbon source, low salinity, oxygen limitation, aerosolization, antibiotics, and exposure to a variety of toxic compounds [208]. Under this state, cells are more resistant to adverse conditions including high or low salinity, heavy metal toxicity, acidity, and antimicrobial compounds like ethanol and chlorine [209, 210]. When conditions become more favourable, cells can reverse back to a non-VBNC state. The specific conditions that induce and reverse the VBNC state are sometimes very specific and multifaceted [208]. Over 100 microbial species have been reported to be capable of entering a VBNC state, including *Escherichia coli*, and the conditions that induce and lead to a recovery from the VBCN state differ from species to species [206–208]. Although the VBNC state has not been studied for bentonite specifically, there is precedent for desiccation and subsequent rehydration-driven induction and recovery from the VBNC state in soil [211, 212].

When the abundance of culturable aerobic heterotrophs increases in hydrated or saturated bentonite, and the experimental design includes replication, there is often high variability in abundance of culturable aerobic heterotrophs between replicates [173]; Chapter 2-4]. For systems like pressure vessels, this can be explained by uneven saturation leading to distinct swelling pressures and water activities throughout the clay plug (Chapter 2, Chapter 3). In microcosms set up using hydrated bentonite that is thoroughly mixed before incubation, it is unclear whether differences in abundance of culturable aerobic heterotrophs between biological replicates are due to actual microbial heterogeneity in the bentonite clay or inconsistent experimental techniques.

Although culturable aerobic heterotrophs increase in abundance in bentonite clay when water is added, most experiments show no evidence of SRB growth in bentonite [123, 124, 126–128]; Chapter 2]. Even in microcosm experiments where bentonite was hydrated with SRB medium and incubated anoxically in the absence of pressure, conditions predicted to promote SRB growth, no increase in SRB abundance was observed [173]. However, in recent anoxic pressure vessel experiments testing the effect of dry density on suppression of microbial growth in Wyoming bentonite (Chapter 3), culturable SRB increased significantly compared to the as-received bentonite starting material for most bentonite samples taken from the outer layers (i.e., in contact with the inner surfaces of the pressure vessel) of the bentonite plug. In this study, it was proposed that SRB could have increased in abundance in anoxic pressure vessels due to a difference in the type of bentonite used, or a difference in setup conditions leading to more anoxic conditions in this study compared to others (Chapter 3). Another difference between studies involving culturing SRB from bentonite is the choice of medium (Chapter 3). In the anoxic pressure vessel study, where an increase in abundance of culturable SRB was observed, an SRB medium from HiMedia Laboratories (M803) was used (Chapter 3). This medium has also been used in other studies where growth of SRB was not observed [127, 151, 173]; Chapter 2, Chapter 4]. A different SRB medium, Postgate's B medium, is also commonly used to culture SRB from bentonite [123–126, 128].

The purpose of this research was to better understand the growth of aerobic heterotrophs and SRB in hydrated bentonite and the conditions that promote and prevent growth. The first goal was to determine if the increase in abundance of culturable aerobic heterotrophs in hydrated

bentonite compared to relatively dry as-received bentonite is due to growth (i.e., cell division) or recovery from the VBNC state. The second goal was to explore heterogeneity of replicate hydrated bentonite microcosms as measured by cultivation. The third goal was to identify conditions that promote growth of SRB in anoxic pressure vessel experiments (Chapter 3), compared to previous experiments where growth of SRB has not been reported [123, 124, 126–128]; Chapter 2, Chapter 4]. This was done by testing differences in the type of bentonite, setup conditions, and choice of SRB medium, as well as the oxygen sensitivity of representative SRB cultures from bentonite clay.

## **6.2 Materials and methods**

### **6.2.1 Recovery from VBNC state versus growth of aerobic heterotrophs**

Four 32.5 g aliquots of Wyoming MX-80 bentonite, referred to throughout as M25, were hydrated to a water activity of 0.98 by adding 8 mL of water and thoroughly mixing by hand using disposable nitrile gloves. Bentonite water activity was confirmed using an Aqualab 4TE water activity meter. Each 32.5 g aliquot of hydrated bentonite was added to a separate sterile 250 mL mason jar (four total). To a separate set of four sterile 250 mL mason jars, 32.5 g of as-received bentonite was added. Duplicate jars of hydrated and of as-received bentonite were incubated at 30°C for one week, and a second set of duplicate hydrated and as-received bentonite microcosms were incubated at 4°C for one week. All setup occurred on the laboratory bench and incubation was under oxic conditions.

Immediately after setup, as well as one, three, and seven days after setup, 2 g aliquots of bentonite were removed from each microcosm for cultivation of aerobic heterotrophs, anaerobic heterotrophs, and SRB. At each time point, a second 2 g aliquot was removed for DNA extraction. From each DNA extract, the V3 region of the 16S rRNA gene was quantified using digital PCR (dPCR). Each 45 µL dPCR mix included 15 µL of 3x QIAcuity EvaGreen Mastermix (Qiagen) and 0.4 µM 341F forward primer and 518R reverse primer [188]. The dPCR mix (35 µL) was combined with 10 µL of template and vortexed for 10 s, then 40 µL of this mixture was transferred to a QIAcuity Nanoplate 26k (Qiagen). The thermal cycling conditions were as follows: initial denaturation at 95°C for 2 min followed by 40 cycles of 95°C denaturation for 15 s, 55°C annealing for 15 s, and 72°C extension for 15 s, followed by a final

extension at 35°C for 5 min. From DNA extracts, the V4-V5 region of the 16S rRNA gene was also amplified and sequenced for the zero and seven-day time points.

### 6.2.2 Bentonite heterogeneity

To test the variability in abundance of culturable aerobic heterotrophs among replicate microcosms, three different methods of bentonite hydration and mixing were tested (Table 6.1), each with six replicate microcosms (Figure 6.1). To test the variability in abundance of culturable aerobic heterotrophs within a microcosm, triplicate microcosms were set up using mixing method one (Figure 6.1). From each, six 2-g aliquots of bentonite were used to establish six different sets of cultures. All microcosms were prepared on the laboratory bench using 10 g of a single batch of Wyoming MX-80 bentonite (referred to throughout as MX9) hydrated with 3 mL of Type I water to achieve a bentonite water activity of 0.99. All microcosms were incubated under oxic conditions for one week at 45°C, as this is the clay and incubation temperature previously observed to have the highest variability among biological replicates (Chapter 4). After incubation, 2 g of bentonite was collected for cultivation of aerobic heterotrophs, and 50 mg for DNA extraction. The V4-V5 region of the 16S rRNA gene was sequenced for all DNA extracts.

**Table 6.1** Methods of hydrating and mixing bentonite for setup of microcosms.

| <b>Mixing method</b> | <b>Description</b>                                                                                                                                         |
|----------------------|------------------------------------------------------------------------------------------------------------------------------------------------------------|
| 1                    | As-received stock bentonite mixed, then six bentonite aliquots weighed and hydrated separately.                                                            |
| 2                    | As-received stock bentonite mixed, then bentonite for six microcosms was weighed together, hydrated together, then separated into six separate microcosms. |
| 3                    | As-received stock bentonite not mixed. Six bentonite aliquots weighed and hydrated separately.                                                             |

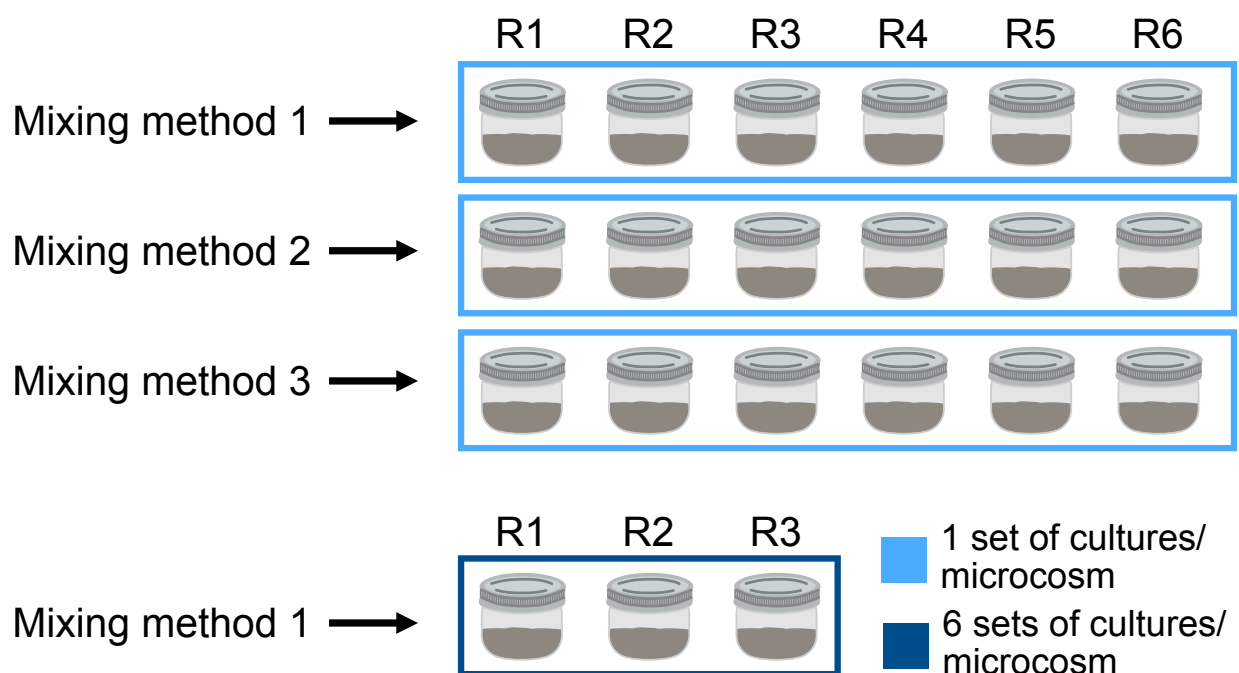


**Figure 6.1** Schematic of bentonite microcosm experiments to test heterogeneity of culturable heterotrophs. R1-R6 refer to replicate numbers.

### 6.2.3 Comparison of SRB medium

As-received Asha, MX6, MX7, MX9, and GFM bentonite (described in Chapter 4) were used to inoculate either M803 SRB medium from HiMedia Laboratories (referred to throughout as “HiMedia”) or Postgate’s medium B (Table 6.2) [213]. Separate cultures inoculated with each clay were incubated at 15, 30, 45, or 60°C for 28 days. Biomass from positive cultures was collected for DNA extraction and subsequent sequencing.

**Table 6.2** Comparison of the ingredients in Postgate's medium B and HiMedia SRB medium. All values are expressed as g/L.

| Medium              | K <sub>2</sub> HPO <sub>4</sub> | Na <sub>2</sub> SO <sub>4</sub> | CaCl <sub>2</sub> | MgSO <sub>4</sub> • 7H <sub>2</sub> O | Na-DL-lactate | NH <sub>4</sub> Cl | Yeast extract | FeSO <sub>4</sub> x7H <sub>2</sub> O | Na-thioglycolate | Ascorbic acid | Peptone | Meat extract B | (NH <sub>4</sub> ) <sub>2</sub> Fe(SO <sub>4</sub> ) <sub>2</sub> • 6H <sub>2</sub> O | Sodium ascorbate |
|---------------------|---------------------------------|---------------------------------|-------------------|---------------------------------------|---------------|--------------------|---------------|--------------------------------------|------------------|---------------|---------|----------------|---------------------------------------------------------------------------------------|------------------|
| Postgate's medium B | 0.5                             | 1.0                             | 0.075             | 2.0                                   | 2.0           | 1.0                | 1.0           | 0.5                                  | 0.1              | 0.1           | NA      | NA             | NA                                                                                    | NA               |
| HiMedia SRB medium  | 0.5                             | 1.5                             | 1.0               | 2.0                                   | 3.5           | NA                 | NA            | NA                                   | NA               | NA            | 2.0     | 1.0            | 0.39                                                                                  | 0.1              |

### 6.2.4 Setup of SRB growth experiment

Triplicate sterile mason jars were filled with either 65 g of Wyoming MX-80 bentonite (MX9) or with 65 g of gapfill material (GFM), prepared using one of four methods (Table 6.3; Figure 6.2). These two types of bentonite were selected because MX9 was used in previous

anoxic microcosms, where SRB growth was not observed [173], and GFM was used in anoxic pressure vessels where an increase in the abundance of culturable SRB was observed (Chapter 3). For MX9, 11.4 mL of Type I water was added to the bentonite and mixed thoroughly by hand with disposable nitrile gloves to achieve a bentonite water activity of 0.96. For GFM, 18.0 mL of water was used to achieve the same water activity. The mass of bentonite was selected to mimic previous anoxic MX9 microcosms where SRB growth was not observed [173]. The water activity of 0.96 was selected as it was the average water activity of previous anoxic pressure vessel samples with the highest measured abundances of culturable SRB (i.e., all 15 MPN tubes positive for SRB growth; Chapter 3) and this was additionally one of three water activities included in MX9 microcosm experiments where SRB growth was not observed [173].

Microcosms set up using method A, B, and D were set up at the University of Waterloo. Method A mimicked the setup conditions of previous microcosms set up at the University of Waterloo [173]. Mason jars set up using method B were set up at Western University and mimicked the setup conditions of previous anoxic pressure vessels set up at Western University (Chapter 3). Microcosms set up using method C were conducted at the University of Waterloo and intended to be at least as strictly anoxic as those set up at Western University (method B). Setup method D was included to test if the mixing of bentonite with water killed microorganisms. This was part of the setup of previous anoxic microcosms, where growth of SRB was not observed, but was not a part of the setup of previous anoxic pressure vessels where growth of SRB was observed. Although all setup conditions were intended to be anoxic, setup method A would be expected to potentially have the highest amounts of trace oxygen. All microcosms were incubated in an anaerobic chamber ( $\sim 25^{\circ}\text{C}$ ) for six weeks. After incubation, 2 g aliquots were collected from each microcosm for cultivation of SRB, aerobic heterotrophs, and anaerobic heterotrophs (6.2.6).

**Table 6.3** Description of microcosm preparation.

| Preparation method | Description                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                        |
|--------------------|----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| A                  | As-received bentonite was transported into the anaerobic chamber one day ahead of microcosm set up. The air lock was flushed 6 times from ambient pressure to -0.68 barr when transporting as-received bentonite into the anaerobic chamber. Water was flushed in a bottle for 30 minutes with N <sub>2</sub> gas. Water was added to bentonite and mixed thoroughly by hand.                                                                                                                                                                                                                                                                                                                      |
| B                  | As-received bentonite was transported into the anaerobic chamber one week ahead of time. The air lock was flushed 3 times from -0.8 barr to -1.0 barr before adding gas to ambient pressure for transportation of as-received bentonite into the anaerobic chamber. Water was flushed in a volumetric flask for 1 hour with Ar gas, then filled with separately flushing water until overflowing and quickly stoppered to prevent oxygen from entering the head space. Water was added to bentonite and mixed thoroughly by hand.                                                                                                                                                                  |
| C                  | As-received bentonite transported into anaerobic chamber 18 days ahead of time. The air lock was cycled 30 times from ambient pressure to -0.68 barr when transporting as-received bentonite into the anaerobic chamber. The air lock was opened from inside the chamber after the 9 <sup>th</sup> , 18 <sup>th</sup> , and 27 <sup>th</sup> round of flushing to shake the bag of bentonite to help remove trace oxygen. Water was flushed in a volumetric flask for 1 hour with N <sub>2</sub> gas, then filled with separately flushing water until overflowing and quickly stoppered to prevent oxygen from entering the headspace. Water was added to bentonite and mixed thoroughly by hand. |
| D                  | Same method as C, but water was added on top of bentonite and not mixed.                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                           |

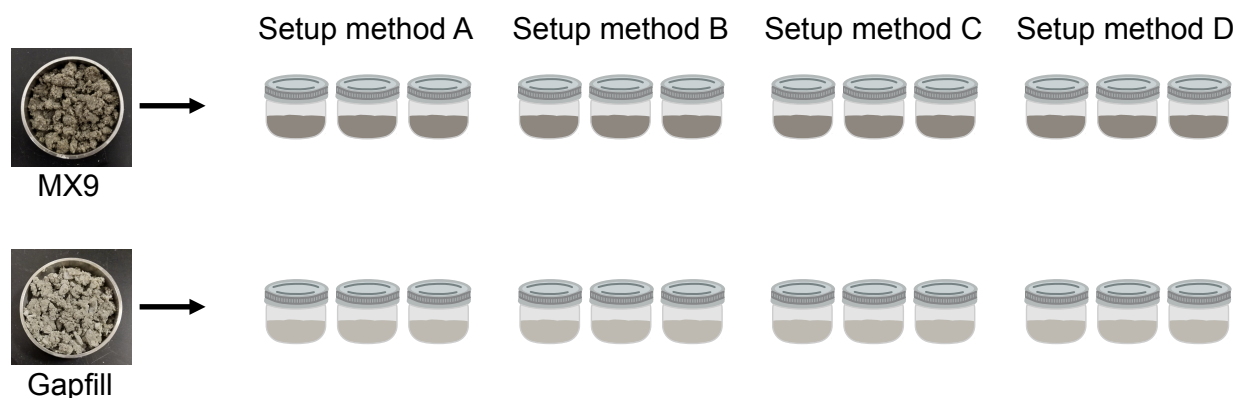


**Figure 6.2** Schematic of the SRB growth experiment.

### 6.2.5 Setup of SRB oxygen sensitivity experiment

A 20 g aliquot of Asha bentonite was hydrated with a pre-determine volume of sterile Type I water to achieve a water activity of 0.96, and thoroughly mixed by hand using disposable nitrile gloves. Hydrated bentonite was placed in a sterile 250 mL mason jar and incubated at 30°C for one week (this is the same microcosm as one of the replicate Asha 30°C microcosm

described in Chapter 4). After incubation, 2 g of bentonite was used to inoculate SRB cultures. These cultures were incubated at 30°C under oxic conditions for 28 days to test if SRB from bentonite would grow under oxic conditions. After incubation, biomass was collected from cultures for DNA extraction and sequencing.

Separately, the oxygen sensitivity of an enrichment culture of *Desulfosporosinus* sp. previously isolated from borehole modules filled with Wyoming bentonite (i.e., mixture of bentonites referred to as MX6 and MX7) [127, 151] was tested. Seven sets of triplicate Hungate tubes, each filled with 9 mL of anoxic SRB medium (M803; HiMedia Laboratories), were inoculated with 200 µL of a *Desulfosporosinus* sp. enrichment culture. Each set of triplicate cultures had a headspace with a different concentration of air (0%, 25%, 50%, 60%, 70%, 80%, or 90%). All manipulations occurred in an anaerobic chamber. Hungate tubes were incubated at 30°C for seven days. Cells were counted in the inoculum as well as at the one-, three-, five-, and seven-day timepoint, using a Petroff-Hausser counting chamber, to prepare a growth curve. The cells in five grids were counted and averaged, unless fewer than ten cells were counted in any of the five grids, in which case the cells in all ten grids were counted and averaged.

#### **6.2.6 Cultivation**

For each sample, 2 g of bentonite was weighed and added to 18 mL of sterile phosphate buffered saline (PBS; 8 g NaCl, 0.2 g KCl, 1.44 g Na<sub>2</sub>HPO<sub>4</sub>, and 0.24 g KH<sub>2</sub>PO<sub>4</sub> in a final volume of 1 L Type I water, with pH adjusted to 7.4 using HCl) solution in a 25 mL tube. These tubes were vortexed horizontally at low speed for 30 min to disperse bentonite within the solution. This dilution is referred to as “dilution A” throughout. Two 10-fold serial dilutions were made in PBS from dilution A, each in a final volume of 10 mL, and are referred to as “dilution B” (1:100 dilution) and “dilution C” (1:1000 dilution), respectively.

For heterotroph cultures, 100 µL of clay suspension was added to Reasoner’s 2A (R2A; M1687; HiMedia Laboratories) agar plates and spread until dry using sterile 2 mm glass beads. For aerobic heterotrophs, three sets of triplicate plates were inoculated with one of dilution A, B, or C. For anaerobic heterotrophs, triplicate plates were inoculated with dilution A. All plates were incubated at 30°C unless otherwise specified (plates from the heterogeneity experiment were incubated at 45°C). Aerobic heterotroph cultures were incubated for 5 days and anaerobic heterotroph cultures were incubated for 28 days. After incubation, colonies were counted on each

plate and an average value of colony forming units (CFU) per g was calculated. When applicable, colonies were swabbed with a sterile foam-tipped applicator (Puritan). Swabs were frozen at -20°C until DNA extraction followed by sequencing.

For SRB cultures, 15 test tubes filled with 9 mL of SRB medium were inoculated for each sample, 5 with dilution A, 5 with dilution B, and 5 with dilution C. These cultures were incubated at 30°C for 28 days. After incubation, tubes positive for SRB growth were identified by a black ferrous sulfide precipitate produced by a reaction between SRB-derived sulfide and ferric ammonium sulfate in the medium. The number of positive tubes of each dilution was compared to a most probable number (MPN) table to determine the MPN/g of each sample [153]. When applicable, 1 mL of each positive SRB culture was collected and centrifuged at 8000 x g for 10 minutes to pellet cells. Cell pellets were frozen at -20°C until DNA extraction followed by sequencing.

All anoxic incubations (i.e., SRB and anaerobic heterotrophs) involved placing agar plates or test tubes in either vacuum chambers (BVV3GSSW, BVV) or bags. In either case, a GasPak EZ anaerobe sachet (BD) was included to remove any residual oxygen and an anaerobic indicator strip (BD) to confirm anoxic conditions throughout incubation. Vacuum chambers were flushed five times with a 90% N<sub>2</sub> and 10% CO<sub>2</sub> gas mixture, and bags were either packed in an anaerobic chamber or flushed with 90% N<sub>2</sub> and 10% CO<sub>2</sub> and then heat sealed. For experiments set up under anoxic conditions, media was flushed prior to cultivation and setup of cultures occurred in an anaerobic chamber. The PBS was flushed with N<sub>2</sub> gas for 30 minutes using sterile tubing downstream of a 0.22 µm PES filter (Sarstedt). The SRB medium was flushed in the same manner for 20 minutes, plus an additional 15 minutes after the addition of parts B and C of the medium. Resazurin was also added to all SRB and R2A agar media at a final concentration of 0.5 mg/mL to confirm anoxic media throughout incubation.

An ANOVA was used to test for differences in abundances of culturable microorganisms between treatments. In all cases, a *p* value threshold of 0.05 was used, and in the case of a significant ANOVA result, a Tukey's HSD post hoc test was used, also with a *p* value threshold of 0.05. For the VBNC versus growth of aerobic heterotroph experiment, samples groups included values (from cultivation or dPCR analysis) from duplicate microcosms. For the

heterogeneity experiments, each sample group included six replicate measurements. Finally, for the SRB growth microcosm experiment, sample groups included culture counts from triplicate mason jars prepared using each setup method.

#### **6.2.7 DNA extraction**

Genomic DNA was extracted from clay using the DNeasy PowerMax soil kit (Qiagen; recovery from VBNC state experiments) or the DNeasy PowerSoil kit (Qiagen; bentonite heterogeneity experiments) and from culture biomass using the DNeasy UltraClean kit (Qiagen). For PowerMax and PowerSoil extractions, 2 g and 50 mg of bentonite were used, respectively. The manufacturer's instructions were followed except for the lysis and elution steps. After addition of lysis solution, PowerBead tubes were incubated at 65°C for 30 minutes (PowerMax extractions) or 70°C for 10 minutes (PowerSoil and UltraClean extractions), followed by bead beating for 10 minutes at 30 Hz using an MM 400 Mixer Mill (Retsch; PowerMax extractions) or for 45 s at 5.5 m/s using a FastPrep-24 Classic Instrument (MP; PowerSoil and UltraClean extractions). To concentrate DNA, final elution volumes were reduced to 2 mL, 60 µL, and 50 µL for PowerMax, PowerSoil, and UltraClean extractions, respectively.

#### **6.2.8 DNA sequencing**

For each genomic DNA sample, the V4-V5 region of the 16S rRNA gene was amplified in triplicate using universal primers 515F-Y [174] and 926R [157], modified to include a six-base index sequence, Illumina flow cell binding, and sequencing primer binding sites [158]. All reactions were set up in a PCR hood (AirClean Systems) with ISO 5-HEPA-filtered air. Surfaces of the hood were first washed with 70% ethanol and then treated with UV light for 15 minutes before each use. All tubes, water, and bovine serum albumin (BSA) were also UV-treated on a UV-transilluminator (ProteinSimple) using 302 nm wavelength light for a minimum of 15 minutes. Each 25 µL PCR included 1x ThermoPol Buffer (New England BioLabs), 0.2 µM forward and reverse primer, 200 µM dNTPs, 15 µg BSA, 0.625 U hot start Taq DNA polymerase (New England BioLabs), and 1 µL of template DNA. The following thermal cycling conditions were used: initial denaturation at 95°C for 5 minutes, then 35 (cultures) or 40 (clay) cycles of 95°C denaturation for 30 s, 50°C annealing for 30s, and 68°C extension for 7 min, followed by a final extension step for 7 min at 68°C. No template controls (NTCs) were included both in the 96

well plate alongside samples, as well as in separate PCR tubes. Positive controls were additional included in separate PCR tubes.

Triplicate PCR products were visualized on a 1% agarose gel stained with ethidium bromide and were then pooled and quantified on a second 1% agarose gel stained with GelRed (Biotium). Based on gel quantification, equimolar amounts of each sample were combined to form a pool, which was excised from a 1.5% agarose gel stained with ethidium bromide. The PCR products were purified from the agarose fragment using the Wizard SV Gel and PCR Clean-Up System (Promega). The purified pool was quantified using the Qubit dsDNA High Sensitivity Assay Kit (Invitrogen) as well as qPCR using P5 and P7 primers (Illumina). The final library was denatured and diluted following Illumina guidelines (Document No. 15039740 v01), then spiked with PhiX Sequencing Control v3 (Illumina).

All clay samples were sequenced on a single MiSeq run with a cluster density of 774,000 clusters/mm<sup>2</sup>, average Q30 of 90.0%, and clusters passing filter of 91.4%. All culture biomass samples were sequenced on a second MiSeq run with a cluster density of 813,000 clusters/mm<sup>2</sup>, average Q30 of 89.5%, and clusters passing filter of 93.2%.

### **6.2.9 Sequence analysis**

Sequences were demultiplexed using Local Run Manager version 4. Demultiplexed reads were analyzed using Quantitative Insights Into Microbial Ecology 2 (QIIME2) [159], including Divisive Amplicon Denoising Algorithm 2 (DADA2) [160]. After importing paired-end reads into QIIME2, DADA2 was used to remove low quality reads and primer sequences, to truncate reads to 250 base pairs, to denoise and dereplicate sequences, and to generate amplicon sequence variants (ASVs). Taxonomy was assigned using a naïve Bayes classifier pre-trained with SILVA database release 138. The Decontam R package [175] was used to identify potential contaminants. Clay and culture samples, which were sequenced in different MiSeq runs, were processed with Decontam separately. A threshold value of 0.5 was selected by examining histograms of Decontam score distributions. Contaminant ASVs were removed from the ASV table prior to subsequent analysis.

All sequence data were deposited in the European Nucleotide Archive. For sequences from the clay heterogeneity study, the accession number is PRJEB7941. For sequences from the VBNC experiments, the accession number is PRJEB79492.

## **6.3 Results and discussion**

### **6.3.1 Recovery from VBNC state versus cell division**

Previous research has suggested that the increase in abundance of culturable aerobic heterotrophs that is commonly observed in hydrated or saturated bentonite [124-127; Chapter 2-4] could be due to a recovery from the VBNC state, rather than actual growth [125]. Although the VBNC state has not been studied for bentonite specifically, desiccation and subsequent rehydration-driven induction and recovery from the VBNC state in soil has been reported [211, 212]. To test whether hydration-induced increases in abundance of culturable aerobic heterotrophs are due to cell division or a recovery from the VBNC state, a series of microcosms were set up under varying water and temperature conditions. The hypothesis was that if the increase in abundance of culturable aerobic heterotrophs, which is commonly observed in bentonite incubated between room temperature and 30°C, was due to a recovery from the VBNC state due to rehydration of the bentonite, this should also occur at 4°C. If it was instead due to actual cell division, this would not be expected to occur (at least at the same rate) in hydrated bentonite incubated at 4°C.

Initially, the abundance of culturable aerobic heterotrophs was the same in all microcosms (Figure 6.3; Tukey's HSD,  $p < 0.05$ ). Similar to previous literature, the abundance of culturable aerobic heterotrophs in hydrated bentonite in the present study increased over the seven-day incubation at 30°C (Tukey's HSD,  $p < 0.05$ ; Figure 6.3A). As predicted, this increase was not observed in hydrated bentonite incubated at 4°C, or in un-hydrated (i.e., as-received) bentonite incubated at either temperature (Tukey's HSD,  $p < 0.05$ ; Figure 6.3A). As a control, anaerobic heterotrophs and SRB, which have not previously been shown to increase in abundance in oxic hydrated bentonite experiments [123, 124, 173]; Chapter 2], were included. Like these previous studies, culturable anaerobic heterotrophs and SRB did not increase in abundance over the seven-day incubation period (Tukey's HSD  $p < 0.05$ ; Figure 6.3A). These results suggest that increase in abundance of aerobic heterotrophs in hydrated bentonite is not due to a recovery from the VBNC state driven by rehydration alone. However, the conditions

that lead to a recovery from the VBNC state can be very specific and multifaceted, so these cultivation results alone do not rule out the possibility that recovery from VBNC state in bentonite heterotrophs is driven by a both increased available water and temperature together [208].

To rule out coupled hydration- and temperature-driven recovery from the VBNC state, the cultivation analysis was coupled to a DNA analysis. Quantification of DNA extracted from each microcosm showed a similar pattern to that of culturable aerobic heterotrophs. A significant increase in abundance of 16S rRNA genes was observed over the seven-day incubation of hydrated bentonite at 30°C, but not 4°C or un-hydrated (i.e., as-received) bentonite at either temperature (Tukey's HSD,  $p < 0.05$ ; Figure 6.3A). Further, 16S rRNA gene sequencing revealed a shift in community composition from as-received bentonite starting material in the 30°C hydrated bentonite microcosms over the seven-day incubation, but not any other microcosms. Because DNA-based analyses are expected to include both culturable and VBNC cells, these results demonstrate that observed increases in abundance of culturable aerobic heterotrophs in hydrated bentonite are likely a result of cell division. In these microcosms, heterotrophic growth was associated primarily with *Streptomyces*, but other heterotrophs may prevail under different conditions.

Consistent with aerobic heterotroph cultivation and dPCR results, the 16S rRNA gene profiles of bentonite collected from each microcosm after incubation showed a temperature- and hydration-dependent shift in community composition (Figure 6.3). After seven days of incubation, the microbial community composition of as-received bentonite microcosms incubated at 30°C and in as-received and hydrated bentonite microcosms incubated at 4°C was similar to that of the as-received bentonite starting material (Figure 6.3). Although there was some overlap in ASVs detected in hydrated bentonite microcosms incubated at 30°C and the as-received bentonite starting material, 30°C hydrated bentonite microcosms were dominated by sequences associated with *Streptomyces*, which were not detected in any other microcosms or the starting material (Figure 6.3). Together these results suggest that the increase in abundance of aerobic heterotrophs is due to growth (i.e., increase in cell number) of these organisms rather than a recovery from the VBNC state.

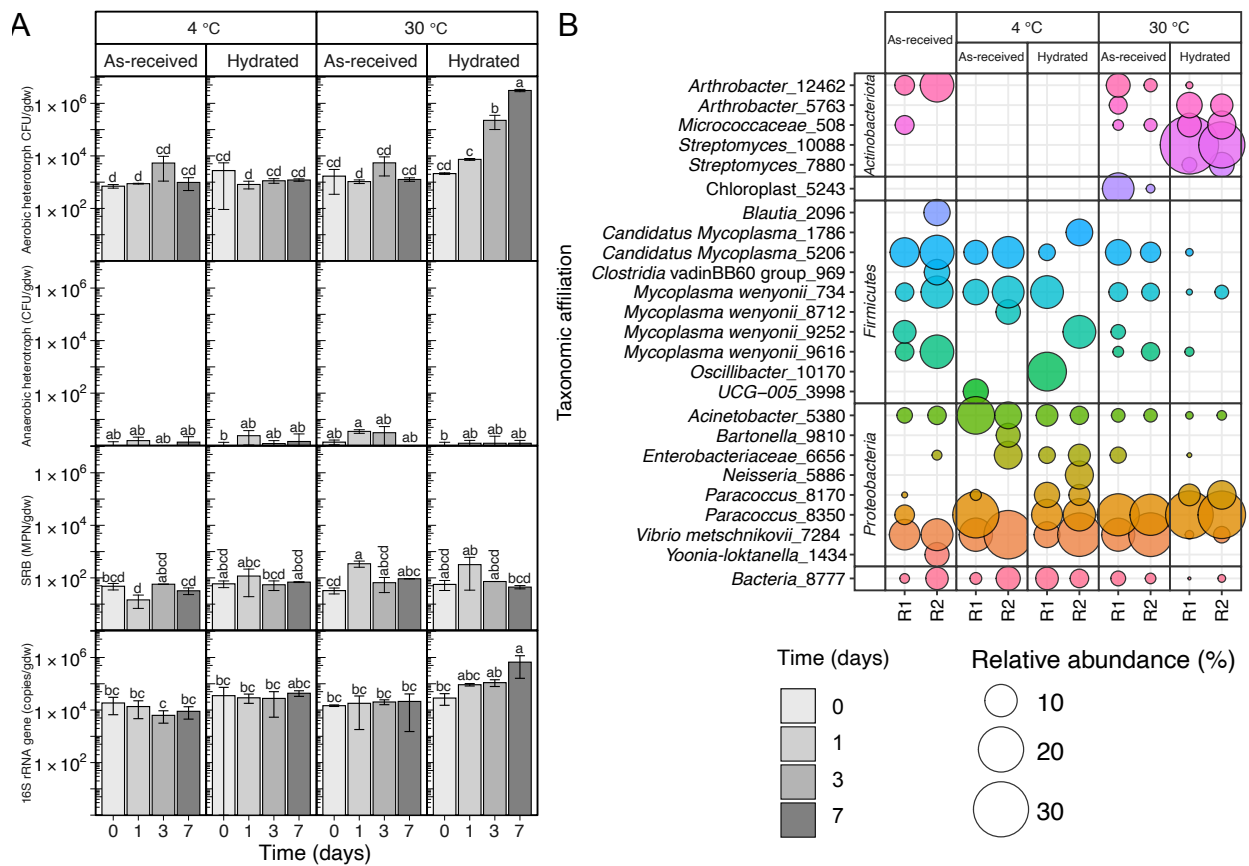


**Figure 6.3** Bar plot showing abundances of culturable microorganisms and 16S rRNA gene copies in microcosms containing either as-received or hydrated MX-80 bentonite (M25) incubated at either 4 or 30°C for one week (A). Corresponding 16S rRNA gene profiles of as-received bentonite starting material and bentonite from microcosms after seven days of incubation (B). Error bars represent standard deviation of duplicate microcosms. Letters above bars represent statistically generated groups, such that bars with the same letter are not statistically different from each other (Tukey's HSD,  $p < 0.05$ ).

### 6.3.2 Bentonite heterogeneity

When growth of aerobic heterotrophs in hydrated bentonite is observed, and biological replicates are included in the experimental design, there can be a relatively high degree of variation in abundance of heterotrophs between biological replicates [173]; Chapter 2-4], even when bentonite is hydrated using thorough mixing [173]. This phenomenon is more or less apparent depending on the type of bentonite used and the temperature at which it is incubated (Chapter 4). A clay and incubation temperature (i.e., a batch of Wyoming MX-80 bentonite referred to as MX9, and 45°C incubation) previously observed (Chapter 4) to result in highly variable abundances of culturable aerobic heterotrophs between biological replicates was selected for additional analysis, including several replicates, to further explore heterogeneity

between and within hydrated bentonite microcosms. Three different methods of mixing as-received bentonite with water were tested (Table 6.1) to determine if inadequate mixing in microcosm setup was the reason for observed variability in abundances of culturable aerobic heterotrophs between replicate hydrated bentonite microcosms. Regardless of mixing method, replicate microcosms had variable abundances of culturable aerobic heterotrophs (Figure 6.4A). In some microcosms, counts of culturable aerobic heterotrophs were in the order of  $10^5$ , and in others they were not detected at all (6.4A). For each mixing method, the standard deviation was proportionally similar (equal to between 1.9 and 2.2 times the average). The variability in abundance of culturable aerobic heterotrophs was smaller between technical replicate plates than between biological replicate microcosms (although some error bars appear large when fewer than 20 colonies were counted on replicate plates; Figure 6.4A). Despite variability, the average abundance of culturable aerobic heterotrophs was the same for all mixing methods (ANOVA,  $p=0.82$ ).

In addition to variability in culturable abundances of aerobic heterotrophs, 16S rRNA gene profiles were also variable between microcosms (Figure 6.4B). Sequence depth was lower for microcosms established using mixing method two (Table 6.1), potentially explaining the more variable profiles between replicates. Bentonite samples from microcosms were most commonly dominated by sequences associated with *Streptomyces* or *Saccharopolyspora*, however, individual microcosms sometimes had little or no representation of these genera in their 16S rRNA gene profiles and were instead dominated by sequences associated with representatives of phyla *Actinobacteria*, *Firmicutes*, or *Proteobacteria* (Figure 6.4B). Although barely detected in the bentonite profiles, the most common taxon dominating 16S rRNA gene profiles of cultures from the bentonite was *Bacillus*, though once again there was variability between replicate microcosms, and some microcosms were instead dominated by sequences associated with other taxa such as *Streptomyces* or *Saccharopolyspora* (Figure 6.4B).

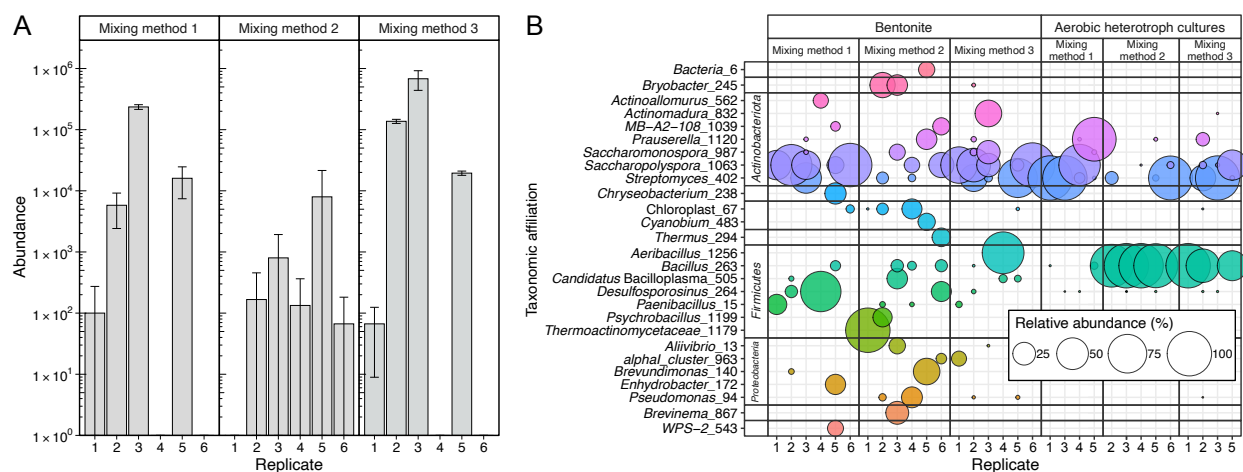


**Figure 6.4** Abundance of culturable aerobic heterotrophs in six replicate microcosms set up using mixing method A (Table 6.1; A), and corresponding 16S rRNA gene profiles of bentonite from each microcosm as well as of biomass from the aerobic heterotroph cultures inoculated with each bentonite sample (B). Error bars in bar plots represent the standard deviation in abundance of culturable heterotrophs on three replicate agar plates. Only genera with a minimum relative abundance of 10% in at least one sample are included in the bubble plot.

Because variation was observed between replicate microcosms, a second experiment was conducted to test if a similar level of variation would be observed if sets of cultures were inoculated using different aliquots of bentonite originating from the same microcosm. To test this, a total of three microcosms, each used to inoculate six separate sets of cultures, were established using mixing method one (Table 6.1). Though to a lesser degree, there was still observed variability in the abundance of culturable aerobic heterotrophs in each set of cultures (Figure 6.5A). Despite variation, the average abundance of culturable aerobic heterotrophs was the same in all three microcosms (ANOVA,  $p=0.41$ ).

The 16S rRNA gene profiles were also variable (Figure 6.5B). In general, 16S rRNA gene profiles of aerobic heterotroph cultures inoculated with aliquots of hydrated bentonite from the same microcosm were usually dominated by similar sequences, but this was not always the case (Figure 6.5B). For example, three replicate bentonite samples from microcosm one had aerobic heterotroph cultures dominated by sequences associated with *Bacillus*, whereas three replicate bentonite samples taken from the same microcosm instead had aerobic heterotroph cultures with high relative abundances of sequences associated with *Prauserella*, *Georgenia*, *Saccharopolyspora*, and *Streptomyces* (Figure 6.5B). Similarly, most cultures inoculated with bentonite from microcosm 2 were dominated by sequences associated with *Prauserella*, but

others instead by sequences associated with *Streptomyces* or *Saccharopolyspora* (Figure 6.5B). Cultures inoculated with bentonite from microcosm 3 had the most consistent 16S rRNA gene profiles, all being dominated by sequences associated with *Streptomyces* (Figure 6.5B).

One explanation for the observed heterogeneity is that the relatively low abundance of cells in as-received bentonite are randomly distributed between and within microcosms such that different species are differentially represented in certain microcosms or areas within a microcosm. These cells could then potentially proliferate with increased water and temperature, resulting in replicate microcosms being dominated by different species. From the results of this experiment, it is possible that there truly are different abundances of culturable aerobic heterotrophs between replicate microcosms and within individual microcosms, but it is also possible that the microorganisms that happen to proliferate in one microcosm are culturable under the specific conditions used, whereas the microorganism that proliferate in another may not be. For example, sequences associated with *Saccharopolyspora* often dominated the 16S rRNA gene profiles of hydrated bentonite after incubation (Figure 6.4B, Figure 6.5B) and are detected in low or no abundance in as-received MX9 (Chapter 4), suggesting that *Saccharopolyspora*-associated cell division occurred in the microcosms. That said, this taxon was rarely detected in the 16S rRNA gene profiles of associated cultures (Figure 6.4B, Figure 6.5B), suggesting that it may not be culturable under the conditions applied. This taxon also dominated the profiles of previously conducted microcosm experiments, and similarly was not detected in 16S rRNA gene profiles of associated cultures [173]. Sequences associated with *Thermoactinomycetaceae* also dominated (3184 or the 3187 total reads were associated with it) the microbial profile of bentonite from one microcosm (replicate one, mixing method two), and this culture had no detectable culturable aerobic heterotrophs, even though members of family *Thermoactinomycetaceae* are typically aerobic heterotrophs [180]. This taxon was also previously found to dominate microbial profiles of bentonite from microcosms, but not those of the associated cultures (Chapter 4), further suggesting that it may not be culturable under the selected conditions. Although speculative, variability in abundance of culturable aerobic heterotrophs between and within replicate hydrated bentonite microcosms could be due to actual differences in abundance of culturable microorganisms, or instead could be due to the proliferation of unculturable (under the selected conditions) microorganisms in some microcosms, and higher proportions of culturable microorganisms in others.

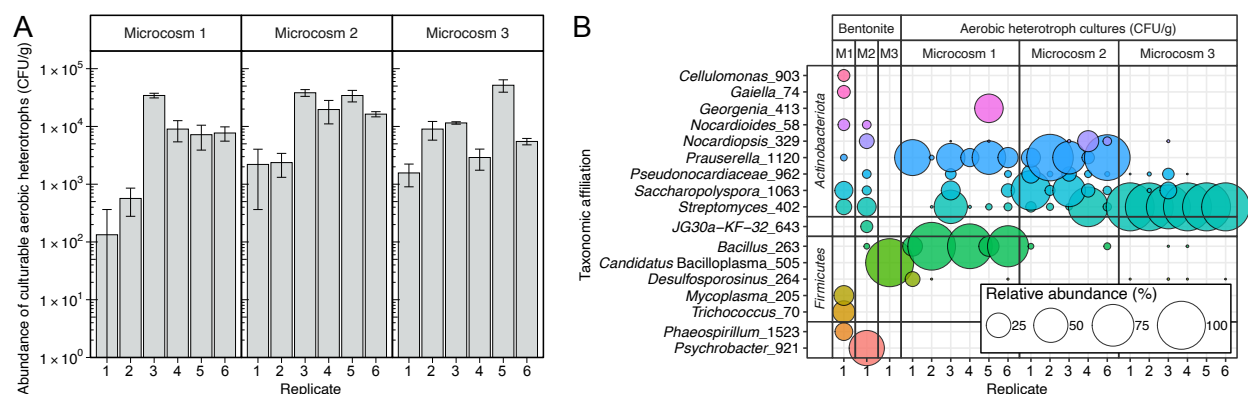


**Figure 6.5** Abundance of culturable aerobic heterotrophs in six subsamples of three replicate microcosms, all set up using mixing method 1 (Table 6.1; A), and corresponding 16S rRNA gene profiles of bentonite from each of the three microcosms as well as of aerobic heterotroph cultures inoculated with each bentonite sample (B). Error bars in bar plots represent the standard deviation in abundance of culturable heterotrophs on three replicate agar plates. Only genera with a minimum relative abundance of 5% in at least one sample are included in the bubble plot.

### 6.3.3 SRB medium comparison

Unlike aerobic heterotrophs, culturable SRB typically do not increase in number in bentonite [123, 124, 126–128]; Chapter 2, Chapter 4], even when supplied with available water and nutrients [173]. However, in a recent set of anoxic pressure vessel experiments set up using gapfill bentonite compacted to varying dry densities and hydrated with sterile Type I water (Chapter 3), SRB did increase in abundance from the as-received bentonite starting material in several samples, especially outer layer samples in contact with the inside of the pressure vessel. In this study, it was proposed that the measured increase in abundance of culturable SRB not previously observed in other studies could be due to differences in SRB medium between studies, differences in the specific bentonite used, or differences in set up that could have resulted in less trace oxygen than in other studies. A series of experiments were conducted to test these factors.

There are two types of SRB medium commonly used in experiments that involve cultivation of SRB from bentonite: Postgate's medium B, and SRB medium from HiMedia (M803). To test if differences in the composition of the two media (Table 6.2) results in different abundances or community compositions of culturable SRB from bentonite, triplicate sets of SRB cultures were inoculated with as-received bentonite incubated at 15, 30, 45, or 60°C for one week. Although too few replicates were included to conduct statistics, the 95% confidence intervals for SRB cultures from a given clay incubated at a specific temperature either set up

with Postgate's medium B or with HiMedia's SRB medium always overlapped (Figure 6.6). This result was consistent for cultures incubated at 15, 30, 45, and 60°C (Figure 6.6).

Regardless of media choice, cultures inoculated with the same clay and incubated at the same temperature had similar 16S rRNA gene profiles (Figure 6.7). The most prevalent genus of putative SRB detected in clay samples was *Desulfosporosinus*, though sequences associated with *Desulfotomaculum* were instead dominant in some samples (Figure 6.7). Most SRB cultures incubated at 15 or 30°C were dominated by sequences associated with SRB, whereas sequences associated with *Bacillus* were more dominant in 45°C cultures (Figure 6.7). Sequences associated with *Desulfospororinus* were dominant in some 60°C cultures, and other dominant genera included *Bacillus*, *Caldibacillus*, *Brevibacillus*, and *Anoxybacillus* (Figure 6.7). At 15 and 30°C, when a HiMedia culture from a particular clay was dominated by sequences associated with genera other than *Desulfosporosinus* (e.g., with *Bacillus* or *Anaerospromus*), the corresponding Postgate's medium B culture generally was as well (Figure 6.7).

Together, these results demonstrate that both media perform similarly, suggesting that experiments conducted using one medium or the other should be comparable. HiMedia's SRB medium, which was used to culture SRB from anoxic pressure vessels where SRB increased in abundance (Chapter 3), was also used to culture SRB from bentonite in other anoxic experiments where they were not observed to increase in abundance, further suggesting that the choice of SRB medium in the anoxic pressure vessel experiment was not the reason for the unexpected increase in abundance of culturable SRB [127, 173].

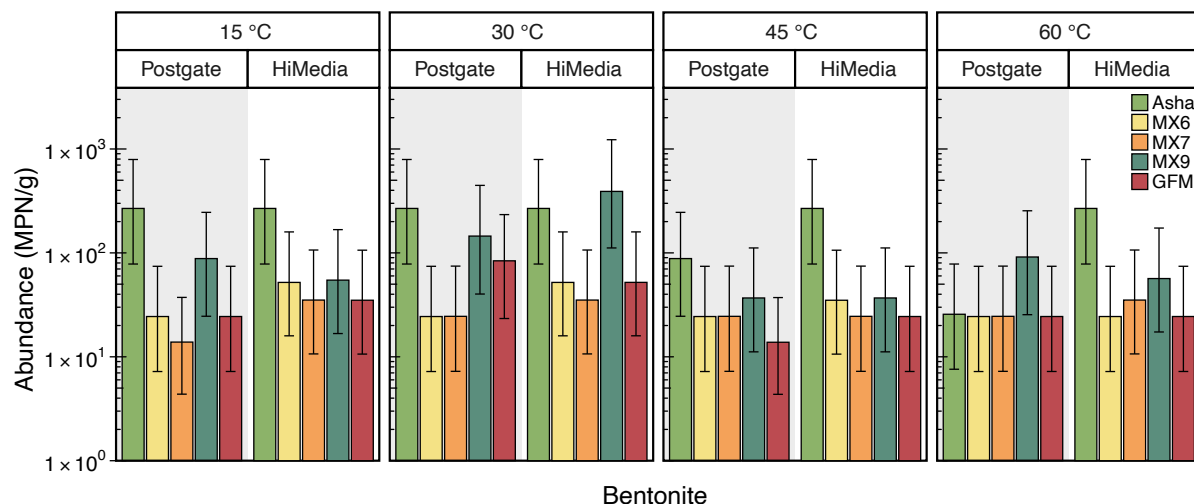


**Figure 6.6** Bar plot showing the abundance of culturable sulfate-reducing bacteria (SRB) in Asha bentonite hydrated to a water activity of 0.99 and incubated under oxic conditions for one week at 15, 30, 45, or 60°C. Replicate SRB cultures were set up with Postgate’s medium B or with SRB medium from HiMedia (M803). Error bars represent the 95% confidence interval provided by the MPN table.

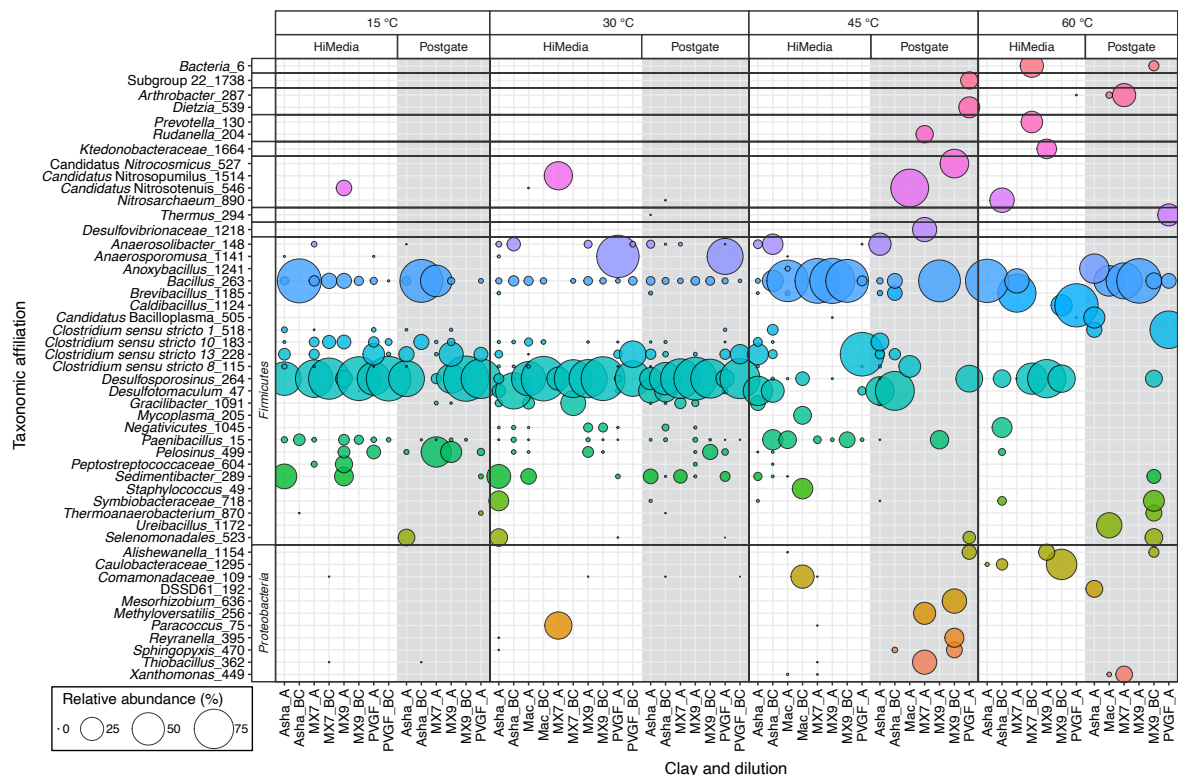


**Figure 6.7** Bubble plot of 16S rRNA gene profiles of SRB cultures inoculated with Asha bentonite hydrated to a water activity of 0.99 and incubated under oxic conditions for one week at 15, 30, 45, or 60°C. Replicate cultures were set up using Postgate’s medium B or with SRB medium from HiMedia (M803). Only genera with a minimum relative abundance of 10% in at least one sample are included. “A” and “BC” at the end of sample names refer to the culture dilution.

### 6.3.3 SRB growth in bentonite

A set of microcosms prepared using different conditions (Table 6.3) was used to test if SRB growth observed in anoxic pressure vessels (Chapter 3), but not a previous microcosm experiment specifically designed to promote the growth of SRB (i.e., bentonite hydrated to a high water activity with SRB medium under anoxic conditions), was due to anoxic pressure vessel set up resulting in a lower concentration of trace oxygen [173]. Setup methods A and B mimicked previous microcosm [173] and anoxic pressure vessel (Chapter 3) conditions, respectively. These two sets of microcosms were set up in different research labs consistent with where the setup of the original experiment (microcosm or anoxic pressure vessel) occurred. Although both conditions were intended to be anoxic, it was expected that method A could result in higher concentrations of trace oxygen. Set up method C was conducted at the same location as method A and was intended to be at least as anoxic as method B. Triplicate microcosms set up using each of these set up methods resulted in the same low abundance of culturable SRB (Tukey's HSD,  $p < 0.05$ ). The abundance of culturable aerobic and anaerobic heterotrophs was also similar between all tested conditions (Figure 6.8). For both aerobic and anaerobic heterotrophs, no increase in abundance was observed over the six-week incubation (Tukey's HSD,  $p < 0.05$ ; Figure 6.8).

One difference between the setup of these experiments and that of anoxic pressure vessels was that the clay was thoroughly mixed with water. To ensure that the mixing process itself did not destroy SRB cells, a fourth condition was included where water was added on top of the bentonite but not mixed. Microcosms set up using this method had the same low abundances of SRB as all other microcosms (Tukey's HSD,  $p < 0.05$ ), suggesting that mixing was not the reason for no SRB growth in microcosms. This present microcosm experiment also tested two different types of bentonite, gapfill bentonite (used in anoxic pressure vessels) and MX9 (used in previous microcosms), to determine if gapfill, which had not previously been used in anoxic microbiology experiments, either contained a higher initial abundance of SRB or was somehow more conducive to SRB growth. The abundance of culturable SRB, once again, was the same for all microcosms, regardless of which bentonite was used (Tukey's HSD,  $p < 0.05$ ). These results suggest that neither clay nor differences in setup method leading to differences in trace oxygen concentration were responsible for SRB growth in anoxic pressure vessels but not in other experiments.

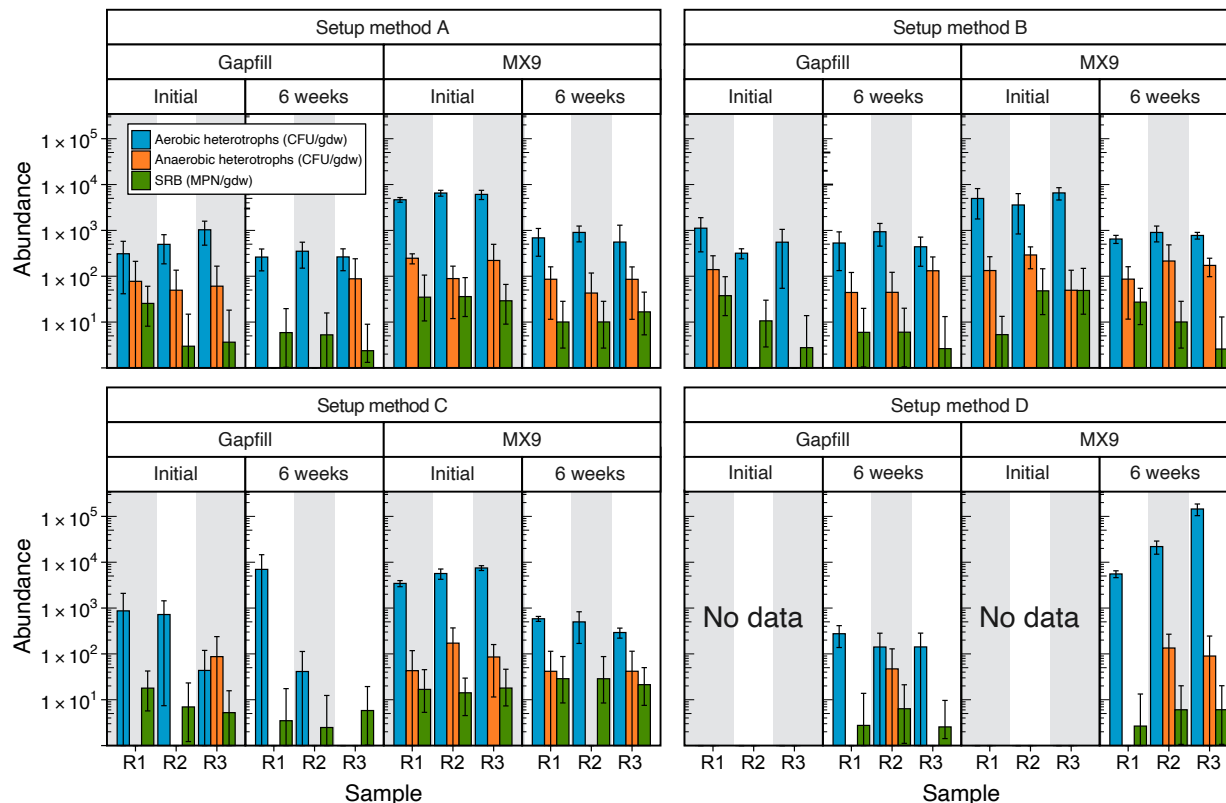


**Figure 6.8** Bar plots of the abundance of culturable aerobic heterotrophs, anaerobic heterotrophs, and SRB in microcosms set up using either gapfill material or Wyoming MX-80 bentonite (MX9) hydrated to a water activity of 0.96 and incubated under anoxic conditions at  $\sim 25^\circ\text{C}$  for six weeks. Replicate microcosms were set up using one of four setup methods (Table 6.3). Error bars represent the standard deviation between triplicate microcosms.

To further test if trace oxygen could be responsible for SRB not growing in some bentonite experiments but growing in others, the oxygen sensitivity of SRB from bentonite was tested. In the one experiment, SRB cultures were inoculated with hydrated Asha bentonite and incubated under oxic conditions. After incubation, five out of five A dilution (i.e., 1:10 dilution of bentonite) cultures, five out of five B dilution (i.e., 1:100 dilution of bentonite) cultures, and one out of five C dilution (i.e., 1:1000 dilution of bentonite) cultures were positive for SRB growth. Biomass was collected from positive cultures of each dilution, and DNA was extracted and sequenced. The 16S rRNA gene profiles of these cultures show presence of sequences associated with the SRB *Desulfotomaculum* in the A and C dilution (Figure 6.9A). Although these cultures did not have airtight caps, they were incubated without shaking and their 16S rRNA gene profiles included relatively high abundances of sequences associated with putative aerobic heterotrophs, so it is possible that an anoxic zone developed in the bottom of the tubes as

aerobes consumed the oxygen, which was not replenished by mixing. Nonetheless, the results of this experiment would suggest that SRB from bentonite, or at least *Desulfotomaculum*, are not killed by exposure to oxygen.

To further explore the oxygen sensitivity of bentonite SRB, an enrichment culture of *Desulfosporosinus* (>90%), a commonly observed putative SRB in bentonite, was subcultured into several new tubes of SRB medium. Triplicate subcultures were incubated with increasing concentrations of air in the headspace. The anoxic cultures exhibited the fastest growth, followed by cultures with a headspace including 25 and 50% air (Figure 6.9B). These cultures were completely black (indicating SRB activity) one, two, and five days after inoculation, respectively. Cultures with 60% air in the headspace or higher had similar cell counts to the initial inoculum at all time points, and these cultures never turned black, indicating no SRB activity (Figure 6.9B). As oxygen concentrations would be expected to be too low to support growth of obligate aerobes (~10% in the headspace of the 50% air cultures and ~5% in the 25% air cultures), and no increase in abundance of cells (SRB or otherwise) was observed in the cultures containing higher concentrations of air where SRB were not detected (i.e., no black precipitate), it is likely that the increase in abundance of cells was reflective of *Desulfosporosinus* growth. Although SRB are typically thought of as obligate anaerobes, *Desulfosporosinus* and *Desulfotomaculum* species are known to form oxygen-tolerant endospores that allow them to survive oxygen exposure [214, 215]. Further, members of the genus *Desulfosporosinus* have been isolated from oxic environments and have genetic evidence of oxygen adaptations typically associated with aerobes [216, 217]. In one study, it was proposed that these organisms may even be performing sulfate reduction in the oxidizing layers of the gold mine tailings in which they were detected [217]. The results of these experiments do not conclusively show that SRB can reduce sulfate in the presence of oxygen (a pure culture would be needed), but they do suggest that SRB in bentonite are likely not inhibited by trace amounts of oxygen that may be introduced during experimental setup.

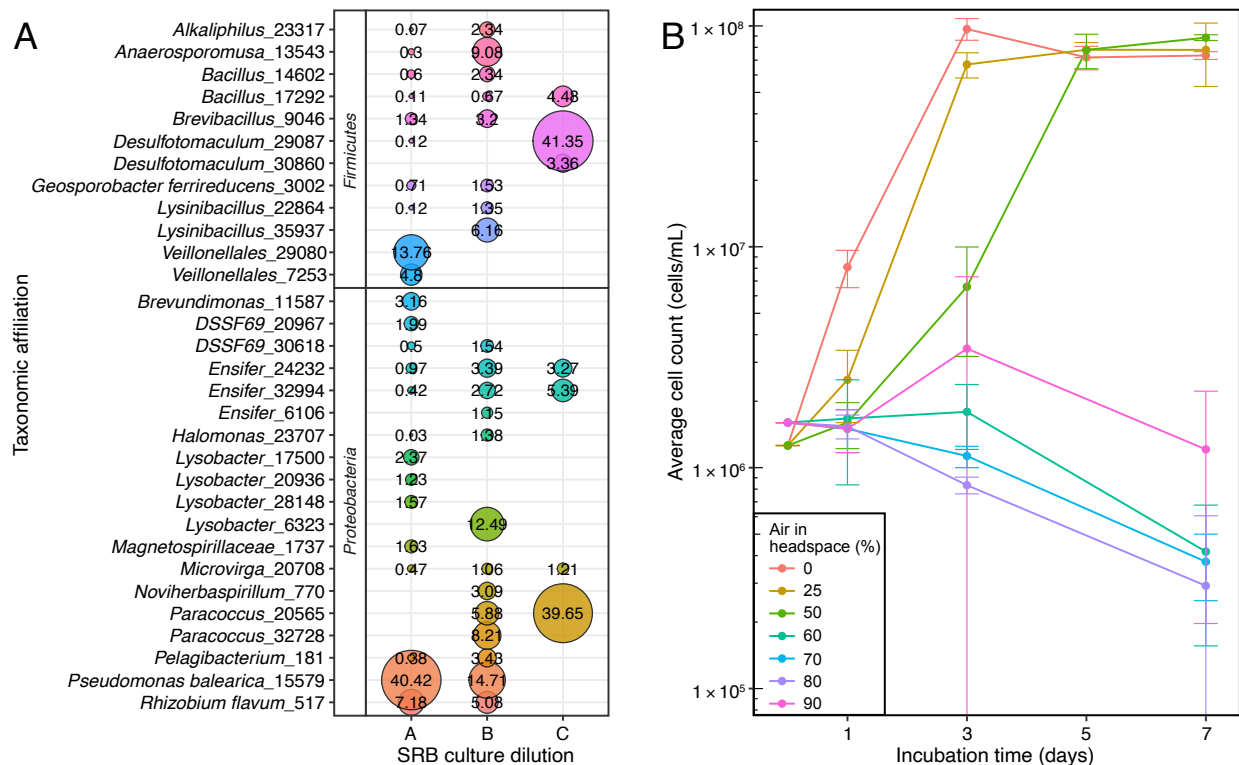


**Figure 6.9** Bubble plot showing 16S rRNA gene profiles of SRB cultures inoculated with three dilutions (A, B, C) of an Asha bentonite clay suspension and incubated under oxic conditions. Asha bentonite was hydrated to a water activity of 0.99 and incubated at 30°C for one week prior to culture inoculation. Only ASVs with a minimum relative abundance of 1% in at least one sample are included (A). Cell count-based growth curve of a *Desulfosporosinus* enrichment culture incubated with varying concentrations of air (0-90%) in the headspace. Error bars represent the standard deviation between triplicate cultures (B).

Overall, it remains unknown why SRB could grow in anoxic pressure vessel experiments (Chapter 3) when a similar increase in abundance has not been observed in previous studies. The results of the experiments presented here suggest that medium, bentonite type, and trace oxygen likely do not account for growth of SRB in anoxic pressure vessels. One other major difference between previous anoxic pressure vessel (Chapter 3) and anoxic microcosm [173] experiments was pressure. That said, SRB were not observed to grow in other experiments where bentonite was exposed to pressure under anoxic conditions, though these studies all used a different type of bentonite and were either conducted in boreholes with lower temperatures and higher concentrations of salt in the saturating water than in anoxic pressure vessels, or were set up using dried clay [126–128, 173]. Another difference between anoxic pressure vessels (Chapter 3) and anoxic microcosms [173] is the presence of a solid, porous substrate (i.e., filter stones directly in

contact with the clay plug) that could potentially promote biofilm formation. Future microcosm studies could be conducted incorporating similar solid substrates to test if SRB grow in their presence.

## **6.4 Conclusion**

The microbiology of bentonite clay is under study in the context of long-term used nuclear fuel storage, as bentonite is a key component of several repository designs. Existing research on the microbiology of bentonite clay has led to questions about how and why aerobic heterotrophs and SRB grow in bentonite. Specific questions regarding the growth of microorganisms in bentonite included if increases in abundance of aerobic heterotrophs commonly measured in bentonite were associated with true growth or instead a recovery from the VBNC state, why culturable abundances of aerobic heterotrophs were so variable in replicate clay samples, and if observed SRB growth in bentonite in one study but not others was due to choice of medium, bentonite type, or more strictly anoxic conditions.

The results of this study demonstrated that the increase in abundance of culturable aerobic heterotrophs commonly observed when bentonite is hydrated under low- or no-pressure conditions is likely due to actual cell division. The abundance of culturable aerobic heterotrophs increased in microcosms of hydrated Wyoming MX-80 bentonite incubated at 30°C but not 4°C, and DNA quantification results mirrored those of aerobic heterotroph culture abundances. To further support that the increase in abundance was due to true growth, a shift in community composition was observed for 30°C hydrated bentonite microcosms but not 4°C hydrated bentonite microcosms. This increase in abundance of culturable aerobic heterotrophs was shown to be highly variable, both in abundance and community composition, between, and to a lesser degree within, replicate microcosms. The microorganisms that proliferated in some hydrated bentonite microcosms were rarely or never detected in cultures inoculated with the same bentonite material, and the microorganisms that proliferated in other microcosms were commonly cultured. As such, it was proposed that the variability in abundance of culturable aerobic heterotrophs may not be an accurate representation of different abundances of aerobic heterotrophs in the microcosms and could instead reflect very different microbial community compositions among replicate microcosms including different proportions of culturable microorganisms.

The experiments presented in this study suggest that SRB medium choice, bentonite type, and more strictly anoxic conditions than used in some previous studies do not account for the increase in abundance of SRB observed in a previous anoxic pressure vessel experiment (Chapter 3). Clay suspensions used to inoculate two different types of SRB medium, and microcosms set up using one of two different types of bentonite and one of four setup methods, potentially associated with introduction of different levels of trace oxygen, all resulted in the same low abundances of culturable SRB. Future studies should continue to explore the conditions that allow for the growth of SRB in bentonite. For example, future hydrated bentonite microcosm studies could include a solid porous substrate, similar to the filter stone in anoxic pressure vessels where SRB were observed to grow, to test if this promotes SRB growth. The experiments presented throughout, as well as previous microcosm and anoxic pressure vessel experiments, all used sterile Type I water to hydrate bentonite. In the context of a DGR, it will be important to test if the increase in abundance of SRB in previous pressure vessel experiments would also occur if those pressure vessels were saturated with relevant groundwater.

## Chapter 7: Conclusions

### 7.1 Summary and context

The study of microbiology in the context of long-term used nuclear fuel storage has been ongoing for over 30 years. Initial research focused on qualitative descriptions of the types of microbial activity that could be relevant to a DGR, often by comparison to relevant subsurface environments and their associated groundwater [218]. More recent research has focused on bentonite specifically, and has aimed to quantitatively identify parameters that promote and suppress microbial growth. Because a goal in designing DGRs is to suppress microbial growth by increased bentonite dry density, studying microbial suppression in bentonite compacted to a high dry density has been a major focus of study [123, 124, 126–129, 151, 219]. An initial study demonstrated suppression of microbial growth in powdered Wyoming MX-80 bentonite compacted to a dry density of  $1.6 \text{ g/cm}^3$  under oxic conditions [123], and subsequent studies have corroborated this [124, 126–129, 151, 219]. In all cases, gapfill material was not studied and bentonite was either pre-saturated prior to compaction or early sampling time points (i.e., < 1 year) were not incorporated into experimental designs. As such, existing studies have overlooked any microbial growth that may occur during the process of saturation, which represents thousands of years in a DGR [20] and could be associated with more favourable conditions for microbial growth due to decreased swelling pressure. Additionally, prior to the research presented in this thesis, intermediate dry densities (i.e.,  $1.4\text{--}1.5 \text{ g/cm}^3$ ), which could more realistically be achieved for gapfill material in a DGR, had not been assessed for their ability to suppress microbial growth. To address gaps in the literature, Chapters 2 and 3 focused on identifying bentonite dry density conditions that suppress microbial growth in powdered and gapfill bentonite under oxic conditions and in gapfill bentonite under anoxic conditions. In both studies, intermediate dry densities were included to test if microbial growth was suppressed by dry densities lower than  $1.6 \text{ g/cm}^3$ . Further, bentonite was not pre-saturated and was sampled at early time points to study microbial growth throughout the saturation phase.

To accurately model microbial growth in a DGR, it is important to not only study microbial response to increased dry density, but also to the range of other physical conditions expected in a DGR. As a DGR is expected to maintain elevated temperatures ( $>70^\circ\text{C}$ ) for over one thousand years [119, 220], it is critical to understand the potential for thermophilic microbial

growth. In the few studies that had been conducted prior to the research presented in this thesis, it was demonstrated that some microorganisms in bentonite could survive a temperature of 60°C but did not grow (i.e., increase in number) during this time [129, 130]. In one study, although microbial activity was not observed, DNA sequencing did demonstrate a shift in microbial community composition [130]. The work presented in Chapter 4 builds on these initial observations by using a coupled cultivation and DNA sequencing-based approach to look for evidence of microbial growth at elevated temperature, and additionally built on previous research by incorporating five additional DGR-relevant temperatures (15, 45, 75, and 105°C) beyond the previously studied 30 and 60°C.

Although lab-scale experiments are crucial for studying the response of bentonite microbial communities to changes in specific DGR-relevant physical properties, they are very short term (i.e., usually one year or less) in comparison to the very long timeframes a DGR is expected to remain active (i.e., one hundred thousand to one million years). A solution to this is to couple lab-scale experiments to the study of natural bentonite deposits (i.e., natural analogues), which have existed in nature for DGR-relevant timeframes. Few natural analogues have been studied [133, 135, 136, 204], and half of the studies in this area predate the era of DNA sequencing [136, 204]. Nonetheless, these studies have demonstrated the presence of culturable microorganisms in all clays studied, sometimes including sulfate-reducing bacteria in low numbers [133, 135, 136, 204]. For the studies that incorporated DNA-sequencing, microbial communities were often dominated by sequences associated with *Proteobacteria* or *Actinobacteria* phyla, and the factors that determine which microorganisms exist in one bentonite sample versus another remain poorly understood [133, 135]. In the existing literature, too little data exists to make broad-scale conclusions or predictions about the potential state of bentonite in a DGR far into its future. The data presented in Chapter 5 adds the study of several bentonite core samples from the Tsukinuno mine (Japan), incorporating cultivation, DNA sequencing, and measurement of a variety of geochemical components to attempt to explain factors that drive microbial community composition and abundance in natural bentonite deposits.

The work presented throughout this thesis raised additional questions regarding the growth of microorganisms in bentonite and the factors that influence it. These questions were addressed in Chapter 6. The first uncertainty was regarding the increase in abundance of

culturable aerobic heterotrophs, compared to as-received bentonite, in hydrated bentonite that is uncompacted or compacted to a relatively low dry density (i.e.,  $<1.4 \text{ g/cm}^3$ ), which was observed in Chapters 2, 3, and 4 as well as in previous literature [123, 124, 126, 127]. One study proposed that this increase in abundance was more likely associated with a recovery from the VBNC state rather than a reflection of microbial growth [125]. When these increases in abundance of culturable heterotrophs are observed, and the experimental design includes replication, the abundances among replicates are often highly variable, even when caution is taken to make replicates as homogeneous as possible [173]. Finally, when growth of SRB was observed in anoxic pressure vessels in Chapter 3, a series of experiments were conducted to explain the factors that may have allowed for this growth, as it had not been observed in previous experiments [123, 124, 126–128].

## **7.2 Dry density considerations for a future DGR**

The results of the work presented in Chapter 2 support that the previously identified dry density threshold of  $1.6 \text{ g/cm}^3$  [123] does suppress microbial growth under oxic conditions in powdered Wyoming MX-80 bentonite, and further demonstrates that this same high dry density leads to suppression of microbial growth in gapfill material, which was previously untested under these conditions. Although intermediate dry densities were not tested for gapfill material under oxic conditions, a dry density as low as  $1.4 \text{ g/cm}^3$  suppressed microbial growth in powdered bentonite once saturation neared completion (Chapter 2). Prior to saturation, partially hydrated pressure vessels (i.e., at early sampling time points) were generally associated with growth of aerobic heterotrophs in the outer layers (i.e., the surfaces in contact with the inside of the pressure vessel). This increase in abundance of culturable heterotrophs has previously been observed [123, 124, 126, 127] and was hypothesized to be due to a recovery from the VBNC state for existing cells rather than microbial growth [125]. The results presented in Chapter 6 demonstrate that the increase in abundance is instead likely due to growth by cell division (i.e., increase in cell number).

A similar initial increase in abundance of aerobic heterotrophs, and anaerobic heterotrophs in this case, was observed in the outer layers of anoxic pressure vessels with bentonite compacted to a dry density of  $1.40 \text{ g/cm}^3$  or less (Chapter 3). Although no increase in abundance of culturable heterotrophs was observed in the inner layer of pressure vessels with a

minimum bentonite dry density of 1.45 g/cm<sup>3</sup>, culturable SRB higher in abundance than the starting material persisted throughout all pressure vessel dry density conditions (1.25-1.60 g/cm<sup>3</sup>) over the one-year experiment. That said, these results do not necessarily imply that SRB were active at all time points, and it is instead possible that they proliferated in outer layer samples at early time points with low saturation and swelling pressure, then persisted as inactive spores in the saturated bentonite. Follow-up experiments suggested that this SRB growth, which had not been observed in previous studies, was likely not inherent to gapfill material, nor due to choice of SRB medium, nor differences in trace oxygen between anoxic experiments (Chapter 6). Additional experiments will be needed to better understand the conditions that allow for the growth of SRB in bentonite.

Together, results of pressure vessel experiments suggest that a dry density of 1.40-1.45 g/cm<sup>3</sup> would suppress microbial growth in the powdered and gapfill bentonite in a future DGR, in both the initial oxic phase and the long-term anoxic phase, once the DGR is fully saturated. Prior to saturation, growth of heterotrophs and SRB may be expected, especially at the bentonite-rock wall interfaces. It is also important to note that, unlike in the current experiments, saturation in a DGR will be linked to temperature. This means that for most of the low saturation period in a DGR, the temperature will be much higher than the ~25°C temperature that these pressure vessel experiments were conducted at. This could be expected to limit microbial growth, although additional experimentation would be helpful to confirm these observations.

### **7.3 Temperature limits of microbial growth in bentonite**

The results of this thesis corroborate previous literature which demonstrated survival of microorganisms in bentonite after incubation at 60°C [129, 130], and further demonstrates that microorganisms can persist in a viable and culturable state in bentonite after incubation at 15, 30, or 45°C, suggesting that the as-received bentonite microbial community includes thermotolerant microorganisms (Chapter 4). Although an increase in the abundance of culturable microorganisms, usually used to define microbial growth in bentonite, was not observed in hydrated bentonite incubated at 60°C, 16S rRNA gene profiles of these microcosms were dominated by sequences associated with *Thermoactinomycetaceae*, which were not detected in as-received bentonite samples and rarely detected in associated cultures. These results highlighted a limitation of culture-based microbial abundance estimates. It is likely that members

of family *Thermoactinomycetaceae* did grow in these microcosms based on DNA sequencing results, but from the cultivation results alone it appeared as though microbial growth did not occur, and further that very few cells from the initial community survived. Although DNA was detected in bentonite incubated at temperatures up to 105°C, microbial profiles between replicates were variable and did not reflect a change in community composition from the as-received bentonite starting material, suggesting that there was no or minimal microbial growth in bentonite at these temperatures.

Overall, the microcosm experiments demonstrate that as-received bentonite includes microorganisms that can grow in hydrated bentonite incubated at 15, 30, 45, or 60°C. These results do not support microbial growth in bentonite incubated at temperatures of 75°C or higher. Importantly, sequences associated with *Thermoactinomycetaceae* were not detected in the as-received bentonite starting material, highlighting the importance of these types of microcosm experiments to test for the presence of certain microbial groups rather than using analysis of as-received clay alone to make conclusions about the microbial potential within. Similarly, the results of Chapter 4 cannot be used to make any statements about whether thermophilic microorganisms would proliferate in bentonite under anoxic conditions, in compacted bentonite, or in bentonite saturated with relevant groundwater rather than Type I water, but all three represent valuable follow-up experiments.

#### **7.4 Microbiology of a future DGR**

Making statements about the types of microorganisms that may be present temporally in a DGR is challenging. Existing literature that used DNA-based analyses suggested that microbial communities within compacted bentonite in a DGR may be associated with taxa such as *Pseudomonas* and *Bacillus*, as they have been shown to proliferate in compacted bentonite [127, 151]. Although there was evidence for growth of microorganisms associated with these taxa under certain conditions, results of the experiments presented within show that as-received bentonite includes several different species of viable microorganisms that can proliferate in bentonite under suitable conditions (Chapter 2, 3, 4, 6). The specific microorganisms that proliferate in a given bentonite sample are likely related to the distribution of low abundance microorganisms and condition-specific selection. Under all conditions tested (i.e., oxygen concentration, temperature, bentonite type, and dry density), sequences associated with phyla

*Proteobacteria*, *Firmicutes*, and *Actinobacteria* dominated. Generally, these sequences were associated with putative heterotrophs, though the sulfur oxidizer *Thiobacillus* was dominant in several high dry density oxic pressure vessels (Chapter 2), and the sulfate-reducing bacterium *Desulfosporosinus* was detected at high relative abundance in the bentonite-pressure vessel interfaces of anoxic pressure vessels (Chapter 3).

All tested physical conditions were found to influence the microorganisms that grew in bentonite (Chapter 2, 3, 4, 6). Correlating physical properties to microbial community composition and abundance was more challenging for natural analogue samples where conditions were not modified throughout an experiment but instead had been stable over very long timeframes (Chapter 5). Nonetheless, nitrate, which could act as an electron acceptor in denitrification, was positively correlated with the abundance of culturable anaerobic heterotrophs, and montmorillonite, which influences the degree of clay swelling, was negatively correlated with the abundance of culturable aerobic and anaerobic heterotrophs. Several different geochemical properties were weakly or moderately correlated with microbial community composition of Tsukinuno bentonite cores. These results suggest that additional physical or chemical properties that have not been considered in the present experiments (e.g., ground water chemical composition and pH) could influence the microorganisms that grow in bentonite beyond the known influence of dry density, temperature, water availability, and oxygen.

Based on the results presented, a DGR, initially oxic and moderate in temperature, may be associated with growth of putative heterotrophs such as *Streptomyces*, *Nocardiosis*, *Saccharopolyspora*, and *Micrococcaceae*, as water becomes available and swelling pressure remains relatively low (Chapter 4 and 6). In areas of increasing temperature, thermophiles like those from family *Thermoactinomycetaceae* may instead dominate (Chapter 4). Eventually, as saturation is complete and oxygen depleted, microbial growth should be suppressed, provided that the dry density is at least 1.4 g/cm<sup>3</sup> (Chapter 2-3). In any areas with locally lower dry densities, growth of microorganisms such as *Mycoplasma*, *Acinetobacter*, and *Pseudomonas* may be expected if oxygen is fully depleted (Chapter 3). If some oxygen remains, growth could instead be associated with *Pseudomonas*, *Bacillus*, or *Thiobacillus*, and additionally *Streptomyces* and *Cupriavidus* if dry density is locally below target (i.e., 1.1-1.25 g/cm<sup>3</sup>; Chapter 2). In general, the abundance of viable microorganisms is expected to be highest at interfaces

(e.g., between bentonite and the host rock; Chapter 2 and 3), where growth of anaerobic sulfate-reducing bacteria and other microorganisms such as *Bacillus* and *Anaerobacillus* could be expected if conditions are anoxic (Chapter 3). These interface zones are more likely to be associated with aerobic microorganisms such as *Pseudomonas* if oxygen persists (Chapter 3).

In the long-term, natural analogue research suggests that at least some of the taxa detected in the lab-scale experiments could prevail in subsurface clay. All bentonite cores from the Tsukinuno mine were dominated by sequences associated with family *Comamonadaceae*, *Thiobacillus*, and *Pseudomonas* (Chapter 5). On a broader taxonomic level, phylum *Proteobacteria* dominated Tsukinuno clay profiles, and additionally phylum *Actinobacteria* in the cultures inoculated with Tsukinuno clay. These represent two of three phyla that dominated all lab-scale experiments presented here (Chapter 2, 3, 4, 6), and the third, *Firmicutes*, is a common inhabitant of subsurface communities in general. Although the conditions of the Tsukinuno bentonite do not perfectly mimic the expected conditions of DGR bentonite, the results of the natural analogue experiment (Chapter 5) did not suggest that SRB would be dominant members of long-term bentonite-associated communities.

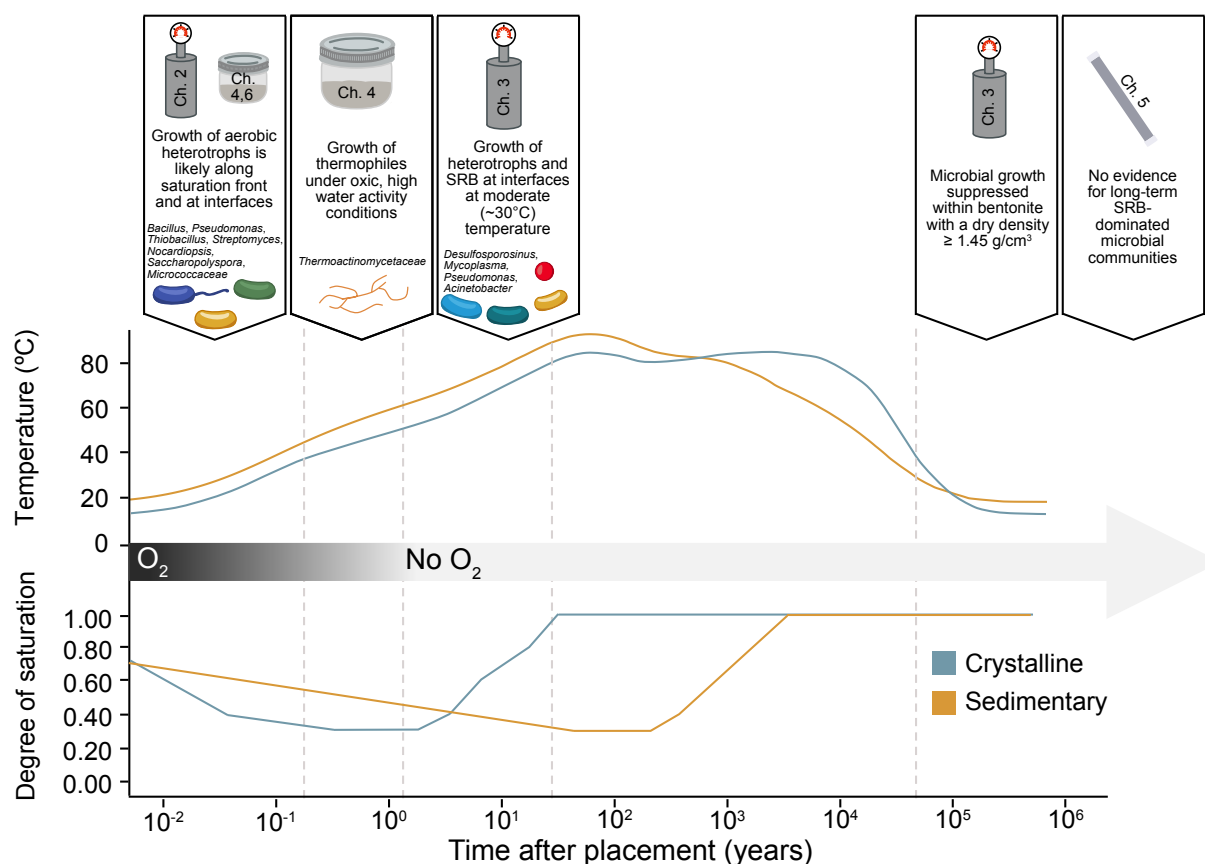


**Figure 7.1** Predicted microbiology of a future DGR throughout time and its relation to research in this thesis.

## 7.5 Future outlook

Although our understanding of microbial growth in bentonite in the context of long-term used nuclear fuel storage is increasing, there remain some outstanding questions. For example, many of the microorganisms that are detected in bentonite via DNA sequencing are from taxa with a high degree of metabolic versatility. With 16S rRNA gene sequencing alone, it is only possible to make broad predictions about the types of metabolisms that the microorganisms detected may be capable of conducting. To achieve a more detailed understanding of the specific microbial metabolisms that occur in bentonite under different conditions, metagenomics and metatranscriptomics should ideally be incorporated, although both are challenging for bentonite, from which DNA and RNA are rarely extracted in sufficient quantities for these analyses.

Another limitation of current DNA-based analysis is the inability to distinguish between relic DNA and DNA belonging to living cells. In the case of natural analogue samples, for example, it is unknown if any of the DNA detected represents currently living organisms. This

can be mitigated to a degree by incorporating a cultivation-based approach and sequencing biomass from the cultures, but not all viable microorganisms are culturable. As such, when clay and culture profiles have a high degree of overlap, it implies that the DNA detected in clay represents viable microorganisms, but when they overlap poorly, the opposite conclusion is not appropriate (i.e., the DNA in clay may represent viable microorganisms that are not culturable under the specific conditions applied). In other experiments, where a shift in microbial community composition is observed after incubation under a specific set of condition (e.g., hydration coupled to increased temperature or dry density), it is likely that the DNA detected represents microorganisms that grew throughout the course of the experiment. That said, it is not known how long this DNA persists after a cell may die. For example, growth of certain microorganisms was observed in the initial low-saturation, low-swelling pressure phase of pressure vessel experiments. Sequences associated with these same microorganisms were also detected in pressure vessels incubated beyond the point of saturation, but it is not known if those cells were still intact and viable at that point or if the high swelling pressure had killed them. Although again challenging due to the low biomass, swelling, and adsorptive nature of bentonite, methods such as propidium monoazide (PMA) pre-treatment prior to DNA extraction, which has been developed for several other environments [221–223], should be developed for bentonite to allow for the study of only the viable portion of the microbial community.

Dry density is an important parameter in DGR design given that it is what is expected to suppress microbial growth. Other conditions that could also influence microbial growth (e.g., temperature, saturation, water activity, groundwater composition, and presence of geogenic gases like hydrogen that could act as electron sources) that are either inherent to the host rock or that are expected to change over the lifetime of a DGR are relatively underexplored, as are combinations of conditions that will co-occur in a DGR (e.g., high dry density coupled to incomplete saturation and high temperature). The results presented throughout show that the microorganisms that grow in bentonite are not always the same and depend on the specific conditions of the bentonite. Although most microorganisms observed to grow in bentonite are putative heterotrophs, further experiments are needed to fully understand the conditions that promote the growth of SRB in bentonite. To accurately model the microbiology of a DGR environment, it is important to know which microorganisms are likely to persist at different times in a DGR. Especially when the physical conditions applied promote the growth of

microorganisms that do not grow under commonly used cultivation conditions (e.g., high temperature), incorporating a multifaceted experimental design (e.g., not relying on cultivation alone, but instead incorporating DNA-based analyses as well) is critical to avoid making incorrect conclusions.

## **7.6 Research significance**

Overall, the research presented throughout this thesis corroborates previous dry density thresholds for suppression of microbial growth in Wyoming MX-80 bentonite under oxic conditions and further presents minimum dry density thresholds for gapfill material under oxic and anoxic conditions, which had not previously been reported. It further explored a range of lower dry densities that represent more realistic targets to achieve in a DGR, through which a lower minimum dry density threshold of 1.40 g/cm<sup>3</sup> (oxic) to 1.45 g/cm<sup>3</sup> (anoxic) was demonstrated to be sufficient to suppress heterotrophic growth, though SRB persisted in anoxic experiments (potentially as inactive spores) at higher dry densities. By using an experimental design involving initially un-hydrated (i.e., as-received) bentonite and early sampling time points (i.e., one month), microbial growth during the saturation phase, which could represent thousands of years in a DGR, was studied. Through this, it was shown that heterotrophs may initially increase in abundance in compacted bentonite prior to complete saturation, and a follow-up experiment demonstrated that this increase in abundance is likely attributed to true growth rather than a recovery from the VBNC state, as previous literature has suggested [125]. This research also adds to existing literature on natural analogue samples, and the effect of elevated temperature on microbial growth and survival in bentonite. Through the temperature experiments, it was shown that as-received bentonite contains thermophilic microorganisms that can proliferate in uncompacted hydrated bentonite incubated at a high temperature (i.e., 60°C). The natural analogue study suggests that bentonite within a DGR, once conditions stabilize, could be associated with low abundance microbial communities without detectable SRB representation. Together these results can be incorporated into models to better understand the impact that microorganisms may have in a DGR over the very long timeframes it needs to remain active, which will ultimately be used to inform DGR design.

## References

1. NWMO. Canada's used nuclear fuel. 2024. Toronto.
2. Hall DS, Keech PG. An overview of the Canadian corrosion program for the long-term management of nuclear waste. *Corros Eng Sci Technol* 2017; **52**: 2–5.
3. Abu-Jdayil B. Rheology of sodium and calcium bentonite-water dispersions: effect of electrolytes and aging time. *Int J Miner Process* 2011; **98**: 208–213.
4. Lagaly G, Ziesmer S. Colloid chemistry of clay minerals: the coagulation of montmorillonite dispersions. *Adv Colloid Interface Sci* 2003; **100–102**: 105–128.
5. Zango ZU, Garba A, Garba ZN, Zango MU, Usman F, Lim J-W. Montmorillonite for adsorption and catalytic elimination of pollutants from wastewater: a state-of-the-arts review. *Sustainability* 2022; **14**: 16441.
6. Ballarini E, Graupner B, Bauer S. Thermal–hydraulic–mechanical behavior of bentonite and sand-bentonite materials as seal for a nuclear waste repository: numerical simulation of column experiments. *Appl Clay Sci* 2017; **135**: 289–299.
7. Bengtsson A, Pedersen K. Microbial sulphide-producing activity in water saturated Wyoming MX-80, Asha and Calcigel bentonites at wet densities from 1500 to 2000kgm<sup>-3</sup>. *Appl Clay Sci* 2017; **137**: 203–212.
8. King F. Microbiologically influenced corrosion of nuclear waste containers. *Corrosion* 2009; **65**: 233–251.
9. Masurat P, Eriksson S, Pedersen K. Evidence of indigenous sulphate-reducing bacteria in commercial Wyoming bentonite MX-80. *Appl Clay Sci* 2010; **47**: 51–57.
10. Vachon MA, Engel K, Beaver RC, Slater GF, Binns WJ, Neufeld JD. Fifteen shades of clay: distinct microbial community profiles obtained from bentonite samples by cultivation and direct nucleic acid extraction. *Sci Rep* 2021; **11**: 22349.
11. Bagnoud A, de Bruijn I, Andersson AF, Diomidis N, Leupin OX, Schwyn B, et al. A minimalistic microbial food web in an excavated deep subsurface clay rock. *FEMS Microbiol Ecol* 2016; **92**: fiv138.
12. Gu T, Jia R, Unsal T, Xu D. Toward a better understanding of microbiologically influenced corrosion caused by sulfate-reducing bacteria. *J Mater Sci Technol* 2019; **35**: 631–636.
13. Boylan AA, Perez-Mon C, Guillard L, Burzan N, Loreggian L, Maisch M, et al. H<sub>2</sub>-fuelled microbial metabolism in Opalinus Clay. *Appl Clay Sci* 2019; **174**: 69–74.
14. Bell E, Lamminmäki T, Alneberg J, Andersson AF, Qian C, Xiong W, et al. Biogeochemical cycling by a low-diversity microbial community in deep groundwater. *Front Microbiol* 2018; **9**: 2129.
15. Bell E, Lamminmäki T, Alneberg J, Andersson AF, Qian C, Xiong W, et al. Active sulfur cycling in the terrestrial deep subsurface. *ISME J* 2020; **325**: 184–187.
16. Wu X, Holmfeldt K, Hubalek V, Lundin D, Åström M, Bertilsson S, et al. Microbial metagenomes from three aquifers in the Fennoscandian shield terrestrial deep biosphere reveal metabolic partitioning among populations. *ISME J* 2016; **10**: 1192–1203.
17. Hernsdorf AW, Amano Y, Miyakawa K, Ise K, Suzuki Y, Anantharaman K, et al. Potential for microbial H<sub>2</sub> and metal transformations associated with novel bacteria and archaea in deep terrestrial subsurface sediments. *ISME J* 2017; **11**: 1915–1929.

18. Purkamo L, Bomberg M, Nyssönen M, Kukkonen I, Ahonen L, Itävaara M. Heterotrophic communities supplied by ancient organic carbon predominate in deep Fennoscandian bedrock fluids. *Microb Ecol* 2014; **69**: 319–332.
19. Purkamo L, Bomberg M, Kietäväinen R, Salavirta H, Nyssönen M, Nuppenen-Puputti M, et al. Microbial co-occurrence patterns in deep Precambrian bedrock fracture fluids. *Biogeosciences* 2016; **13**: 3091–3108.
20. King F, Hall DS, Keech PG. Nature of the near-field environment in a deep geological repository and the implications for the corrosion behaviour of the container. *Corros Eng Sci* 2017; **52**: 25–30.
21. Flemming H-C, Wurtz S. Bacteria and archaea on Earth and their abundance in biofilms. *Nat Rev Microbiol* 2019; **17**: 247–260.
22. Bar-On YM, Phillips R, Milo R. The biomass distribution on Earth. *Proc Natl Acad Sci USA* 2018; **115**: 6506–6511.
23. Whitman WB, Coleman DC, Wiebe WJ. Prokaryotes: the unseen majority. *Proc Natl Acad Sci USA* 1998; **95**: 6578–6583.
24. Jones S. What lives beneath? *Nat Rev Microbiol* 2005; **3**: 668.
25. Hellerschmied C, Schritter J, Waldmann N, Zaduryan AB, Rachbauer L, Scherr KE, et al. Hydrogen storage and geo-methanation in a depleted underground hydrocarbon reservoir. *Nat Energy* 2024; **9**: 333–344.
26. Boston PJ, Ivanov M V, McKay CP. On the possibility of chemosynthetic ecosystems in subsurface habitats on Mars. *Icarus* 1992; **95**: 300–308.
27. Lollar BS, Lacrampe-Couloume G, Slater GF, Ward J, Moser DP, Gihring TM, et al. Unravelling abiogenic and biogenic sources of methane in the Earth's deep subsurface. *Chem Geol* 2006; **226**: 328–339.
28. Onstott TC. Deep life: The hunt for the hidden biology of Earth, Mars, and beyond. 2017. Princeton University Press.
29. Michalski JR, Onstott TC, Mojzsis SJ, Mustard J, Chan QHS, Niles PB, et al. The Martian subsurface as a potential window into the origin of life. *Nat Geosci* 2018; **11**: 21–26.
30. Lollar GS, Warr O, Telling J, Osburn MR, Lollar BS. 'Follow the water': Hydrogeochemical constraints on microbial investigations 2.4 km below surface at the Kidd Creek deep fluid and deep life observatory. *Geomicrobiol J* 2019; **36**: 859–872.
31. Lollar BS, Heuer VB, McDermott J, Tille S, Warr O, Moran JJ, et al. A window into the abiotic carbon cycle—Acetate and formate in fracture waters in 2.7 billion year-old host rocks of the Canadian Shield. *Geochim Cosmochim Acta* 2021; **294**: 295–314.
32. Purkamo L. Life in the deep subsurface and its implications for astrobiology. *Oxford Research Encyclopedia of Planetary Science*. 2023.
33. McMahon S, Parnell J. Weighing the deep continental biosphere. *FEMS Microbiol Ecol* 2014; **87**: 113–120.
34. Escudero C, Oggerin M, Amils R. The deep continental subsurface: the dark biosphere. *Int Microbiol* 2018; **21**: 3–14.
35. Phelps TJ, Murphy EM, Pfiffner SM, White DC. Comparison between geochemical and biological estimates of subsurface microbial activities. *Microb Ecol* 1994; **28**: 335–349.
36. Chivian D, Brodie EL, Alm EJ, Culley DE, Dehal PS, DeSantis TZ, et al. Environmental genomics reveals a single-species ecosystem deep within Earth. *J Sci* 2008; **322**: 275–278.
37. Hoehler TM, Jørgensen BB. Microbial life under extreme energy limitation. *Nat Rev Microbiol* 2013; **11**: 83–94.

38. Biddle JF, Lipp JS, Lever MA, Lloyd KG, Sørensen KB, Anderson R, et al. Heterotrophic archaea dominate sedimentary subsurface ecosystems off Peru. *Proc Natl Acad Sci USA* 2006; **103**: 3846–3851.
39. Lomstein BA, Langerhuus AT, D'Hondt S, Jørgensen BB, Spivack AJ. Endospore abundance, microbial growth and necromass turnover in deep sub-seafloor sediment. *Nature* 2012; **484**: 101–104.
40. Whitman WB, Coleman DC, Wiebe WJ. Prokaryotes: the unseen majority. *Proc Natl Acad Sci USA* 1998; **95**: 6578–6583.
41. Anderson RE. Tracking microbial evolution in the subseafloor biosphere. *mSystems* 2021; **6**: e00731-21.
42. Government of Canada. Water sources: groundwater. *Government of Canada*.
43. Xia J, Zhang S, Zhao X, Liu J, Chen Y. Effects of different groundwater depths on the distribution characteristics of soil-*Tamarix* water contents and salinity under saline mineralization conditions. *Catena (Amst)* 2016; **142**: 166–176.
44. Dong Y, Kumar CG, Chia N, Kim P, Miller PA, Price ND, et al. *Halomonas sulfidaeris*-dominated microbial community inhabits a 1.8 km-deep subsurface Cambrian sandstone reservoir. *Environ Microbiol* 2014; **16**: 1695–1708.
45. Nyyssönen M, Hultman J, Ahonen L, Kukkonen I, Paulin L, Laine P, et al. Taxonomically and functionally diverse microbial communities in deep crystalline rocks of the Fennoscandian Shield. *ISME J* 2014; **8**: 126–138.
46. Stevens TO, McKinley JP. Lithoautotrophic microbial ecosystems in deep basalt aquifers. *J Sci* 1995; **270**: 450–455.
47. Parnell J, McMahon S. Physical and chemical controls on habitats for life in the deep subsurface beneath continents and ice. *Proc Natl Acad Sci USA* 2016; **374**: 20140293.
48. Ruff SE, Humez P, de Angelis IH, Diao M, Nightingale M, Cho S, et al. Hydrogen and dark oxygen drive microbial productivity in diverse groundwater ecosystems. *Nat Commun* 2023; **14**: 3194.
49. Ivarsson M, Bengtson S, Drake H, Francis W. Fungi in deep subsurface environments. *Adv Appl Microbiol* 2018; **102**: 83–116.
50. Fredrickson JK, McKinley JP, Bjornstad BN, Long PE, Ringelberg DB, White DC, et al. Pore-size constraints on the activity and survival of subsurface bacteria in a late cretaceous shale-sandstone sequence, northwestern New Mexico. *Geomicrobiol J* 1997; **14**: 183–202.
51. Fredrickson JK, Balkwill DL. Geomicrobial processes and biodiversity in the deep terrestrial subsurface. *Geomicrobiol J* 2006; **23**: 345–356.
52. Fredrickson JK, McKinley JP, Nierzwicki-Bauer SA, White DC, Ringelberg DB, Rawson SA, et al. Microbial community structure and biogeochemistry of Miocene subsurface sediments: implications for long-term microbial survival. *Mol Ecol* 1995; **4**: 619–626.
53. Chapelle FH, Lovley DR. Rates of microbial metabolism in deep coastal plain aquifers. *Appl Environ Microbiol* 1990; **56**: 1865–1874.
54. Itävaara M, Salavirta H, Marjamaa K, Ruskeeniemi T. Geomicrobiology and metagenomics of terrestrial deep subsurface microbiomes. *Adv Appl Microbiol* 2016; **94**: 1–77.
55. Krumholz LR. Microbial communities in the deep subsurface. *Hydrogeol J* 2000; **8**: 4–10.

56. Chapelle FH, O'Neill K, Bradley PM, Methé BA, Ciufo SA, Knobel LL, et al. A hydrogen-based subsurface microbial community dominated by methanogens. *Nature* 2002; **415**: 312–315.
57. Berg IA. Ecological aspects of the distribution of different autotrophic CO<sub>2</sub> fixation pathways. *Appl Environ Microbiol* 2011; **77**: 1925–1936.
58. Momper L, Jungbluth SP, Lee MD, Amend JP. Energy and carbon metabolisms in a deep terrestrial subsurface fluid microbial community. *ISME J* 2017; **11**: 2319–2333.
59. Magnabosco C, Ryan K, Lau MCY, Kuloyo O, Sherwood Lollar B, Kieft TL, et al. A metagenomic window into carbon metabolism at 3 km depth in Precambrian continental crust. *ISME J* 2016; **10**: 730–741.
60. Bell E, Lamminmäki T, Alneberg J, Andersson AF, Qian C, Xiong W, et al. Active sulfur cycling in the terrestrial deep subsurface. *ISME J* 2020; **14**: 1260–1272.
61. Wang L, Jin Z, Chen X, Su Y, Huang X. The origin and occurrence of natural hydrogen. *Energies (Basel)* 2023; **16**: 2400.
62. Li S, Mosier D, Kouris A, Humez P, Mayer B, Strous M, et al. High diversity, abundance, and expression of hydrogenases in groundwater. *ISME commun* 2024; **4**: ycae023.
63. Hermsdorf AW, Amano Y, Miyakawa K, Ise K, Suzuki Y, Anantharaman K, et al. Potential for microbial H<sub>2</sub> and metal transformations associated with novel bacteria and archaea in deep terrestrial subsurface sediments. *ISME J* 2017; **11**: 1915–1929.
64. Haveman SA, Pedersen K, Ruotsalainen P. Distribution and metabolic diversity of microorganisms in deep igneous rock aquifers of Finland. *Geomicrobiol J* 1999; **16**: 277–294.
65. Fu B, Jin X, Conrad R, Liu H, Liu H. Competition between chemolithotrophic acetogenesis and hydrogenotrophic methanogenesis for exogenous H<sub>2</sub>/CO<sub>2</sub> in anaerobically digested sludge: impact of temperature. *Front Microbiol* 2019; **10**: 2418.
66. Ye R, Jin Q, Bohannan B, Keller JK, Bridgham SD. Homoacetogenesis: A potentially underappreciated carbon pathway in peatlands. *Soil Biol Biochem* 2014; **68**: 385–391.
67. Bell E, Lamminmäki T, Alneberg J, Andersson AF, Qian C, Xiong W, et al. Active sulfur cycling in the terrestrial deep subsurface. *ISME J* 2020; **325**: 184–187.
68. Lin LH, Hall J, Lippmann-Pipke J, Ward JA, Lollar BS, DeFlaun M, et al. Radiolytic H<sub>2</sub> in continental crust: nuclear power for deep subsurface microbial communities. *Geochem Geophys* 2005; **6**: 1525–2027.
69. Haveman SA, Pedersen K. Distribution of culturable microorganisms in Fennoscandian Shield groundwater. *FEMS Microbiol Ecol* 2002; **39**: 129–137.
70. Wu X, Pedersen K, Edlund J, Eriksson L, Åström M, Andersson AF, et al. Potential for hydrogen-oxidizing chemolithoautotrophic and diazotrophic populations to initiate biofilm formation in oligotrophic, deep terrestrial subsurface waters. *Microbiome* 2017; **5**: 37.
71. Boylan AA, Perez-Mon C, Guillard L, Burzan N, Loreggian L, Maisch M, et al. H<sub>2</sub>-fuelled microbial metabolism in Opalinus clay. *Appl Clay Sci* 2019; **174**: 69–76.
72. Bagnoud A, Chourey K, Hettich RL, de Bruijn I, Andersson AF, Leupin OX, et al. Reconstructing a hydrogen-driven microbial metabolic network in Opalinus Clay rock. *Nat Commun* 2016; **7**: 12770.
73. Vinsot A, Appelo CAJ, Lundy M, Wechner S, Lettry Y, Lerouge C, et al. In situ diffusion test of hydrogen gas in the Opalinus Clay. *Geol Soc Spec Publ* 2014; **400**: 563–578.
74. Pedersen K. Microbial life in deep granitic rock. *FEMS Microbiol Rev* 1997; **20**: 399–414.

75. Pedersen K. Metabolic activity of subterranean microbial communities in deep granitic groundwater supplemented with methane and H<sub>2</sub>. *ISME J* 2013; **7**: 839–849.
76. Ettwig KF, Zhu B, Speth D, Keltjens JT, Jetten MSM, Kartal B. Archaea catalyze iron-dependent anaerobic oxidation of methane. *Proc Natl Acad Sci USA* 2016; **113**: 12792–12796.
77. Chang YH, Cheng TW, Lai WJ, Tsai WY, Sun CH, Lin LH, et al. Microbial methane cycling in a terrestrial mud volcano in eastern Taiwan. *Environ Microbiol* 2012; **14**: 895–908.
78. Beal EJ, House CH, Orphan VJ. Manganese- and iron-dependent marine methane oxidation. *Science (1979)* 2009; **325**: 184–187.
79. Ettwig KF, Zhu B, Speth D, Keltjens JT, Jetten MSM, Kartal B. Archaea catalyze iron-dependent anaerobic oxidation of methane. *Proc Natl Acad Sci USA* 2016; **113**: 12792–12796.
80. Colwell FS, Onstott TC, Delwiche ME, Chandler D, Fredrickson JK, Yao Q-J, et al. Microorganisms from deep, high temperature sandstones: constraints on microbial colonization. *FEMS Microbiol Rev* 1997; **20**: 425–435.
81. Teske AP. The deep subsurface biosphere is alive and well. *Trends Microbiol* 2005; **13**: 402–404.
82. Parkes RJ, Webster G, Cragg BA, Weightman AJ, Newberry CJ, Ferdelman TG, et al. Deep sub-seafloor prokaryotes stimulated at interfaces over geological time. *Nature* 2005; **436**: 390–394.
83. Harrison BK, Zhang H, Berelson W, Orphan VJ. Variations in archaeal and bacterial diversity associated with the sulfate-methane transition zone in continental margin sediments (Santa Barbara Basin, California). *Appl Environ Microbiol* 2009; **75**: 1487–1499.
84. Onstott TC, Phelps TJ, Colwell FS, Ringelberg D, White DC, Boone DR, et al. Observations pertaining to the origin and ecology of microorganisms recovered from the deep subsurface of Taylorsville Basin, Virginia. *Geomicrobiol J* 1998; **15**: 353–385.
85. Orphan VJ, Taylor LT, Hafenbradl D, Delong E. Culture-dependent and culture-independent characterization of microbial assemblages associated with high-temperature petroleum reservoirs. *Appl Environ Microbiol* 2000; **66**: 700–711.
86. Soares A, Edwards A, An D, Bagnoud A, Bradley J, Barnhart E, et al. A global perspective on bacterial diversity in the terrestrial deep subsurface. *Microbiol* 2023; **169**: 001172.
87. Jungbluth SP, Amend JP, Rappé MS. Metagenome sequencing and 98 microbial genomes from Juan de Fuca Ridge flank subsurface fluids. *Sci Data* 2017; **4**: 170037.
88. Jungbluth SP, del Rio TG, Tringe SG, Stepanauskas R, Rappé MS. Genomic comparisons of a bacterial lineage that inhabits both marine and terrestrial deep subsurface systems. *PeerJ* 2017; **5**: e3134.
89. Kadnikov V, Mardanov A, Beletsky A, Karnachuk O, Ravin N. Microbial life in the deep subsurface aquifer illuminated by metagenomics. *Front Microbiol* 2020; **11**: 572252.
90. Kadnikov V v, Mardanov A v, Beletsky A v, Banks D, Pimenov N v, Frank YA, et al. A metagenomic window into the 2-km-deep terrestrial subsurface aquifer revealed multiple pathways of organic matter decomposition. *FEMS Microbiol Ecol* 2018; **94**: fiy152.
91. Lin L-H, Wang P-L, Rumble D, Lippmann-Pipke J, Boice E, Pratt LM, et al. Long-term sustainability of a high-energy, low-diversity crustal biome. *J Sci* 2006; **314**: 479–482.

92. Becraft ED, Lau Vetter MCY, Bezuidt OKI, Brown JM, Labonté JM, Kauneckaitė-Griguole K, et al. Evolutionary stasis of a deep subsurface microbial lineage. *ISME J* 2021; **15**: 2830–2842.
93. Moser DP, Gihring TM, Brockman FJ, Fredrickson JK, Balkwill DL, Dollhopf ME, et al. *Desulfotomaculum* and *Methanobacterium* spp. dominate a 4-to 5-kilometer-deep fault. *Appl Environ Microbiol* 2005; **71**: 8773–8783.
94. Moser DP, Onstott TC, Fredrickson JK, Brockman FJ, Balkwill DL, Drake GR, et al. Temporal shifts in the geochemistry and microbial community structure of an ultradeep mine borehole following isolation. *Geomicrobiol J* 2003; **20**: 517–548.
95. Davidson MM, Silver BJ, Onstott TC, Moser DP, Gihring TM, Pratt LM, et al. Capture of planktonic microbial diversity in fractures by long-term monitoring of flowing boreholes, Evander Basin, South Africa. *Geomicrobiol J* 2011; **28**: 275–300.
96. Gihring TM, Moser DP, Lin L-H, Davidson M, Onstott TC, Morgan L, et al. The distribution of microbial taxa in the subsurface water of the Kalahari Shield, South Africa. *Geomicrobiol J* 2006; **23**: 415–430.
97. Lin L-H, Hall J, Onstott TC, Gihring T, Lollar BS, Boice E, et al. Planktonic microbial communities associated with fracture-derived groundwater in a deep gold mine of South Africa. *Geomicrobiol J* 2006; **23**: 475–497.
98. Itävaara M, Nyssönen M, Kapanen A, Nousiainen A, Ahonen L, Kukkonen I. Characterization of bacterial diversity to a depth of 1500 m in the Outokumpu deep borehole, Fennoscandian Shield. *FEMS Microbiol Ecol* 2011; **77**: 295–309.
99. Brazelton WJ, Morrill PL, Szponar N, Schrenk MO. Bacterial communities associated with subsurface geochemical processes in continental serpentinite springs. *Appl Environ Microbiol* 2013; **79**: 3906–3916.
100. Daly RA, Borton MA, Wilkins MJ, Hoyt DW, Kountz DJ, Wolfe RA, et al. Microbial metabolisms in a 2.5-km-deep ecosystem created by hydraulic fracturing in shales. *Nat Microbiol* 2016; **1**: 16146.
101. Wu X, Holmfeldt K, Hubalek V, Lundin D, Åström M, Bertilsson S, et al. Microbial metagenomes from three aquifers in the Fennoscandian Shield terrestrial deep biosphere reveal metabolic partitioning among populations. *ISME J* 2016; **10**: 1192–1203.
102. Mielke RE, Pace DL, Porter T, Southam G. A critical stage in the formation of acid mine drainage: Colonization of pyrite by *Acidithiobacillus ferrooxidans* under pH-neutral conditions. *Geobiology* 2003; **1**: 81–90.
103. Li L, Wing BA, Bui TH, McDermott JM, Slater GF, Wei S, et al. Sulfur mass-independent fractionation in subsurface fracture waters indicates a long-standing sulfur cycle in Precambrian rocks. *Nat Commun* 2016; **7**: 13252.
104. Kieft TL, Fredrickson JK, Onstott TC, Gorby YA, Kostandarites HM, Bailey TJ, et al. Dissimilatory reduction of Fe(III) and other electron acceptors by a *Thermus* isolate. *Appl Environ Microbiol* 1999; **65**: 1214–1221.
105. Uroz S, Calvaruso C, Turpault M-P, Frey-Klett P. Mineral weathering by bacteria: ecology, actors and mechanisms. *Trends Microbiol* 2009; **17**: 378–387.
106. Gadd GM. Geomycology: biogeochemical transformations of rocks, minerals, metals and radionuclides by fungi, bioweathering and bioremediation. *Mycol Res* 2007; **111**: 3–49.
107. Osburn MR, LaRowe DE, Momper LM, Amend JP. Chemolithotrophy in the continental deep subsurface: Sanford Underground Research Facility (SURF), USA. *Front Microbiol* 2014; **5**: 610.

108. Soares A, Edwards A, An D, Bagnoud A, Bradley J, Barnhart E, et al. A global perspective on bacterial diversity in the terrestrial deep subsurface. *Microbiol* 2023; **169**: 001172.
109. Casar CP, Kruger BR, Flynn TM, Masterson AL, Momper LM, Osburn MR. Mineral-hosted biofilm communities in the continental deep subsurface, Deep Mine Microbial Observatory, SD, USA. *Geobiology* 2020; **18**: 508–522.
110. Gleeson DB, Kennedy NM, Clipson N, Melville K, Gadd GM, McDermott FP. Characterization of bacterial community structure on a weathered pegmatitic granite. *Microb Ecol* 2006; **51**: 526–534.
111. Rempfert KR, Miller HM, Bompard N, Nothaft D, Matter JM, Kelemen P, et al. Geological and geochemical controls on subsurface microbial life in the Samail Ophiolite, Oman. *Front Microbiol* 2017; **8**: 56.
112. Fomina M, Skorochod I. Microbial interaction with clay minerals and its environmental and biotechnological implications. *Minerals* 2020; **10**: 861.
113. Zhang L, Gadd GM, Li Z. Microbial biomodification of clay minerals. *Adv Appl Microbiol* 2021; **114**: 111–139.
114. Zhang Y, Horne RN, Hawkins AJ, Primo JC, Gorbatenko O, Dekas AE. Geological activity shapes the microbiome in deep-subsurface aquifers by advection. *Proc Natl Acad Sci USA* 2022; **119**: e2113985119.
115. Leupin OX, Bernier-Latmani R, Bagnoud A, Moors H, Leys N, Wouters K, et al. Fifteen years of microbiological investigation in Opalinus Clay at the Mont Terri rock laboratory (Switzerland). *Swiss J Geosci* 2017; **5**: 345–356.
116. Wersin P, Spahiu K, Bruno J. Time evolution of dissolved oxygen and redox conditions in a HLW repository. 1994. Swedish Nuclear Fuel and Waste Management Co, Stockholm.
117. Müller HR, Garitte B, Vogt T, Köhler S, Sakaki T, Weber H, et al. Implementation of the full-scale emplacement (FE) experiment at the Mont Terri rock laboratory. *Swiss J Geosci* 2017; **110**: 287–306.
118. Guo R. Thermal response of a conceptual deep geological repository in sedimentary rock. 2018. Nuclear Waste Management Organization, Toronto, ON.
119. Guo R. Thermal response of a mark II conceptual deep geological repository in crystalline rock. 2016. Nuclear Waste Management Organization, Toronto, ON.
120. Noronha J. Deep geological repository conceptual design report crystalline/sedimentary rock environment. 2016. Nuclear Waste Management Organization, Toronto, ON.
121. Schröder C, Burkhardt C, Antranikian G. What we learn from extremophiles. *ChemTexts* 2020; **6**: 1–6.
122. King F. Microbially influenced corrosion of copper nuclear fuel waste containers in a Canadian disposal vault. 1996. Atomic Energy of Canada Ltd, Chalk River, ON.
123. Stroes-Gascoyne S, Hamon CJ, Maak P, Russell S. The effects of the physical properties of highly compacted smectitic clay (bentonite) on the culturability of indigenous microorganisms. *Appl Clay Sci* 2010; **47**: 155–162.
124. Jalique DR, Stroes-Gascoyne S, Hamon CJ, Priyanto DG, Kohle C, Evenden WG, et al. Culturability and diversity of microorganisms recovered from an eight-year old highly-compacted, saturated MX-80 Wyoming bentonite plug. *Appl Clay Sci* 2016; **126**: 245–250.

125. Stroes-Gascoyne S, Hamon CJ, Maak P. Limits to the use of highly compacted bentonite as a deterrent for microbiologically influenced corrosion in a nuclear fuel waste repository. *Phys Chem Earth* 2011; **36**: 1630–1638.
126. Burzan N, Murad Lima R, Frutschi M, Janowczyk A, Reddy B, Rance A, et al. Growth and persistence of an aerobic microbial Community in Wyoming Bentonite MX-80 despite anoxic in situ conditions. *Front Microbiol* 2022; **13**: 858324.
127. Engel K, Ford SE, Binns WJ, Diomidis N, Slater GF, Neufeld JD. Stable microbial community in compacted bentonite after 5 years of exposure to natural granitic groundwater. *mSphere* 2023; **8**: e00048-23.
128. Martinez-Moreno MF, Povedano-Priego C, Morales-Hidalgo M, Mumford AD, Ojeda JJ, Jroundi F, et al. Impact of compacted bentonite microbial community on the clay mineralogy and copper canister corrosion: a multidisciplinary approach in view of a safe Deep Geological Repository of nuclear wastes. *J Hazard Mater* 2023; **458**: 131940.
129. Martinez-Moreno MF, Povedano-Priego C, Mumford AD, Morales-Hidalgo M, Mijnenonckx K, Jroundi F, et al. Microbial responses to elevated temperature: Evaluating bentonite mineralogy and copper canister corrosion within the long-term stability of deep geological repositories of nuclear waste. *Sci Total Environ* 2024; **915**: 170149.
130. Matschiavelli N, Kluge S, Podlech C, Standhaft D, Grathoff G, Ikeda-Ohno A, et al. The year-long development of microorganisms in uncompacted Bavarian bentonite slurries at 30 and 60 C. *Environ Sci Technol* 2019; **53**: 10514–10524.
131. Fukunaga S, Jintoku T, Iwata Y, Nakayama M, Tsuji T, Sakaya N, et al. Investigation of microorganisms in bentonite deposits. *Geomicrobiol J* 2005; **22**: 361–370.
132. Takagi T. Bentonite in Japan - geology and industries. 2005. National Institute of Advanced Industrial Science and Technology, Tsukuba.
133. Beaver RC, Engel K, Binns WJ, Neufeld JD. Microbiology of barrier component analogues of a deep geological repository. *Can J Microbiol* 2021; **68**: 73–90.
134. Villar M V, Fernández-Soler JM, Delgado Huertas A, Reyes E, Linares J, Jiménez de Cisneros C, et al. The study of Spanish clays for their use as sealing materials in nuclear waste repositories: 20 years of progress. 2006.
135. Lopez-Fernandez M, Cherkouk A, Vilchez-Vargas R, Jauregui R, Pieper D, Boon N, et al. Bacterial diversity in bentonites, engineered barrier for deep geological disposal of radioactive wastes. *Microb Ecol* 2015; **70**: 922–935.
136. Stroes-Gascoyne S, FAJ, VP. In: Cramer, j., Smelle, J.A.T. (Eds.), Final Report of the AECL/SKB Cigar Lake Analog Study. Atomic Energy of Canada Limited Report AECL-10951. 1994.
137. Francis AJ, J-TG, GJB, DCJ. Enumeration and characterization of microorganisms associated with the uranium ore deposit at Cigar Lake, Canada. 1994. New York.
138. Weber WJ, Navrotsky A, Stefanovsky S, Vance ER, Vernaz E. Materials science of high-level nuclear waste immobilization. *MRS Bull* 2009; **34**: 46–53.
139. Noronha J. Deep geological repository conceptual design report. 2016. Nuclear Waste Management Organization, Toronto, ON.
140. Gaines RH. Bacterial activity as a corrosive influence in the soil. *Ind Eng Chem* 1910; **2**: 128–130.
141. Von Wolzogen Kühr CAH, van der Vlugt LS. Graphitization of cast iron as an electro-biochemical process in anaerobic soils. *Water (Basel)* 1934; **18**: 147–165.

142. Sarioğlu F, Javaherdashti R, Aksöz N. Corrosion of a drilling pipe steel in an environment containing sulphate-reducing bacteria. *Int J Press Vessel* 2002; **73**: 127–131.
143. Al-Jaroudi SS, Ul-Hamid A, Al-Gahtani MM. Failure of crude oil pipeline due to microbiologically induced corrosion. *Corros Eng Sci* 2010; **46**: 568–579.
144. Yang Y, Khan F, Thodi P, Abbassi R. Corrosion induced failure analysis of subsea pipelines. *Reliab Eng Syst Saf* 2017; **159**: 214–222.
145. Herisson J, Guéguen-Minerbe M, van Hullebusch ED, Chaussadent T. Influence of the binder on the behaviour of mortars exposed to H<sub>2</sub>S in sewer networks: a long-term durability study. *Mater Struct* 2017; **50**: 8.
146. Bagnoud A, Leupin O, Schwyn B, Bernier-Latmani R. Rates of microbial hydrogen oxidation and sulfate reduction in Opalinus Clay rock. *Appl Geochem* 2016; **72**: 42–50.
147. Behazin M, Pedersen K, Li L, Abrahamsen-Mills L, Boylan A, Bryan N. State of science review of sulfide production in deep geological repositories for used nuclear fuel. 2021.
148. Kieft TL, Kovacik WP, Ringelberg DB, White DC, Haldeman DL, Amy PS, et al. Factors limiting microbial growth and activity at a proposed high-level nuclear repository, Yucca Mountain, Nevada. *Appl Environ Microbiol* 1997; **63**: 3128–3133.
149. Pedersen K, Motamedi M, Karnland O, Sandén T. Mixing and sulphate-reducing activity of bacteria in swelling, compacted bentonite clay under high-level radioactive waste repository conditions. *J Appl Microbiol* 2000; **89**: 1038–1047.
150. Motamedi M, Karland O, Pedersen K. Survival of sulfate-reducing bacteria at different water activities in compacted bentonite. *FEMS Microbiol Lett* 1996; **141**: 83–87.
151. Engel K, Ford SE, Coyotzi S, McKelvie J, Diomidis N, Slater G, et al. Stability of microbial community profiles associated with compacted bentonite from the Grimsel underground research laboratory. *mSphere* 2019; **4**: e00601-19.
152. Rietveld HM. The rietveld method. *Phys Scr* 2014; **89**: 098002.
153. U.S. Food & Drug Administration. BAM Appendix 2: Most Probable Number for Serial Dilutions.
154. Engel K, Coyotzi S, Vachon MA, McKelvie JR, Neufeld JD. Validating DNA extraction protocols for bentonite clay. *mSphere* 2019; **4**: e00334-19.
155. Muyzer G, De Waal EC, Uitterlinden AG. Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. *Appl Environ Microbiol* 1993; **59**: 695–700.
156. Parada AE, Needham DM, Fuhrman JA. Every base matters: assessing small subunit rRNA primers for marine microbiomes with mock communities, time series and global field samples. *Environ Microbiol* 2016; **18**: 1403–1414.
157. Quince C, Lanzen A, Davenport RJ, Turnbaugh PJ. Removing noise from pyrosequenced amplicons. *BMC Bioinformatics* 2011; **12**: 38.
158. Bartram AK, Lynch MDJ, Stearns JC, Moreno-Hagelsieb G, Neufeld JD. Generation of multimillion-sequence 16S rRNA gene libraries from complex microbial communities by assembling paired-end Illumina reads. *Appl Environ Microbiol* 2011; **77**: 3834–3852.
159. Bolyen E, Rideout JR, Dillon MR, Bokulich NA, Abnet CC, Al-Ghalith GA, et al. Reproducible, interactive, scalable and extensible microbiome data science using QIIME 2. *Nat Biotechnol* 2019; **37**: 852–857.
160. Callahan BJ, McMurdie PJ, Rosen MJ, Han AW, Johnson AJA, Holmes SP. DADA2: high-resolution sample inference from Illumina amplicon data. *Nat Methods* 2016; **13**: 581–583.

161. Min D, Doxey AC, Neufeld JD, Neufeld JD. AXIOME3: Automation, eXtension, and Integration Of Microbial Ecology. *Gigasci* 2021; **10**: giab006.
162. Heidrich V, Karlovsky P, Beule L. ‘SRS’R Package and ‘q2-srs’ QIIME 2 Plugin: normalization of microbiome data using scaling with ranked subsampling (SRS). *Appl Sci* 2021; **11**: 11473.
163. Davis NM, Proctor DiM, Holmes SP, Relman DA, Callahan BJ. Simple statistical identification and removal of contaminant sequences in marker-gene and metagenomics data. *Microbiome* 2018; **6**: 226.
164. Pedersen K, Motamedi M, Karnland O, Sandén T. Cultivability of microorganisms introduced into a compacted bentonite clay buffer under high-level radioactive waste repository conditions. *Eng Geol* 2000; **58**: 149–161.
165. Dandurand LM, Morra MJ, Chaverra MH, Orser CS. Survival of *Pseudomonas spp* in air-dried mineral powders. *Soil Biol Biochem* 1994; **26**: 1423–1430.
166. Watanabe M, Kojima H, Fukui M. Review of *Desulfotomaculum* species and proposal of the genera *Desulfallas* gen. nov., *Desulfofundulus* gen. nov., *Desulfofarcimen* gen. nov. and *Desulfohalotomaculum* gen. nov. *Int J Syst Evol Microbiol* 2018; **68**: 2891–2899.
167. Nandasena K, Yates R, Tiwari R, O ’hara G, Howieson J, Ninawi M-H, et al. Genome analyses of the carboxydophilic sulfate-reducers *Desulfotomaculum nigrificans* and *Desulfotomaculum carboxydivorans* and reclassification of *Desulfotomaculum caboxydivorans* as a later synonym of *Desulfotomaculum nigrificans*. *Stand Genomic Sci* 2014; **9**: 462–472.
168. Kaksonen AH, Spring S, Schumann P, Kroppenstedt RM, Puhakka JA. *Desulfurispora thermophila* gen. nov., sp. nov., a thermophilic, spore-forming sulfate-reducer isolated from a sulfidogenic fluidized-bed reactor. *Int J Syst Evol Microbiol* 2007; **57**: 1089–1094.
169. Haynes HM, Pearce CI, Boothman C, Lloyd JR. Response of bentonite microbial communities to stresses relevant to geodisposal of radioactive waste. *Chem Geol* 2018; **501**: 58–67.
170. Miettinen H, Bomberg M, Bes R, Tiljander M, Vikman M. Transformation of inherent microorganisms in Wyoming-type bentonite and their effects on structural iron. *Appl Clay Sci* 2022; **221**: 106465.
171. Alzamel M, Haruna S, Fall M. Saturated hydraulic conductivity of bentonite–sand barrier material for nuclear waste repository: effects of physical, mechanical thermal and chemical factors. *Environ Earth Sci* 2022; **81**: 223.
172. Martinez-Moreno MF, Povedano-Priego C, Mumford AD, Morales-Hidalgo M, Mijndonckx K, Jroundi F, et al. Microbial responses to elevated temperature: Evaluating bentonite mineralogy and copper canister corrosion within the long-term stability of deep geological repositories of nuclear waste. *Sci Total Environ* 2024; **915**: 170149.
173. Punch R. Impact of water activity on microbial growth in bentonite clay. 2023. University of Waterloo.
174. Parada AE, Needham DM, Fuhrman JA. Every base matters: assessing small subunit rRNA primers for marine microbiomes with mock communities, time series and global field samples. *Environ Microbiol* 2016; **18**: 1403–1414.
175. Davis C. Enumeration of probiotic strains: Review of culture-dependent and alternative techniques to quantify viable bacteria. *J Microbiol Methods* 2014; **103**: 9–17

176. Kirk S, Avignone-Rossa CA, Bushell ME. Growth limiting substrate affects antibiotic production and associated metabolic fluxes in *Streptomyces clavuligerus*. *Biotechnol Lett* 2000; **22**: 1803–1809.
177. Odeberg G, Bläckberg A, Sunnerhagen T. Infection or contamination with *Rothia*, *Kocuria*, *Arthrobacter* and *Pseudoglutamicibacter*—a retrospective observational study of non-*Micrococcus Micrococcaceae* in the clinic. *J Clin Microbiol* 2023; **61**: e01484-22.
178. Shi T, Wang Y-F, Wang H, Wang B. Genus *Nocardiopsis*: a prolific producer of natural products. *Mar Drugs* 2022; **20**: 374.
179. Vodickova P, Suman J, Benesova E, Strejcek M, Neumann-Schaal M, Cajthaml T, et al. *Arthrobacter polaris* sp. nov., a new cold-adapted member of the family *Micrococcaceae* isolated from Antarctic fellfield soil. *Int J Syst Evol Microbiol* 2022; **72**: 005541.
180. Jiang Z, Xiao M, Yang L-L, Zhi X-Y, Li W-J. Genome-based taxonomic classification within the family *Thermoactinomycetaceae*. *Int J Syst Evol Microbiol* 2019; **69**: 2028–2036.
181. Fardeau M-L, Barsotti V, Cayol J-L, Guasco S, Michotey V, Joseph M, et al. *Caldinitratiruptor microaerophilus*, gen. nov., sp. nov. isolated from a French hot spring (Chaudes-Aigues, Massif Central): a novel cultivated facultative microaerophilic anaerobic thermophile pertaining to the *Symbiobacterium* branch within the *Firmicutes*. *Extremophiles* 2010; **14**: 241–247.
182. Hendershot WH, Duquette M. A simple barium chloride method for determining cation exchange capacity and exchangeable cations. *Soil sci soc Amer J* 1986; **50**: 605–608.
183. Hafez AA, Goyal SS, Rains DW. Quantitative determination of total sulfur in plant tissues using acid digestion and ion-chromatography. *Agron J* 1991; **83**: 148–153.
184. Schollenberger CJ, Simon RH. Determination of exchange capacity and exchangeable bases in soil—ammonium acetate method. *Soil Sci* 1945; **59**: 13–24.
185. Lindsay WL, Norvell Wa. Development of a DTPA soil test for zinc, iron, manganese, and copper. *Soil sci soc Amer J* 1978; **42**: 421–428.
186. Olsen SR. Estimation of available phosphorus in soils by extraction with sodium bicarbonate. 1954. US Department of Agriculture.
187. Prince AL. Determination of total nitrogen, ammonia, nitrates, and nitrites in soils. *Soil Sci* 1945; **59**: 47–52.
188. Muyzer G, De waal EC, Uitterlinden AG. Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. *Appl Environ Microbiol* 1993; **59**: 695–700.
189. Liu CM, Kachur S, Dwan MG, Abraham AG, Aziz M, Hsueh P-R, et al. FungiQuant: a broad-coverage fungal quantitative real-time PCR assay. *BMC Microbiol* 2012; **12**: 255.
190. Stahl DA, Flesher B, Mansfield HR, Montgomery L. Use of phylogenetically based hybridization probes for studies of ruminal microbial ecology. *Appl Environ Microbiol* 1988; **54**: 1079–1084.
191. Sun L, Tanskanen JT, Hirvi JT, Kasa S, Schatz T, Pakkanen TA. Molecular dynamics study of montmorillonite crystalline swelling: Roles of interlayer cation species and water content. *Chem Phys* 2015; **455**: 23–31.
192. Fowler L, Engqvist H, Öhman-Mägi C. Effect of copper ion concentration on bacteria and cells. *Materials* 2019; **12**: 3798.
193. Cela S, Sumner ME. Critical concentrations of copper, nickel, lead, and cadmium in soils based on nitrification. *Commun Soil Sci Plant Anal* 2002; **33**: 19–30.

194. Carlson CA, Ingraham JL. Comparison of denitrification by *Pseudomonas stutzeri*, *Pseudomonas aeruginosa*, and *Paracoccus denitrificans*. *Appl Environ Microbiol* 1983; **45**: 1247–1253.
195. Kämpfer P, Schulze R, Jäckel U, Malik KA, Amann R, Spring S. *Hydrogenophaga defluvii* sp. nov. and *Hydrogenophaga atypica* sp. nov., isolated from activated sludge. *Int J Syst Evol Microbiol* 2005; **55**: 341–344.
196. Han Y-L, Zhang X-Z, Liu H-B, Rittmann BE, Zhao H-P. Novel sulfate reduction coupled to simultaneous nitrification and autotrophic denitrification process for removing nitrogen and organics from saline wastewater. *Environ Sci Technol* 2023; **57**: 10733–10744.
197. Zhou L, Dong N, Fu M, Yue X, Jian Y, Li H, et al. Dissimilatory sulfate reduction in an anaerobic biofilm reactor for tofu processing wastewater treatment: Bacterial community and their functional genes. *Sci Total Environ* 2023; **892**: 164579.
198. Yuan H, Yuan J, You Y, Zhang B, Wu Y, Huang S, et al. Simultaneous ammonium and sulfate biotransformation driven by aeration: Nitrogen/sulfur metabolism and metagenome-based microbial ecology. *Sci Total Environ* 2021; **794**: 148650.
199. Li Y, Guo L, Yang R, Yang Z, Zhang H, Li Q, et al. *Thiobacillus* spp. and *Anaeromyxobacter* spp. mediate arsenite oxidation-dependent biological nitrogen fixation in two contrasting types of arsenic-contaminated soils. *J Hazard Mater* 2023; **443**: 130220.
200. Zhou L, Lai Y, Shao Z, Jian Y, Zhuang W-Q. Keystone bacteria in a thiosulfate-driven autotrophic denitrification microbial community. *Chem Eng J* 2023; **470**: 144321.
201. Beaver R, Vachon M, Tully C, Engel K, Spasov E, Binns WJ, et al. Impact of dry density and incomplete saturation on microbial growth in bentonite clay for nuclear waste storage. *J Appl Microbiol* 2024; **135**: Ixae053.
202. Khan ST, Horiba Y, Yamamoto M, Hiraishi A. Members of the family *Comamonadaceae* as primary poly (3-hydroxybutyrate-co-3-hydroxyvalerate)-degrading denitrifiers in activated sludge as revealed by a polyphasic approach. *Appl Environ Microbiol* 2002; **68**: 3206–3214.
203. Kämpfer P, Kroppenstedt RM. *Pseudonocardia benzenivorans* sp. nov. *Int J Syst Evol Microbiol* 2004; **54**: 749–751.
204. Fukunaga S, Jintoku T, Iwata Y, Nakayama M, Tsuji T, Sakaya N, et al. Investigation of microorganisms in bentonite deposits. *Geomicrobiol J* 2005; **22**: 361–370.
205. Colwell RR, Brayton PR, Grimes DJ, Roszak DB, Huq SA, Palmer LM. Viable but non-culturable *Vibrio cholerae* and related pathogens in the environment: implications for release of genetically engineered microorganisms. *Biotechnol* 1985; **3**: 817–820.
206. Fakruddin M, Mannan KS Bin, Andrews S. Viable but nonculturable bacteria: food safety and public health perspective. *Int Sch Res Notices* 2013; **2013**: 703813.
207. Liu J, Yang L, Kjellerup BV, Xu Z. Viable but nonculturable (VBNC) state, an underestimated and controversial microbial survival strategy. *Trends Microbiol* 2023; **31**: 1013–1023.
208. Dong K, Pan H, Yang D, Rao L, Zhao L, Wang Y, et al. Induction, detection, formation, and resuscitation of viable but non-culturable state microorganisms. *Compr Rev Food Sci Food Saf* 2020; **19**: 149–183.
209. Nowakowska J, Oliver JD. Resistance to environmental stresses by *Vibrio vulnificus* in the viable but nonculturable state. *FEMS Microbiol Ecol* 2013; **84**: 213–222.

210. Su C-P, Jane W-N, Wong H. Changes of ultrastructure and stress tolerance of *Vibrio parahaemolyticus* upon entering viable but nonculturable state. *Int J Food Microbiol* 2013; **160**: 360–366.
211. Vriezen JAC, de Bruijn FJ, Nüsslein KR. Desiccation induces viable but non-culturable cells in *Sinorhizobium meliloti* 1021. *AMB Express* 2012; **2**: 6.
212. Pedersen JC, Jacobsen CS. Fate of *Enterobacter cloacae* JP120 and *Alcaligenes eutrophus* AEO106 (pRO101) in soil during water stress: effects on culturability and viability. *Appl Environ Microbiol* 1993; **59**: 1560–1564.
213. Postgate JR. Versatile medium for the enumeration of sulfate-reducing bacteria. *Appl Microbiol* 1963; **11**: 265–267.
214. Ramamoorthy S, Sass H, Langner H, Schumann P, Kroppenstedt RM, Spring S, et al. *Desulfosporosinus lacus* sp. nov., a sulfate-reducing bacterium isolated from pristine freshwater lake sediments. *Int J Syst Evol Microbiol* 2006; **56**: 2729–2736.
215. Cypionka H, Widdel F, Pfennig N. Survival of sulfate-reducing bacteria after oxygen stress, and growth in sulfate-free oxygen-sulfide gradients. *FEMS Microbiol Ecol* 1985; **1**: 39–45.
216. Karnachuk O V, Kurganskaya IA, Avakyan MR, Frank YA, Ikkert OP, Filenko RA, et al. An acidophilic *Desulfosporosinus* isolated from the oxidized mining wastes in the Transbaikal area. *Microbiol* 2015; **84**: 677–686.
217. Sato Y, Hamai T, Hori T, Aoyagi T, Inaba T, Kobayashi M, et al. *Desulfosporosinus* spp. were the most predominant sulfate-reducing bacteria in pilot-and laboratory-scale passive bioreactors for acid mine drainage treatment. *Appl Microbiol Biotechnol* 2019; **103**: 7783–7793.
218. Stroes-Gascoyne S, West JM. An overview of microbial research related to high-level nuclear waste disposal with emphasis on the Canadian concept for the disposal of nuclear fuel waste. *Can J Microbiol* 1996; **42**: 349–366.
219. Stroes-Gascoyne S, Kohle CL, Maak P, Hamon CJ, Dixon DA. The effects of dry density and porewater salinity on the physical and microbiological characteristics of compacted 100% bentonite. *MRS Proceedings* 2011; **985**: 505–510.
220. Guo R. Thermal response of a Mark II conceptual deep geological repository in crystalline rock. 2016. Nuclear Waste Management Organization, Toronto, ON.
221. Tian S, Tian Z, Yang H, Yang M, Zhang Y. Detection of viable bacteria during sludge ozonation by the combination of ATP assay with PMA-Miseq sequencing. *Water (Switzerland)* 2017.
222. Nocker A, Sossa-Fernandez P, Burr MD, Camper AK. Use of propidium monoazide for live/dead distinction in microbial ecology. *Appl Environ Microbiol* 2007; **73**: 5111–5117.
223. Zhou GW, Yang XR, Rønn R, Su JQ, Cui L, Zheng BX, et al. Metabolic inactivity and re-awakening of a nitrate reduction dependent iron(II)-oxidizing bacterium *Bacillus ferrooxidans*. *Front Microbiol* 2019; **10**: 1494.