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Bacterial diversity and production of sulfide in microcosms containing uncompacted bentonites

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Abstract

Aims: This study examined the diversity and sulfide-producing activity of microorganisms in microcosms containing commercial clay products (*e.g.*, MX-80, Canaprill and National Standard) similar to materials which are currently considered for use in the design specifications for deep geologic repositories (DGR) for spent nuclear fuel.

Methods and results: In anoxic microcosms incubated for minimum of 60 days with 10 g l^{-1} NaCl, sulfide production varied with temperature, electron donor and bentonite type. Maximum specific sulfide production rates of 0.189 d^{-1}, 0.549 d^{-1} and 0.157 d^{-1} occurred in lactate-fed MX-80, Canaprill and National Standard microcosms, respectively. In microcosms with 50 g l^{-1} NaCl, sulfide production was inhibited. Denaturing gradient gel electrophoresis (DGGE) profiling of microcosms revealed the presence of bacterial classes *Clostridia, Bacilli, Gammaproteobacteria, Deltaproteobacteria, Actinobacteria, Sphingobacteriia* and *Erysipelotrichia*. Spore-forming and non-spore-forming bacteria were confirmed in

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microcosms using high-throughput 16S rRNA gene sequencing. Sulfate-reducing bacteria of the genus *Desulfosporosinus* predominated in MX-80 microcosms; whereas, *Desulfotomaculum* and *Desulfovibrio* genera contributed to sulfate-reduction in National Standard and Canaprill microcosms.

Conclusions: Commercial clays microcosms harbour a sparse bacterial population dominated by spore-forming microorganisms. Detected sulfate- and sulfur-reducing bacteria presumably contributed to sulfide accumulation in the different microcosm systems.

Significance and impact of study: The use of carbon-supplemented, clay-in-water microcosms offered insights into the bacterial diversity present in as-received clays, along with the types of metabolic and sulfidogenic reactions that might occur in regions of a DGR (*e.g.*, interfaces between the bulk clay and host rock, cracks, fissures, *etc.*) that fail to attain target parameters necessary to inhibit microbial growth and activity.

Keywords: Environmental science, Geochemistry, Microbiology

1. Introduction

Spent nuclear fuel (SNF) contains a complex mixture of highly-radioactive and toxic nuclear fission and activation products that require special handling and storage. With about 240,000 metric tons of SNF having been stockpiled worldwide as of 2009, and approximately 10,500 tons of SNF generated annually (Feiveson et al., 2011), the need for a long-term solution for SNF storage is an acute concern faced by Canada and other nations that use nuclear power for electricity generation. The deep geologic repository (DGR) concept is an option favored by many countries for the long-term management of SNF and other highly-radioactive waste forms (McKinley et al., 2007; Nash and Dowdeswell, 2005). Most radioactive waste management concepts developed by SNF management agencies include a system of natural and engineered barriers that function to contain and isolate the waste from the environment (Villagran et al., 2011). These barriers consist of the host rock and the engineered barrier system (EBS), which itself is comprised of different components, including highly-compacted bentonite clay surrounding corrosion-resistant metal containers that together would encapsulate the SNF (McKinley et al., 2007). The barrier capacity of the clay-based EBS relies on the ability of bentonites to swell and seal voids in porous media, while adsorbing hazardous constituents (Pusch, 2015). Nuclear regulators require extensive testing of all EBS elements for stability and integrity, and to take into account processes that could potentially disturb the integrity and functionality of the EBS, as part of licencing requirements (Chapman and McCombie, 2003; Villagran et al., 2011).

Microbial implications on the performance of the EBS in DGR have been the subject of considerable study (Stroes-Gascoyne et al., 2006, 2010; Wang and Francis, 2005;

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Humphreys et al., 2010; Mulligan et al., 2009; Behrends et al., 2012; Pedersen, 2010, 2013; Pedersen et al., 2012; Lopez-Fernandez et al., 2015; Stone et al., 2016). As genuine bio-catalysts, bacteria are capable of vigorously modifying their environments through their growth and metabolite production. Some of these transformations may cause substantial changes in the structure and stability of certain components of the EBS, with deleterious effects on the overall integrity and functionality of the barrier system (McKelvie et al., 2016). Sulfate-reducing bacteria (SRB) are of particular concern since biogenic sulfide is expected to be a main corrosive agent for copper canisters under anoxic conditions in a DGR (e.g., King, 2009). Because of the inherent physical characteristics of highly-compacted bentonites, including small pore size, low water activity (a_w) and high swelling pressure, these clays have been credited with suppressing microbial proliferation and activity within the EBS (Stroes-Gascoyne and West, 1997; Pedersen et al., 2000; Stroes-Gascoyne et al., 2006, 2010). The suppression of microbial activity in sealing materials surrounding waste containers is predicted to minimize the contribution of microbially-influenced corrosion to the overall corrosion process to acceptable levels (King, 2009). Indigenous microorganisms within deep groundwater and host rock, along with bacteria introduced with fluid seepage during construction and maintenance of a DGR, will be an unavoidable source of microbial contamination in the EBS (McKelvie et al., 2016). In addition, commercially-available bentonites, including Wyoming MX-80 bentonite - the reference clay for DGR concepts in Canada, Sweden and Finland – have been demonstrated to harbour a small set of bacteria (Haveman et al., 1995; Pedersen et al., 2000; Stroes-Gascoyne et al., 2006). While some data regarding limited microbial activity in dense clays has been generated over the past 25 years, the initial abundance and diversity of microorganisms in *as-received* bentonites and their potential contribution to bacterial production of sulfide in those areas of the EBS where swelling pressures and water activities fail to attain inhibitory target values (at least transiently, during the repository resaturation process after closure) remains poorly-characterized. In this study, a relatively-specialized bacterial community consisting principally of spore-forming fermenting and sulfate- and metal-respiring microorganisms was found to be responsible for sulfide production in clay in water (10% w/v) microcosms containing uncompacted bentonites and stimulated with labile carbon. While not intended to model the highly-compacted bulk clay of the EBS, the clay-in-water slurry conditions within microcosms offer a glimpse into how and which microbes might proliferate at interfaces or voids/fissues, providing cues as to their potential local effects.

2. Materials and methods

2.1. Source of clays

Commercial clay products were obtained from American Colloid Company (Volclay MX-80®, Wyoming clay, lot number 17912), Canadian Clay Product Inc.

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(Canaprill®, Avonlea clay) and Bentonite Performance Minerals LLC (National® Standard, Wyoming clay, lot number 119289). Information on the geological origin and elemental composition of studied clays are summarised in the supplementary materials (Table S1).

2.2. Media, cultivation techniques, and growth conditions

Sulfate-reducing and other anaerobic bacteria were enriched from commercial clays in anoxic microcosms containing 10 g of bentonite in 90 ml of modified Zhilina's medium, consisting of (g l^{-1} indicated in brackets) NH_4Cl (1.0), K_2HPO_4 (0.2), KCl (0.2), NaCl (10 g l^{-1}, unless otherwise specified) and 0.15 mL of 1% (w/v) resazurin (Zhilina et al., 1997). To prepare microcosms, aliquots of as-received clays were dispensed into pre-autoclaved 160-mL borosilicate glass bottles and equilibrated in an anaerobic chamber under 80% (v/v) N_2, 10% (v/v) CO_2 and 10% (v/v) H_2 atmosphere. After a minimum of 12 h, bottles were sealed with butyl-rubber stoppers before mixing with aqueous solutions. Modified Zhilina's medium was then autoclaved and cooled in a dispensing system under a 90% (v/v) N_2 and 10% (v/v) CO_2 atmosphere (hereafter referred to as anoxic gas), according to Widdel and Bak (1992). Prior to dispensing, 90 ml of 0.8 M $NaHCO_3$/0.2 M Na_2CO_3, 1 ml l^{-1} of trace elements (Pfennig and Lippert, 1966), 0.5 ml l^{-1} of 5% yeast extract, 20 mmol l^{-1} sodium sulfate, 0.25 mmol l^{-1} sodium sulfide, 10 mmol l^{-1} sodium lactate (optional) and 10 mmol l^{-1} sodium acetate (optional) were added to the Zhilina's medium from anoxic sterile stock solutions. The pH was adjusted to 8.0 using either NaOH or HCl solutions before the medium was dispensed under anoxic atmosphere into bottles containing clays. Microcosms were then incubated in an anaerobic chamber in the dark without agitation at both 15 °C and 37 °C. Experiments with different clays were performed sequentially. The length of incubation was initially limited to 60−70 days (*i.e.*, MX-80); however, in order to record sulfide accumulation over a longer period of time, the incubation time was increased to a minimum of 120 days (*i.e.*, National Standard and Canaprill). At defined intervals, 1 ml aliquots from the various microcosms were aseptically removed under anoxic gas for chemical analysis. Inorganic species (*e.g.*, sulfide and sulfates) were assayed immediately after sampling, or stored at -80 °C for subsequent analysis of organic compounds (*e.g.*, lactate and acetate).

For stimulation of sulfide-producing bacteria under lithotrophic conditions, basal clay-medium mixtures, prepared without either lactate or acetate, were incubated at 22 °C in the anaerobic chamber under 10% (v/v) H_2 atmosphere as indicated above. Unless indicated otherwise, the lithotrophic microcosms were shaken briefly once per day but otherwise then incubated without agitation.

Viable cultivable anaerobic and aerobic organotrophic microorganisms were estimated at room temperature (RT) after either 10 days (aerobic growth) or after

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10 and 30 days (anaerobic growth in the anaerobic chamber) using R2A agar (BD; Franklin Lake, NJ) and plate counting following standard methods (9215 C, APHA, 2005).

Sulfate-reducing microorganisms in the bulk MX-80 clay were enumerated according to the most probable number (MPN) procedure (Koch, 1994) by the marked increase in sulfide content in the anoxic modified Postgate's medium B (Postgate, 1984) containing (g l^{-1}) NaCl (10.0), KH_2PO_4 (0.5), NH_4Cl (1.0), $CaSO_4$*$2H_20$ (1.0), $MgSO_4$*$7H_2O$ (2.0), $FeSO_4$*$7H_2O$ (0.5 g l^{-1}), yeast extract (0.5 g l^{-1}), sodium lactate (20 mmol l^{-1}), sodium sulfide (0.25 mmol l^{-1}) and supplemented with micro-elements (Pfennig and Lippert, 1966) and selenate/tungstate stock solution (Widdel and Bak, 1992) after 90 days of incubation at RT.

2.3. Analytical procedures

Aqueous sulfide concentrations were determined using a colorimetric method modified from Trüper and Schlegel (1964). The basic sulfide assay was performed as follows: a 40 μl clay-free sample was added to 640 μl 2% zinc acetate (in 0.1% w/v glacial acetic acid) to trap sulfide. Thereafter, 790 μl of the "paraphenylenediamine reagent" (0.2% w/v of N,N-dimethyl-p-phenylenediamine sulfate [Eastman Kodak No. 1333] in 20% w/v H_2SO_4) was added. The mixture was briefly vortexed and left for 1.5 min at RT. Then, it was centrifuged at 16000 × g for 2 min and 40 μl of 10% $FeNH_4(SO_4)_2$*$10H_2O$ in 2% w/v H_2SO_4 was added to the solid-free supernatant. After 20 min, the absorbance was measured at 670 nm against the blank.

Dissolved sulfate in clay microcosms was measured using an adapted turbidometric method (Cypionka and Pfennig, 1986). Accordingly, a 150 μl volume of centrifuged, turbidity-free sample was added to 350 μl of deionized water, 500 μl of citric acid-glycerol solution (5%, w/v, citric acid*H_20 in 60%, v/v, glycerol) and 125 μl barium reagent (1%, w/v, $BaCl_2$*$2H_20$ in 10%, w/v, citric acid). After mixing, the solution was incubated for 30 min at RT after which the turbidity was measured at 436 nm against a no-sulfate control.

Concentrations of acetic and lactic acids were determined using a Varian ProStar HPLC system (Varian Inc., Palo Alto, CA) equipped with a Varian Prostar 410 autosampler, Varian Prostar 212 pump system, and Varian Prostar 325 UV-Vis detector. Each sample was measured at 210 nm after elution through a Hi-Plex H column (6 × 316 mm; Agilent Technologies, Santa Clara, CA) with 0.01 M H_2SO_4 in ddH_2O as the mobile phase.

2.4. Assessment of sulfide accumulation rates

To approximate sulfide generation in clays, we assumed that microbial sulfidogenesis at the beginning of the exponential phase is an irreversible, first-order

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single-batch biochemical reaction with an excess of substrates. Further assuming that the amount of sulfide adsorbed from solution onto clay particles is constant and negligible, we calculated a specific rate constant of the dissolved sulfide accumulation in clay microcosms per unit of time (Eq. 1):

$$SRSA = 2.303* \frac{\log_{10}C2 - \log_{10}C1}{t2 - t1} \quad (1)$$

where C1 and C2 are equal to the concentration of dissolved sulfide at Time 1 (t1) and Time 2 (t2). The abbreviation SRSA in Eq. (1) stands for Specific Rate of Sulfide Accumulation.

2.5. Molecular biological procedures

2.5.1. DNA extraction

Lactate- and acetate-enriched clay microcosms, developed over a 90-day period, were centrifuged at 4000 × g in 3-ml aliquots for 20 min at 4 °C. The combined pellets from experimental replicates of each type of microcosm were resuspended in 2 ml of NaCl-EDTA, aliquoted in 1-ml fractions and stored at −20 °C until DNA extraction. DNA from two fractions of the same type of experiment were recovered independently using two different protocols: 1) the FastDNA SPIN kit for soil (MP Biomedicals, Inc., Carlsbad, CA) and 2) Marmur's modified procedure, as described elsewhere (Marmur, 1961; Voordouw et al., 1990), but adapted in this case to include two final repeat steps to remove phenolic residuals by diethyl ether extraction (Sambrook et al., 1999). Subsequently, DNA preparations that originated from the same experiment were pooled together from protocols 1 and 2 for further processing.

2.5.2. PCR amplification of 16S rRNA genes

PCR amplification of pooled DNA recovered from clay microcosms for denaturing gradient gel electrophoresis (DGGE) was conducted with Bacteria domain-specific primers 27f-GC (5′-ccgcgccgcccggcggcggggcggggcgggggCAGAGTTTGATCCTGGCTCAG-3′; lowercase letters designate the GC clamp) and 534R (5′-ATTACCGCGGCTGCTGG-3′; Grigoryan et al., 2008; Muyzer et al., 1993). Isolated DNA from pure cultures were PCR-amplified with the universal bacterial primers 27F and 1389R (Lane, 1991). The PCR reactions were performed in 50 μl volumes containing 1−2 μl of template DNA, 0.5 μmol of reverse and forward primers, 25 μl Taq 2X Master Mix (New England Bio Labs, MA) and 22 μl of molecular-grade water (MO BIO Laboratories, Carlsbad, CA). Cycle conditions included an initial 5 min denaturing step at 94 °C, followed by 30 cycles of 45 s at 95 °C, 45 s at 60 °C, and 45 s at 72 °C, and a final elongation step of 5 min at 72 °C using a Techne TC-412 thermal cycler (Techne Inc., Burlington, NJ). PCR products were separated by electrophoresis on 1.5% agarose

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gel plates (Invitrogen, Carlsbad, CA) stained with ethidium bromide (Sigma−Aldrich, St. Louis, MO) and digitally-photographed.

2.5.3. DGGE fingerprinting

Bacterial 16S rDNA amplicons (~500 bp) were separated by the DGGE method (Muyzer et al., 1993) using the DCODE system (Bio-Rad, Hercules, CA). Aliquots (10 μl) of PCR product were mixed with 10 μl of loading dye buffer and resolved on an 8% w/v polyacrylamide gel in 1× TAE buffer using denaturing gradients from 35 to 60% (where 100% denaturant contained 7 M urea and 40% deionized formamide). Reference markers containing PCR-products amplified with similar primers from known bacterial species included *Acidithiobacillus* sp., *Gallionella* sp., *Streptomyces* sp., *Vibrio* sp., *Nitrosomonas* sp., *Shewanella* sp., *Desulfovibrio* sp. and *Salmonella* sp. DGGE was carried out at 70 V and 60 °C for 14 h. After electrophoresis, gels were stained with SYBR Green I (1:10,000 dilution in 1× TAE buffer) for 20−30 min and photographed. In total, two DGGE gels harbouring two repetitive DNA extractions from the same samples were analyzed. Bands were excised from polyacrylamide gels and DNA was extracted in 50 μl TE overnight at RT. The extraction mixtures were then centrifuged for 5 min at 10000 × g, and 2 μl of the supernatant was aliquoted for reamplification using primers 27f (lacking the GC clamp) and 534R. The PCR products were purified using the GeneJET Genomic DNA Purification Kit (Thermo Fisher Scientific, Waltham, MA).

2.5.4. Phylogenetic analysis of DGGE fingerprints

Sequencing of PCR products from excised DGGE bands was performed using a 3730xl DNA Analyzer (Applied Biosystems, Thermo Fisher Scientific Corp., Foster City, CA) housed at the Plant Biotechnology Institute of the National Research Council of Canada (Saskatoon, SK, Canada). The unprocessed DNA sequences were manually edited using Bioedit Sequence Alignment Editor software (Hall, 1999). Recovered sequences were checked for chimeras using the DECIPHER package (Wright et al., 2012) and assembled using Staden GAP4 (Staden et al., 1999). Homologous sequences were retrieved from GenBank using BLAST (Altschul et al., 1990) and the Ribosomal Database Project database (RDP release 11: https://rdp.cme.msu.edu). For group analysis, sequences from the DGGE bands were processed using the multiple alignment tool MAFFT (Katoh et al., 2002) followed by trimming and filtration with TrimAl (Capella-Gutierrez et al., 2009). Shortened and aligned nucleotides (ca. 300 bp) were further assigned into operational taxonomic units (OTUs) via the web server-based CD HIT Suite (Huang et al., 2010) at a clustering threshold of 95% and local alignment coverage of 0.75.

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2.5.5. Bacterial 16S rRNA gene library preparation and high throughput amplicon sequencing

Five milliliter aliquots from lactate-sulfate microcosms containing the same type of clay, but incubated at different temperatures (15 °C or 37 °C), were pooled after 9 months of incubation and DNA extracted with the FastDNA SPIN kit for soil (MP Biomedicals, Inc., Carlsbad, CA). Sequence libraries were prepared by PCR amplification of the V3−V4 conserved regions of bacterial 16S rRNA gene using the primers Bakt_341F and Bakt_805R (Herlemann et al., 2011) that included 5′ overhangs complementary to priming sequences for Nextera XT-indexed adapters (Illumina, Inc., San Diego, CA). The PCR reaction was comprised of 5 μl template, 12.5 μl 2x KAPA HiFi HotStart ReadyMix (KAPA Biosystems, Wilmington, MA), 1.0 μmol reverse and forward primers, 0.5 μl 25 mmol l^{-1} $MgCl_2$ with a final volume adjusted to 25 μl using molecular-grade water. Thermal cycling involved initial denaturation at 96 °C (4 min), followed by 30 cycles of denaturation at 96 °C (30 s), primer annealing at 56 °C (30 s) and primer extension at 72 °C (30 s), followed by extension at 72 °C for 5 min. Amplicons were visualized by gel electrophoresis to verify size (ca. 425 bp), purified by Agencourt AMPure XP system (Beckman Coulter, Brea, CA), eluted in PCRapace elution buffer (STRATEC Molecular GmbH, Berlin, Germany), and quantified using a Qubit dsDNA HS kit (Invitrogen, Carlsbad, CA). PCR amplicons were barcoded with sequencing adapters using the Nextera XT index kit. Obtained libraries were normalized to 4 nM and pooled prior to sequencing and further processing. Libraries (6 pM final concentration) were sequenced using MiSeq Reagent Kit V3 (600 cycles) on the MiSeq System (Illumina, Inc., San Diego, CA).

2.5.6. High-throughput sequence data analysis

Paired-end MiSeq reads from the three samples examined were aligned and transformed to contigs (2 × 300 bp) yielding a total of 61514 reads. Contig sequences were further subjected to error elimination, chimera checking, and contaminant removal by using Mothur Ver. 1.32.2 with SILVA database Ver. 123 as a bacterial taxonomy reference (Schloss et al., 2009; Kozich et al., 2013). SILVA bacterial reference sequence datasets were normalized to 3498 sequences (the size of the smallest library of filtered reads) per sample for community data analysis to ensure consistent sequencing depth. OTUs were generated using average-neighbor clustering with a 3% cutoff using algorithms implemented in Mothur Ver. 1.31.2.

2.5.7. Nucleotide sequence accession numbers

All Illumina sequence data from this study were submitted to the NCBI Sequence Read Archive under accession numbers SRR6342323, SRR6337247 and SRR6337245. Sanger sequencing data for DGGE clones, reported in the current manuscript, have been submitted to GenBank and were assigned accession numbers MG650197,

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MG650198, MG650199, MG650200, MG650201, MG650202, MG650203, MG650204, MG650205, MG650206, MG650207, MG650208, MG650209, MG650210, MG650211, MG650212, MG650213, MG650214, MG650215, MG650216, MG650217, MG650218, MG650219, MG650220, MG650221, MG650222, MG650223, MG650224, MG650225, MG650226, MG650227, MG650228, MG650229, MG650230, MG650231, MG650232, MG650233, MG650234, MG650235, MG650236, MG650237, MG650238, MG650239, MG650240, MG650241, MG650242, MG650243, MG650245, MG650246, MG650247, MG650248, MG650249, MG650250, MG650251, MG650252, MG650253, MG650254, MG650255, MG650256, MG650257 and MG650258.

3. Results

3.1. Quantification of bacteria in MX-80 clay

The routine R2A plate counting protocol for enumerating viable cells revealed that the reference uncompacted MX-80 clay contained 1.64×10^5 Colony-Forming Units per gram dry weight (CFU gdw^{-1}) and 2.5×10^3 CFU gdw^{-1} of aerobic and anaerobic heterotrophs, respectively. MPN estimations, based on sulfide production in Postgate's medium B with lactate, yielded a maximum of no greater than 4.24×10^1 cells gdw^{-1}.

3.2. Sulfide production in clay microcosms

Sulfide was detected in long-term MX-80 microcosms supplemented with lactate (unless otherwise specified, the microcosms described below contained 10 g l^{-1} NaCl) only after 42–44 days regardless of incubation temperature, with daily production rates of 0.189 d^{-1} and 0.078 d^{-1} at 15 °C and 37 °C, respectively (Table 1), and

Table 1. Average specific rates of sulfide production (d^{-1}) in clay microcosms maintained under different temperatures and salinities. Standard error in parentheses.

Clay and Temp., °C	Acetate		Lactate	
	10 g NaCl l^{-1}	50 g NaCl l^{-1}	10 g NaCl l^{-1}	50 g NaCl l^{-1}
MX-80				
15	0.167 (0.035)	0	0.189 (0.054)	0.09
37	0.062 (0.087)	0	0.078 (0.034)	0
Canaprill				
15	0.147 (0.008)	0	0.317 (0.052)	0.064
37	0.185 (0.002)	0	0.548 (0.092)	0
National Standard				
15	0.072 (0.034)	0	0.035 (0.009)	0.047
37	0	0	0.157 (0.085)	0

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totaling 8.9 mmol l^{-1} and 2.3 mmol l^{-1} sulfide, respectively, after 65 days (Fig. 1A and B). In MX-80 microcosms, 10 mmol l^{-1} of lactate was oxidized with transient production of acetate (Fig. 1A and B).

Incremental sulfide production in MX-80 microcosms occurred at a rate of 0.167 d^{-1} when acetate was added, producing a total of 4.5 mmol l^{-1} at 15 °C after 65 days (Fig. 1C). Acetate-amended MX-80 microcosms incubated at 37 °C resulted in a negligible increase in sulfide (Fig. 1D). Although MX-80 microcosms initially contained 10 mmol l^{-1} acetate, only ca. 5 mmol l^{-1} could be detected after a short equilibration period (~1.5 h) with clay and 0.6 mmol l^{-1} and 1.6 mmol l^{-1} of acetate were detected in microcosms after ca. 70 days of incubation at 15 °C and 37 °C, respectively (Fig. 1C and D).

Dihydrogen gas was also found to support as much as 5.6−5.8 mmol l^{-1} of sulfide production in MX-80 microcosms after 250 days at RT (data not shown), although no sulfide was detected over the initial 24 days. Typically, sulfide accumulation was marked by the appearance of a distinct black layer at the clay-liquid interface, which

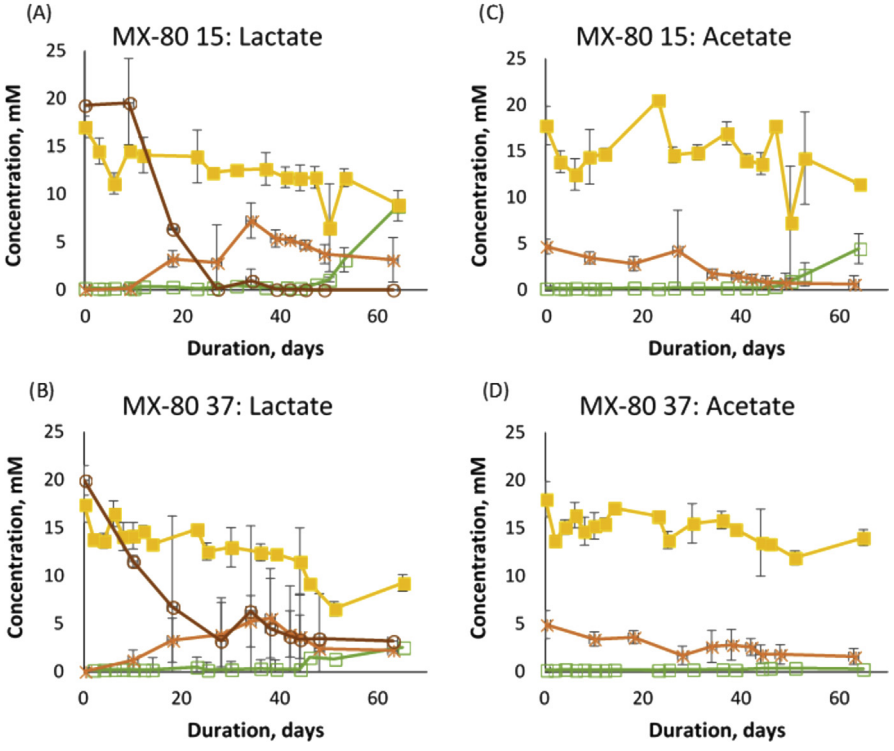

Fig. 1. Comparative analysis of sulfate to sulfide reduction coupled with oxidation of either lactate or acetate in MX-80 microcosms with 10 g l^{-1} NaCl at different temperatures. Concentrations of sulfide (□), sulfate (■), lactate (○) and acetate (∗) are indicated as a function of time for microcosms incubated at 15 °C (A and C) and 37 °C (B and D) with lactate (A and B) and acetate (C and D). Error bars represent standard deviations.

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subsequently penetrated more deeply into the clay body. The black colour of this layer was presumably caused by transformation of trace metallic elements (*e.g.*, iron) in clays to insoluble black metal sulfides (*e.g.*, pyrite) in the presence of hydrosulfide ions, and accumulated at neutral and alkaline pH due to bacterial reduction of sulfate to sulfide (Lin et al., 2009).

The reduction of sulfate corresponded with sulfide production in most cases (Fig. 1). Our measurements also indicated a decrease in sulfate (1−4 mmol l^{-1}) in reference microcosms that did not contain any exogenous donors of electrons required for biochemical sulfate-reduction, possibly indicating the adsorption of sulfate by clays particles. Neither measurable sulfide nor blackening of clay slurries were observed in these reference samples (data not shown).

In contrast, a slower sulfide production rate was observed in microcosms containing National Standard clay, with total sulfide increases in lactate-amended systems amounting to ca. 4.9 mmol l^{-1} and 1.1 mmol l^{-1} after 110 days of incubation at 15 °C and 37 °C, respectively (Fig. 2A and B, Table 1). Acetate supported the production of ca. 3.8 mmol l^{-1} of sulfide at 15 °C within 110 days; whereas, no sulfide

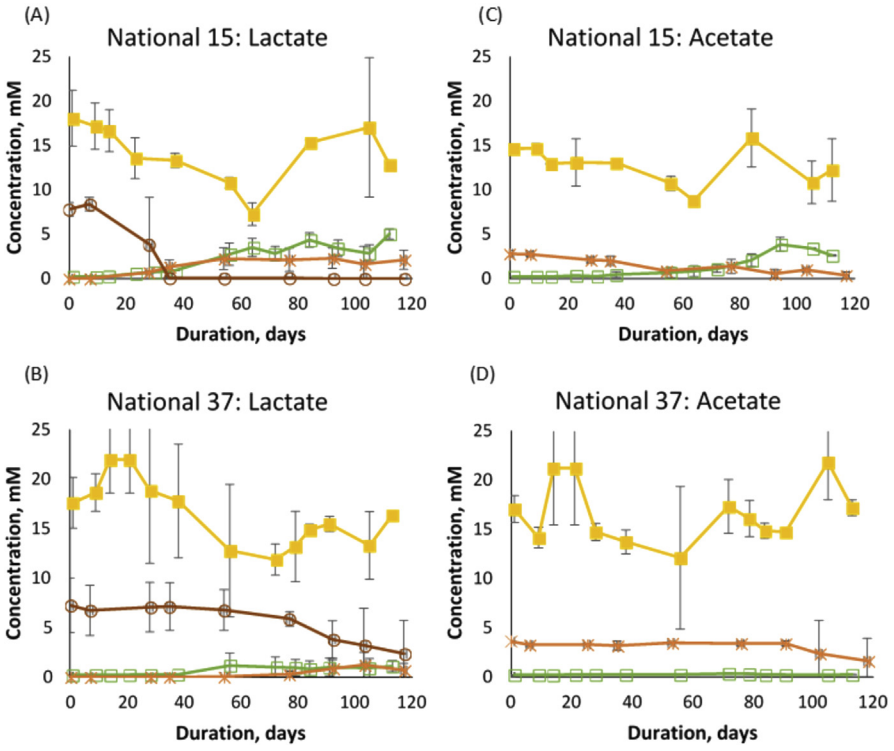

Fig. 2. Comparative analysis of sulfate to sulfide reduction coupled with oxidation of either lactate or acetate in National Standard microcosms with 10 g l^{-1} NaCl at different temperatures. Concentrations of sulfide (□), sulfate (■), lactate (○) and acetate (⁎) are indicated as a function of time for microcosms incubated at 15 °C (A and C) and 37 °C (B and D) with lactate (A and B) and acetate (C and D). Error bars represent standard deviations.

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was produced in acetate-amended microcosms at 37 °C over the same period (Fig. 2C and D). Rates of sulfide production with National Standard bentonite averaged at 0.035 d^{-1} and 0.157 d^{-1} in microcosms with lactate correspondingly incubated at 15 °C and 37 °C, and 0.072 d^{-1} in microcosms with acetate incubated at 15 °C (Table 1). Notably, in National Standard microcosms, sulfide was initially detected after only 20 days of incubation at 15 °C; whereas, in lactate-amended microcosms incubated at 37 °C, sulfide accumulation was detected after 28 days.

In lactate-amended National Standard microcosms incubated at 15 °C (Fig. 2A), lactate became completely exhausted by 35 days, with a concurrent accumulation of 2 mmol l^{-1} acetate. At 37 °C, only about 70% of introduced lactate was oxidized with subsequent production of acetate (Fig. 2B). No detectable increase in soluble sulfide in the aqueous phase was observed in microcosms amended with acetate and incubated at 37 °C. However, there was a 1.5 mmol l^{-1} drop in acetate, suggesting that either physicochemical (sorption) or biochemical degradation of acetate occurred (Fig. 2B). Sulfate determinations in National Standard microcosms yielded inconsistent data, likely due to limitations of turbidometric methods with fine clays – the data in Fig. 2 are therefore displayed for illustrative purposes only.

Assessment of Canaprill microcosms revealed faster sulfide generation than Wyoming-type bentonites. About 3.0 mmol l^{-1} and 10 mmol l^{-1} of sulfide was produced in lactate-stimulated microcosms with Canaprill clay at 15 °C and 37 °C, respectively, after 120 days (Fig. 3A and B). Accumulation of sulfide in Canaprill samples reached 0.549 d^{-1} and 0.317 d^{-1} in microcosms with lactate correspondingly incubated at 15 °C and 37 °C (Table 1). Lactate in these microcosms was oxidized completely (consumed slowly over 65 days) in conjunction with transient acetate production (2.0 mmol l^{-1} and 4.0 mmol l^{-1}, respectively, at 15 °C and 37 °C) (Fig. 3A and B). In acetate-stimulated Canaprill microcosms, approximately 1.5 mmol l^{-1} and 2.0 mmol l^{-1} of sulfide was produced over the first 38 days at 15 °C and 37 °C, respectively, during which time all acetate was oxidized (Fig. 3C and D). Sulfide generation rates in Canaprill microcosms with acetate were 0.147 d^{-1} and 0.185 d^{-1} at 15 °C and 37 °C, respectively (Table 1). Though some variability was experienced in interpreting sulfate assay data, sulfate-reduction trends were readily observable in most Canaprill clay microcosm experiments, particularly when lactate was added.

Sulfide production in Canaprill clays under 10% hydrogen showed unexpected trends. Replicate (x4) Canaprill microcosms incubated under H_2/CO_2 atmosphere initially displayed (over the first 21 days) transient production of sulfide (<1 mmol l^{-1}). In one case, up to 1.7 mmol l^{-1} of sulfide gradually accumulated (data not shown) over 250 days. In another case, the microcosm turned yellow, likely due to partial sulfate disproportionation to elemental sulfur, and then back to blackish with <0.3 mmol l^{-1} of measureable soluble sulfide. Such a back-and-

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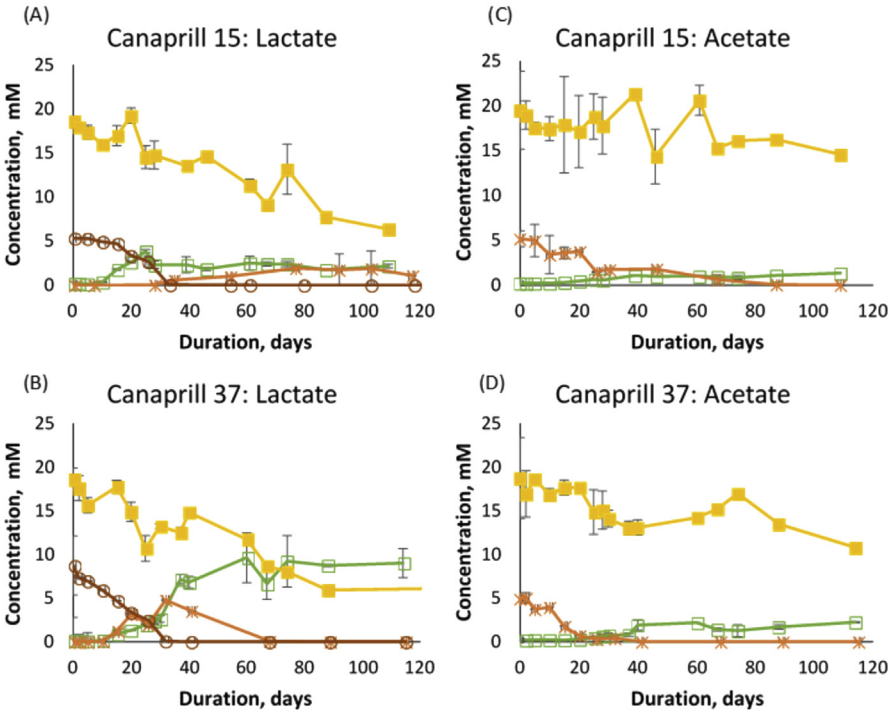

Fig. 3. Comparative analysis of sulfate to sulfide reduction coupled with oxidation of either lactate or acetate in Canaprill microcosms with 10 g l^{-1} NaCl at different temperatures. Concentrations of sulfide (□), sulfate (■), lactate (○) and acetate (∗) are indicated as a function of time for microcosms incubated at 15 °C (A and C) and 37 °C (B and D) with lactate (A and B) and acetate (C and D). Error bars represent standard deviations.

forth succession of coloration repeated once again after another 5−7 days, with the aqueous phase in these bottles eventually remaining consistently pink without any yellowish-grey precipitation. Formation of yellow-grey precipitate, along with turning solution from colourless to slightly-pink due to oxidation of resazurin, suggested that some sulfur and polysulfide were being produced transiently in Canaprill microcosms. No development of yellow-white precipitate was observed in any microcosms containing MX-80 under similar conditions, or in control microcosms without specially-introduced organics or hydrogen gas.

A five-fold increase of salinity (from 10 g l^{-1} to 50 g l^{-1} of sodium chloride) in the base medium suppressed sulfide production in acetate-amended microcosms regardless of clay source or temperature (see Fig. S1 in Supplementary materials). No incremental sulfide production was noted in 50 g l^{-1} NaCl microcosms with lactate incubated for 120 days at 37 °C. Some sulfide accumulation was seen in 50 g l^{-1} NaCl microcosms amended with lactate incubated at 15 °C; however, sulfide production was delayed until after 90 days. As a result, approximately 1.5 mmol l^{-1}, 1.8 mmol l^{-1} and 0.9 mmol l^{-1} of soluble sulfide was detected in lactate-amended microcosms with MX-80, National Standard and Canaprill clays, respectively

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(Supplementary Fig. S1). Relative rates of sulfide production were (respectively) 0.09 d^{-1}, 0.064 d^{-1} and 0.047 d^{-1} for the above clays in lactate-amended microcosms (Table 1).

3.3. Comparative analysis of bacterial communities in clay microcosms

Comparative analysis of band distribution in DGGE fingerprints (see Fig. S2 in Supplementary materials) revealed a substantial difference in microbial abundance and diversity between the bentonites studied, and substrate- and temperature-dependent variation in the composition of microbial communities that developed from the same type of clay (Fig. S3 in Supplementary materials). Extrapolation of the DGGE-based diversity results from the different clay microcosms suggests that microorganisms of the genera *Desulfosporosinus, Desulfurispora, Desulfobulbus, Desulfuromonas, Bacillus, Pseudomonas* (and other genera capable of anaerobic respiratory metabolism) would populate anoxic, water-saturated uncompacted clays lacking fermentable organics. Whereas, fermenting clostridia will flourish in the presence of fermentable substrates. Description of DGGE patterns and statistical analysis of related data can be found in supplementary materials (See Figs. S2−S5, and Tables S2-S3; Supplementary materials).

Using high-throughput sequencing, 16S rRNA gene amplicons from relevant late-stage, lactate-amended clay microcosms incubated at 37 °C were sequenced. Approximately 25,000 paired-end 16S rDNA reads per sample were obtained, revealing *Firmicutes* (77%, 80% and 36.6% for MX-80, National Standard and Canaprill, respectively) and *Proteobacteria* (22.6%, 9.7% and 38.4% for MX-80, National Standard and Canaprill, respectively) as the most-abundant phyla. Bacteria of the phylum *Chloroflexi* made up 24% of detected sequences in microcosms containing Canaprill clay. Bacteria from the orders *Bacillales* and *Clostridiales* were the most abundant among representatives of the phylum *Firmicutes* in either microcosms with MX-80 (8.5% and 64%, respectively), National Standard (21.9% and 57.8%, respectively), and Canaprill (3.8% and 31.9%, respectively). High-throughput sequencing results revealed the predominance of the clostridia-affiliated sequences within bacterial 16S rRNA gene libraries in all clay microcosms examined (Fig. 4B). In particular, the clostridia in MX-80 microcosms were represented by the families *Clostridiaceae* (6.2%), *Peptococcaceae* (31.4%) and *Clostridiales* Family *XI Incertae Sedis*, "class *Tissierella*" (4.3%). The genus-level assessment of *Clostridia*-affiliated sequences in MX-80 microcosms included bacteria from the genus *Desulfosporosinus*, and constituted a substantial part of bacterial reads in MX-80 (30%), along with minor contributions of the genera *Alkaliphilus* (6.2%), *Tissierella* (4.3%), *Desulfitobacterium* (1%) and others (Fig. 4C). Bacilli of cluster 1 within the family *Bacillaceae*, most of which related to the genus

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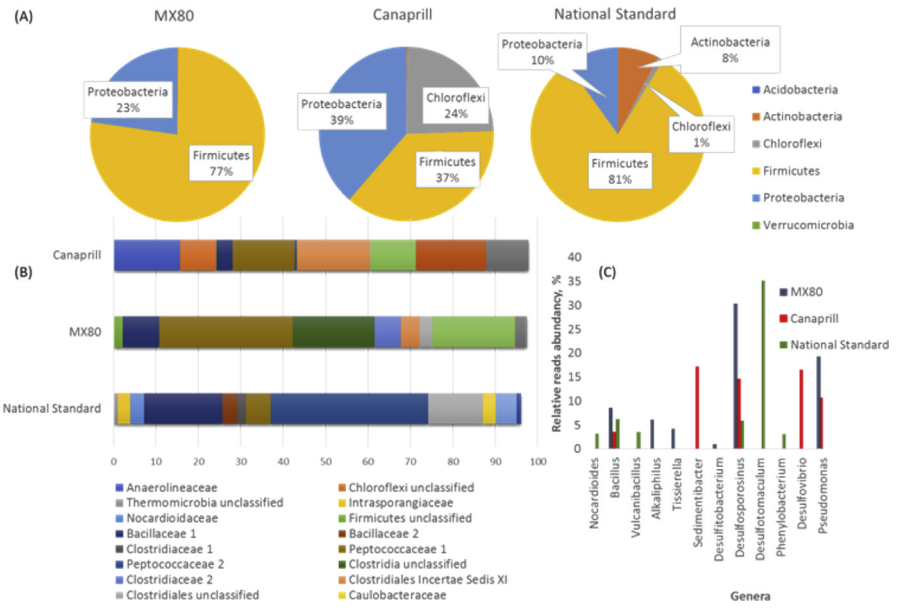

Fig. 4. Phylum- (A), family- (B) and genera (C) -level taxonomic diversity and relative abundancy of 16S rRNA gene fragments in the high-throughput library retrieved from clay microcosms.

Bacillus (8.6%), also contributed to the dominance of *Firmicutes*-affiliated sequences in the 16S rDNA library from MX-80 clay microcosms. Bacteria of the classes *Gammaproteobacteria* (order *Pseudomonadales*, family *Pseudomonadaceae*) and *Deltaproteobacteria* (order *Desulfuromonadales*) of the phylum *Proteobacteria* comprised a minor fraction (19.5% and 2.7%, respectively) of bacteria in MX-80 microcosms. As was the case for DGGE results, the majority of detected *Pseudomonadaceae* reads were affiliated with the genus *Pseudomonas*.

Similarly, clostridia of families *Peptococcaceae* cluster 1 (15.1%) and *Clostridiales* Family *XI Incertae Sedis*, "class *Tissierella*" (17.2%), and bacilli of family *Bacillaceae* (genus *Bacillus*, 3.5%) made up fractions of firmicutes within the 16S rDNA library from Canaprill microcosm. Noticeably, most of the *Peptococcaceae* from Canaprill microcosms were related to the genus *Desulfosporosinus* (14.7%) of *Peptococcaceae* cluster 1, with the remaining *Peptococcaceae* sequences belonging to the genus *Desulfatomaculum* (0.4%, data not shown) of cluster 2. Notably, *Clostridiales* Family *XI Incertae Sedis* reads were closely-related to the genus *Sedimentibacter*. *Desulfovibrionales* (family *Desulfovibrionaceae*, genus *Desulfovibrio* 16.5%) and *Desulfuromonadales* (unclassified, 10%) of *Deltaproteobacteria* and *Pseudomonadales* (family *Pseudomonadaceae*, genus *Pseudomonas* 10.7%) of the *Deltaproteobacteria* were the most abundant orders affiliated with the *Proteobacteria* among the sequences recovered from Canaprill microcosms. The family *Anaerolineaceae* (15.6%), along with a large fraction of unclassified *Chloroflexi* (8.4%), contributed to *Chloroflexi* being the third-most abundant phylum within the microbial community discovered in Canaprill microcosms.

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For National Standard microcosms, the most abundant taxa were again *Clostridiales* and *Bacillales* (Fig. 4). Consisting mostly of bacteria of clusters 1 and 2 of the family *Peptococcaceae* (5.9% and 37%), and cluster 1 of the family *Clostridiaceae* (2%), along with some unclassified *Clostridiales* (19%), the *Clostridiales* made up to 57.8% of rDNA reads in National Standard microcosms. Most of these Clostridia-affiliated reads were closely-related with the genera *Desulfotomaculum* (35%) and *Desulfosporosinus* (5.6%). Bacilli belonging to clusters 1 and 2 of the family *Bacillaceae* (18.3% and 3.6%, respectively) represented 21.9% of total reads, with bacteria of genera *Bacillus* (6.3%) and *Vulcanibacillus* (3.6%) being most significant among them. Minor amounts of 16S rDNA reads from the National Standard microcosms were associated with microorganisms of the phyla *Actinobacteria, Proteobacteria* and *Chloroflexi* (Fig. 4A). Furthermore, *Actinobacteria* of a National Standard sample were represented by microorganisms of the orders *Micrococcales* (family *Intrasporangiaceae*, 3%) and *Propionibacterineae* (family *Nocardioidaceae*, 3.3%).

4. Discussion

4.1. Environmental conditions in clays select for adapted bacteria

As-purchased commercial bentonites (*e.g.,* MX-80) are unfavourable environments for microbial colonization because of scarce nutrients, low water activity, and the presence of toxic elements (*e.g.,* Hg, Zr, Cu, Co, etc.). As outlined in Supplementary materials section 1, bentonites consist mainly of montmorillonite-type clays, along with some other minerals, which can supply potassium, sodium, calcium, magnesium, iron, aluminium, silica and other micronutrients for microbial growth. However, the availability of biogenic elements, *e.g.,* carbon, phosphorous and nitrogen, in clays is limiting. Because MX-80 and other marketed bentonites are commonly heat-dried prior to packaging, they contain 5-11 wt % moisture and their water activity is reduced to 0.4−0.55 (see Supplementary materials section 1), making them poorly-habitable since most prokaryotes are unable to proliferate below 0.9 a_w (Stevenson and Hallsworth, 2014).

Despite challenging conditions, clay products are not sterile, with numbers of culturable aerobes varying between $>10^2$ CFU gdw^{-1} and 10^3−10^4 CFU gdw^{-1}, along with fewer or no viable SRB (Haveman et al., 1995; Pedersen et al., 2000). Occurrence of viable microorganisms in bentonites has been also confirmed by detection of chemotaxonomic (*e.g.,* phospholipid fatty acids) and genetic (*e.g., rrs*, *apsA* genes) markers in corresponding clay samples (Stroes-Gascoyne et al., 2010; Persson et al., 2011). Our current study revealed the presence of 10^5 CFU gdw^{-1} and 10^3 CFU gdw^{-1} of aerobic and anaerobic organotrophic bacteria in as-received MX-80, respectively, with no more SRB than 10 cells gdw^{-1}. While recent

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values are marginally higher than previously reported for MX-80, our samples were obtained from commercial lots produced much more recently.

Under stress and starvation conditions like those in dry bentonites, microorganisms exhibit a number of physiological responses, including cell dewatering, mineralization, rebuilding of specific cell ultrastructures, down-sizing, accumulation of protective intracellular metabolites, reduction or alteration in physiological activity and entry into long-term dormancy (Mulyukin et al., 2001; Suzina et al., 2006). Formation of specialized endospores by bacilli and clostridia (Paredes-Sabja et al., 2011), cyanobacterial akinetes (Billi, 2012), and other dormant forms of Gram-positive and Gram-negative bacteria also enhances microbial stress tolerance (Soina et al., 2004; Mulyukin et al., 2008, 2009). Reliable reports exist of the resurrection of dormant cells, for instance clostridial and bacilli endospores, from various desiccated and heat-treated environmental samples (Kennedy and Swierczynski, 1994; O'Sullivan et al., 2015).

Consequently, the observed abundance of spore-forming bacilli and clostridia (*e.g., Bacillus* spp., *Desulfosporosinus* spp., *Desulfotomaculum* spp., *Alkaliphilus* spp. and *Sedimentibacter* spp.) in microcosms is compatible with expected selective conditions in examined clays (Supplementary Tables S2 and S4), and likely not due to methodological biases. Non-spore-forming bacteria of the genera *Pseudomonas* and *Desulfovibrio* have also been reported to form dormant cells capable of surviving starvation periods (Motamedi et al., 1996; Masurat et al., 2010; Hosono Honda et al., 2011). However, the detection of sequences of bacteria of the class *Anaerolineae* within the phylum *Cloroflexi* in Canaprill (24% of reads) is unexpected. In clayish environments, bacteria of the class *Anaerolineae* should possess distinct dormancy mechanisms to tolerate low water activity along with a scarcity of resources for fermentative metabolism. Even though sequences related to this taxon have been recently observed in clone libraries from a broad range of ecosystems, *e.g.* anaerobic digesters, bacterial mats and sub-sea sediments (Hanada, 2014; Parkes et al., 2014), it is worth validating the presence of *Anaerolineae*-like bacteria in clays using different approaches to minimize possible biases related to detection and classification of 16S rRNA amplicons (Tremblay et al., 2015).

4.2. Deriving ecophysiological features from bacterial community composition

Though the presence of lactate and sulfate in studied anoxic microcosms created differential conditions for enrichment of specific physiological groups of bacteria, yet the phylogenetic analysis of recovered 16S rRNA clone libraries can be extrapolated to give a potential picture of the functional repertoire of microorganims that proliferated in the different clay systems. While lactate would not be found in natural

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bentonites, addition of this easy-to-assimilate substrate to microcosms has the benefit of stimulating the entire bacterial consortium, including sulfide- and acid-producing bacteria that could cause deteriortation of materials in a DGR (McKelvie et al., 2016), making them more assessable for the purposes of the comprehensive microbiological investigation.

Analysis of our microcosms revealed that microorganisms capable of reducing or disproportionating sulfur oxyanions and elemental sulfur were significantly involved (Supplementary Table S4). Variable trends of sulfide production in the different clay microcosms probably resulted from different initial numbers of microorganisms involved in reduction of sulfate coupled with oxidation of either lactate or acetate along with the unique chemistries of the three different clays (*e.g.,* higher aluminium concentration in Wyoming (21%) vs Avonlea (14%) clays; see Supplementary Table S1). Notably, aluminium in clays has been reported to inhibit activity of SRB (Amonette et al., 2003; Wong et al., 2004) and so the higher aluminium content along lower initial counts of viable bacteria and spores in Wyoming clays (*e.g.,* MX-80, National Standard) could have had a more pronounced inhibitory effect on SRB activity in comparison with Avonlea clay (Canaprill).

Bacteria of the genera *Desulfosporosinus*, *Desulfotomaculum*, *Desulfovibrio* and *Desulfobulbus* (Supplementary Tables S2 and S4) were presumably primarily responsible for sulfide production in clay incubations. Representatives of these genera are known to couple sulfate reduction with incomplete oxidation of organic acids (in this case, lactate) to acetate (Supplementary Table S4) and *Desulfosporosinus, Desulfotomaculum,* and *Desulfovibrio* are capable of utilizing a wider-range of oxidized inorganic sulfur species (*e.g.* SO_3^{2-}, $S_2O_3^{2-}$, S) as alternative electron acceptors, with hydrogen or formate as electron donors. *Desulfotomaculum* spp. and *Desulfosporosinus* spp. can respire sulfate using butyrate and other fatty acids as electron donors, although in a species-dependent manner.

The incidental accumulation of sulfide in the acetate-amended MX-80 microcosms, in the absence of dominatant acetate-oxidizing SRB, probably resulted from the activity of undiscovered acetate-oxidizing sulfidogens. It also might indicate the occurrence of synthrophic H_2-driven sulfidogenesis coupled with acetate oxidation, resembling a recently-described acetate-utilizing, sulfate-reducing binary culture from soda lake sediments (Zhilina et al., 2005a, b). In the presence of bioavailable metal oxyanions, some microorganisms, such as *Desulfosporosinus lacus* and *Desulfosporosinus auripigmenti*, can switch from dissimilatory sulfate reduction to reduction of metal species (*e.g.,* Fe^{3+} and AsO_4^{3-}), providing an additional ecological advantage in water-saturated clay environments (Stackebrandt et al., 2003; Ramamoorthy et al., 2006). The prevalence of *Desulfosporosinus* spp. in microcosms with MX-80 bentonites has previously been reported (Fru and Athar, 2008; Persson et al., 2011).

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In fact, enrichment of bacteria with fermentative or metal-reducing metabolism along with SRB in microcosms containing readily-fermentable substrates (lactate) and bioreducible compounds (*e.g.* Fe^{3+}, Mn^{4+}, Cr^{4+}) other than sulfur oxyanions is highly-anticipated. Consequently, it is not surprising that a large majority of discovered taxa are known to sustain their growth by fermentation in the absence of external electron acceptors. Furthermore, bacteria of the genera *Sedimentibacter*, *Geosporobacter*, *Tissierella* and others (Supplementary Table S4) are unable to utilize external inorganic acceptors of electrons and grow only by fermentation. These bacteria often play a substantial role in the decomposition of complex organics into volatile organic acids and hydrogen, and can potentially provide organotrophic and hydrogenotrophic prokaryotes (*e.g.,* sulfate- or metal-reducers, and methanogens) with hydrogen and light fatty acids (*e.g.,* acetate). In addition, some of the detected clostridia with primarily-fermentative metabolisms can potentially utilize inorganic electron acceptors other than sulfate that may occur in clays. For instance, bacteria of family *Sedimentibacter* can reduce thiosulfate anaerobically while growing on arsenate, and bacteria of the genus *Alkaliphilus* can respire a wide range of substrates (*e.g.,* Fe^{3+}, Co^{3+}, Cr^{4+}, U^{6+}, Se^{6+}, Mn^{4+}), potentially broadening their ability to adapt to bentonite conditions. Similarly, some fermenting proteobacteria detected in clay microcosms, *e.g., Pelobacter* species, are able to use either elemental sulfur or Fe^{3+} as electron acceptor in redox reactions (Sun et al., 2010) in addition to disproportionating organic compounds (*e.g.,* into two different compounds, one of higher and one of lower oxidation states). Overall, considering the abundance of DNA sequences related to other taxa known to respire ferric ions, *e.g., Bacillus subterraneus* and *Desulfuromonas* spp. (Supplementary Table S4), we propose that the capability to reduce Fe^{+3} (and Mn^{+4}) is the third key physiological feature, after reduction of sulfur oxyanions and fermentation, allowing bacteria to sustain activity in anaerobic water-saturated uncompacted clay systems.

The last large group of sequence reads from clay microcosms of note was related to bacteria of the genera *Bacillus* and *Pseudomonas,* facultative anaerobes with versatile, and mostly organotrophic, competences and found in many different ecosystems. In general, these organisms would be active when residual oxygen is present in microcosms. Similar aerobic bacilli and pseudomonads have been identified elsewhere in studies of dense bentonite blocks (Fru and Athar, 2008; Jalique et al., 2016), indicating that these microorganisms may proliferate in DGR buffers to some extent under oxic conditions following repository closure.

Taken together, this study, along with recent discoveries of bacteria of the genera *Desulfosporosinus, Sedimentibacter, Bacillus, Paenibacillus, Pseudomonas* and *Tissierella* in highly-compacted MX-80 (Fru and Athar, 2008; Persson et al., 2011; Jalique et al., 2016), not only indicates that microorganisms of these taxa are ubiquitous in commercial Wyoming bentonite, but also suggests the potential for these prokaryotes to exist transiently during evolution of conditions in the DGR (*e.g.,* when

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sufficient pore space, water, energy and carbon sources are available). The majority of discovered bacteria shared a similar metabolic capacity to obtain energy primarily via fermentation, rather than respiration. However, in the presence of reducible inorganic ions, some of these microorganisms would readily switch from fermentation to anaerobic respiration, *e.g.* sulfate- and iron reduction, as this is thermodynamically-favourable. Detected taxa mainly consisted of mesophilic bacteria potentially able to propagate in brackish and saline waters (<30 g l^{-1}) at circumenutral pH (see Supplementary Table S4). Only certain non-SRB belonging to the genera *Alkaliphilus*, *Bacillus* and *Pseudomonas* were detected, which are not known to pose a risk for EBS elements and can tolerate higher concentrations of salt (50 g l^{-1} NaCl and above) and elevated pH (>10). In oligotrophic habitats such as clay-based buffers lacking fermentable substrates, fermentative metabolism would probably be limited to the syntrophic exchange of hydrogen or acetate that may further drive reductive reactions. Subsequently, chemolithotrophic prokaryotes capable of oxidizing hydrogen and fixing mineral carbon in anabolic reactions would become critical as primary producers of organic matter, similar to processes occurring in other dark, low-energy and low-carbon environments, *e.g.* deep marine and terrestrial ecosystems (Hoehler, 2005). Remarkably, autotrophic growth (*e.g.,* using hydrogen for dissimilatory reduction of sulfate) is a distinguishing characteristic of the genus *Desulfosporosinus* (Stackebrandt et al., 1997), whose representatives were found in this study to prevail among DGGE and high-throughput 16S rRNA gene libraries. In addition, clostridia of the genera *Ruminococcus* and *Morella* detected in this study are also known for their ability to grow autotrophically as acetogens using hydrogen and carbon dioxide, acting as primary producers of organic carbon (Drake and Küsel, 2003). It is noteworthy that the bentonite microcosm model utilized in this study was not intended to mimic the bulk conditions prevailing in the EBS of a DGR, where high salinity/low water activity, low nutrient availability, restricted pore space and high clay swelling pressure would all increasingly constrain microbial activity over time (Stroes-Gascoyne et al., 2006, 2010). That said, the microcosms facilitated the discovery of a diversity of indigenous bacteria present in as-received clays and helped to evaluate their potential behavior within different ecological or technical scenarios (*e.g.*, within low clay-swelling pressure, gel-like interfaces that may temporarily exist between bentonite blocks and host rocks containing groundwater or technical fluids). From an EBS perspective, potential energy sources for microbial consortia in clay buffers would primarily consist of radiolytic dihydrogen gas and organic contaminants introduced along with technical fluids during DGR construction and maintenance (McKelvie et al., 2016), as well as anaerobic container corrosion (which produces hydrogen) after conditions in a DGR evolve to become anoxic. Yet, activity of such an adapted, multipotent indigenous bacterial population, even in hospitable water-saturated clay microcosms, would be anticipated to remain largely-constrained. Moreover, we demonstrated that the addition of 50 g l^{-1} (0.86 M) NaCl to 10% clay microcosms substantially-reduced bacterial sulfate-reduction

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even in lactate-driven microcosms compared with microcosms containing 10 g l^{-1} NaCl, completely inhibiting sulfate reduction during incubation with acetate.

Declarations

Author contribution statement

Alexander A. Grigoryan: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Daphne R. Jalique, Prabhakara Medihala: Performed the experiments.

Simcha Stroes-Gascoyne: Conceived and designed the experiments; Analyzed and interpreted the data; Wrote the paper.

Gideon M. Wolfaardt, Jennifer McKelvie, Darren R. Korber: Conceived and designed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

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Competing interest statement

The authors declare no conflict of interest.

Additional information

Illumina sequence data associated with this study has been deposited at the NCBI Sequence Read Archive under the accession numbers SRR6342323, SRR6337247 and SRR6337245. Sanger sequencing data for DGGE clones reported in this study have been submitted to GenBank under the accession numbers MG650197−MG650243 and MG650245−MG650258.

Supplementary content related to this article has been published online at https://doi.org/10.1016/j.heliyon.2018.e00722.

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