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Deciphering indigenous bacteria in compacted bentonite through a novel and efficient DNA extraction method: Insights into biogeochemical processes within the Deep Geological Disposal of nuclear waste concept

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ABSTRACT

Compacted bentonites are one of the best sealing and backfilling clays considered for use in Deep Geological Repositories of radioactive wastes. However, an in-depth understanding of their behavior after placement in the repository is required, including if the activity of indigenous microorganisms affects safety conditions. Here we provide an optimized phenol:chloroform based protocol that facilitates higher DNA-yields when other methods failed. To demonstrate the efficiency of this method, DNA was extracted from acetate-treated bentonites compacted at 1.5 and 1.7 g/cm^3 densities after 24 months anoxic incubation. Among the 16S rRNA gene sequences identified, those most similar to taxa mediating biogeochemical sulfur cycling included sulfur oxidizing (e.g., *Thiobacillus,* and *Sulfurimonas*) and sulfate reducing (e.g., *Desulfuromonas* and *Desulfosporosinus*) bacteria. In addition, iron-cycling populations included iron oxidizing (e.g., *Thiobacillus* and *Rhodobacter*) plus reducing taxa (e.g., *Geobacillus*). Genera described for their capacity to utilize acetate as a carbon source were also detected such as *Delftia* and *Stenotrophomonas*. Lastly, microscopic analyses revealed pores and cracks that could host nanobacteria or spores. This study highlights the potential role of microbial driven biogeochemical processes in compacted bentonites and the effect of high compaction on microbial diversity in Deep Geological Repositories.

1. Introduction

Many countries including Spain, Finland, Canada, Sweden, and the United States are investigating Deep Geological Repositories (DGRs) for the disposal of high level radioactive wastes (Alexander and McKinley, 2011). In these repositories, the waste will be placed in corrosion-resistant metal canisters surrounded by a compacted bentonite buffer to create an engineered system at approximately 400–500 m underground (Briggs et al., 2017; Pedersen, 1999). Bentonites are swelling clays with a high sorption capacity that results in a very low hydraulic conductivity (Perdrial et al., 2009), making them an ideal backfilling and sealing material in the engineered barrier system (Lopez-Fernandez et al., 2015). As the main functions of the bentonites are to provide mechanical protection to the waste canisters and to reduce the migration of radionuclides to the biosphere, it is of vital importance that these bentonites remain chemically stable (Perdrial et al., 2009).

Bentonites will be utilized as compacted blocks of specific dimensions and dry densities, depending on the national concept, as their low permeability and high swelling capacity will create very harsh conditions for the activity and subsistence of autochthonous and allochthonous microorganisms. These conditions include a very low water activity, swelling pressures in the order of 7–8 MPa at full water saturation, and an average pore diameter of 0.02 μm (Ratto and

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Itavaara, 2012; Stroes-Gascoyne et al., 2011). Despite this challenging environment for microbial growth, an evaluation of microbial activity and diversity is essential for safety assessments of the DGR concept. For example, some microorganisms are expected to survive and maintain their metabolic activity in less compressed areas such as interphases and fractures between the canisters and bentonite blocks (Pedersen et al., 2000a). In the last years, several studies have assessed the behavior of allochthonous bacteria in highly compacted bentonite or culturing of microbes from the bentonite (Jalique et al., 2016; Masurat et al., 2010; Pedersen et al., 2017, 2000a, 2000b; Stroes-Gascoyne et al., 2011). For instance, Masurat et al. (2010) demonstrated bacterial survival by both indigenous and colonizing bacteria from groundwater in compacted bentonite, although the cell numbers of sulfate-reducing bacteria (SRB) decrease with the increasing bentonite density. Jalique et al. (2016) isolated Gram-positive spore-forming bacteria from compacted bentonite and concluded that spore formation could facilitate survival in such a harsh environment. However, several studies reported difficulties in extracting genomic DNA from bentonites due to its swelling upon addition of lysis buffer, hindering the complete release of microbial cells from the bentonite matrix (Perdrial et al., 2009; Stone et al., 2016). In addition, the inherent ion exchange properties of bentonites potentially interfere with nucleic acid extraction by forming charge-based complexes (Stone et al., 2016). This imposes several challenges to standard DNA extraction techniques from bentonites and especially from highly compacted samples. For example, DNA extraction from compacted MX-80 bentonite using the PowerMax DNA isolation kit by Engel et al. (2019) resulted in maximum DNA yields of only 0.15 ng/μL. Therefore, the development and standardization of a DNA extraction protocol for highly compacted bentonites is required to produce high-quality community DNA that is free from inhibitors and able to be amplified by PCR.

SRB and iron-reducing bacteria (IRB) are extensively studied in relation to their impact on DGR performance. For instance, SRB may induce the corrosion of metal canisters housing the nuclear waste by coupling oxidation of lactate or acetate to sulfide generation under anoxic conditions (Bengtsson and Pedersen, 2017; Grigoryan et al., 2018; Necib et al., 2017; Pedersen, 2010; Pedersen et al., 2000a) while IRB may mediate lactate or acetate oxidation coupled to structural Fe (III) bentonite transformations that result in a decrease in surface area, swelling, interlayer spacing, and hydraulic conductivity (Dong, 2012; Haynes et al., 2018; Rickard, 2012). The structure and composition of the bacterial community in Spanish bentonite from El Cortijo de Archidona (Almeria) have been previously characterized using high-throughput sequencing technologies (Lopez-Fernandez et al., 2015, 2018b; Povedano-Priego et al., 2019). These next generation techniques have paved the way for detailed studies of bacterial community shifts due to chemical, physical, and biological influences. They have also been successfully employed to study complex natural communities in bentonite porewater and in the deep biosphere where DGRs will be situated (Jroundi et al., 2020; Lopez-Fernandez et al., 2018a; Smart et al., 2017). However, the microbial diversity in highly compacted bentonite under DGR relevant conditions has remained largely overlooked and yet might highlight issues for DGR safety.

In the present study, an optimized phenol-chloroform based DNA extraction method was used prior to 16S rRNA gene sequencing of the microbial community in compacted Spanish bentonite at different dry densities to determine the effect of acetate amendment. This electron donor's amendment may be prone to stimulate the growth of detrimental bacteria, compromising the safety of DGR. To our knowledge, this is the first study of the impact of highly compacted bentonite (1.5 and 1.7 g/cm^3) and acetate amendment on the native microbial populations. These findings will help to establish an efficient DNA extraction protocol for compacted bentonites and aid in developing appropriate DGR based waste treatment and long-term management strategies.

2. Materials and methods

2.1. Bentonite compaction and anaerobic incubation

2.1.1. Bentonite collection and pre-treatment

Bentonites were collected from "El Cortijo de Archidona" (Almeria, Spain). The collection method and mineralogical characteristics of these bentonites are described in Povedano-Priego et al. (2019). In the laboratory, bentonites were placed in a laminar flow cabinet to air dry for 72 h that facilitated grinding of the samples ($\leq$ 2 mm particle size). Afterwards, the ground bentonite was treated with sterile sodium acetate (30 mM) as electron donor to stimulate microbial growth of e.g. IRB and SRB. A control treatment consisting of the addition of sterile distilled water was also conducted. The treatments were carried out by spraying the sodium acetate and distilled water on bentonites under aseptic conditions.

2.1.2. Compaction process and incubation

The hygroscopic water content of 12% in the bentonite was calculated after desiccation for 48 h at 100 °C. The amount of bentonite needed to obtain the different densities was calculated considering the wet mass and the water content. The bentonite compaction process was carried out under aseptic conditions by placing the clay in a cylindrical-steel mold with a diameter of 30.3 × 12.3 mm, the mold was tightly closed, and a mean pressure of 8 and 28 MPa was applied with a hydraulic press to obtain 8.81 cm^3 blocks of bentonite with densities of 1.5 and 1.7 g/cm^3, respectively (Fig. 1). To establish anoxic conditions, the compacted-bentonite specimens were placed in an anaerobic jar with anaerobiosis generator sachets (AnaeroGen™, Thermo Scientific) and incubated for 6 and 24 months at room temperature. Silica gel was placed inside the jar to avoid moisture generated by the anaerobiosis incubation resulting in swelling of the bentonite. At the end of the incubation time the samples were stored at − 20 °C until analyzed.

2.2. Characterization of the compacted bentonite bacterial diversity

2.2.1. Optimized DNA extraction protocol from compacted bentonite

After the incubation period, the DNA extraction protocol begins with breaking the compacted bentonite into small fragments to create a homogeneous powder (Fig. 2). Then, a sterilized glass bead (3 mm diameter) and 0.25 g sterilized glass beads (0.3 mm diameter) were added to a sterile 2 mL screw-cap tube (SampleBead tube) containing 0.3 g of ground bentonite (Fig. 3) and 400 μL of Na_2HPO_4 (120 mM pH 8.0) was added. DNA strongly interacts with the bentonite clay particles that is desorbed by the Na_2HPO_4 and thus, improves the yield of extracted DNA. Afterwards, chemical lysis was achieved by adding 600 μL of lysis buffer (100 mM Tris-HCl [pH 8.0]; 100 mM EDTA [pH 8.0]; 100 mM NaCl; 1% polyvinylpyrrolidone [PVP]; and 2% SDS), 24 μL freshly thawed lysozyme (10 mg/mL), and 2 μL proteinase K (20 mg/mL). Lysozyme participates in bacterial cell wall breakdown and proteinase K contributes to the degradation of proteins and nucleases that could degrade the DNA. Mechanical lysis of the cells was performed twice using a FastPrep® FP120 (MP Biomedicals) at 5.5 m s^{-1} for 45 s. Afterwards, samples were incubated at 37 °C for 30 min and followed by 60 °C for 45 min. After centrifugation at 14,000*g* for 5 min, supernatants were collected at room temperature and transferred to MaXtract High Density Tubes (Qiagen). An additional FastPrep step was performed to ensure the collection of all DNA trapped in the bentonite by adding 1 mL of lysis buffer in the SampleBead tube. Supernatants from the second centrifugation were mixed with those obtained at the first FastPrep cycle (Fig. 3). One volume of phenol:chloroform:isoamyl alcohol (PCI, 25:24:1, pH 8.0) was added to the MaXtract High Density Tubes, mixed by gently inverting, and centrifuged for 10 min at 1500*g* at 4 °C (Fig. 4). Then, the aqueous phase was washed by adding an equal volume of (1) phenol:chloroform (PC, 1:1, pH 8.0) and (2) chloroform in the same tubes and centrifuged as before between each solution. The

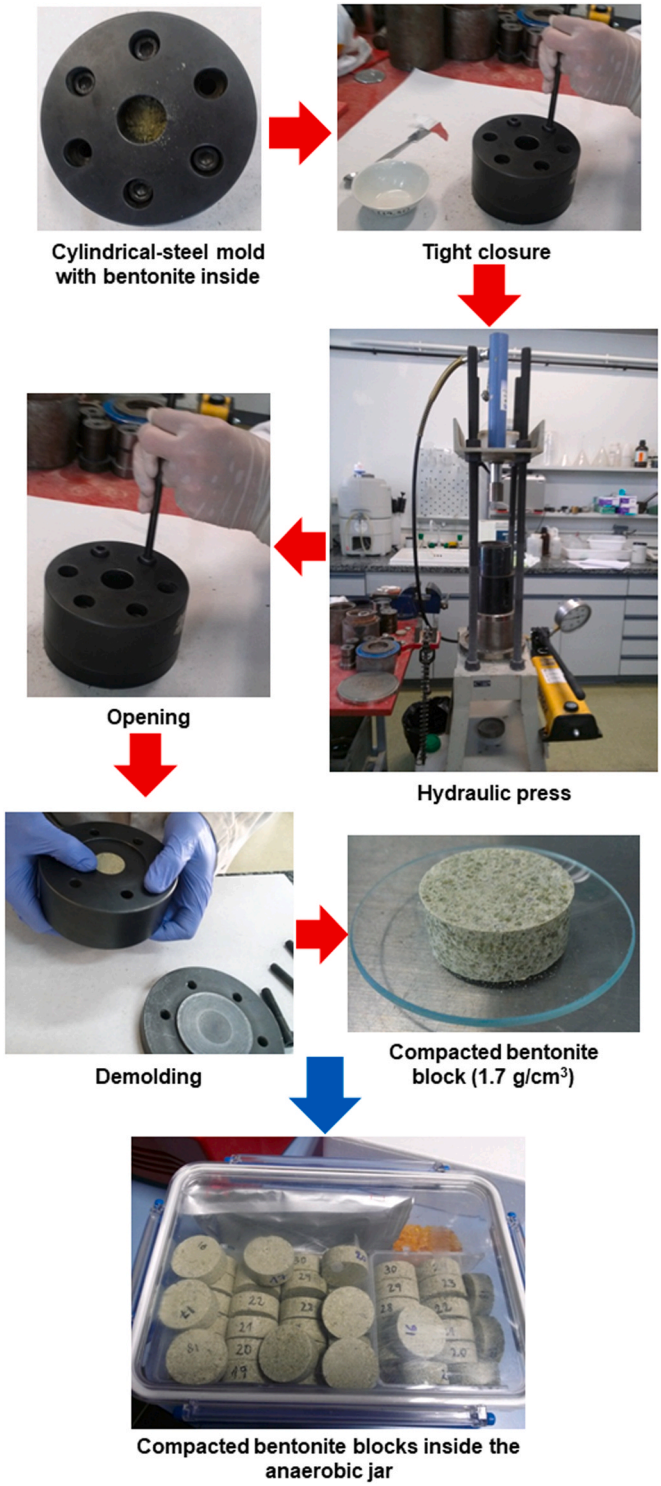

Fig. 1. Workflow of the compaction process, resulting in a compacted block of 1.7 g/cm^3 density.

supernatants were transferred to new 15 mL tubes and DNA was precipitated by adding one volume of ice-cold isopropanol and 0.1 vol of 3 M sodium acetate (pH 5.2) prior to incubation for 1 h at − 80 °C. The genomic DNA was pelleted at 5000*g* for 30 min at 4 °C and the resulting pellets were washed with 1 mL 80% cold ethanol followed by centrifugation at 10,000*g* for 5 min at 4 °C. The ethanol was carefully aspirated to avoid disturbing the pellet and then dried at 37 °C until all ethanol was evaporated. Total DNA was resuspended in 35 μL nuclease-free water. Pre-warming of the solution at ~60 °C is recommended to facilitate resuspension of the dried DNA pellet. A detailed protocol description is provided in Supplementary material S1.

The extracted genomic DNA was quantified on a Qubit 3.0 Fluorometer (Life Technology) and stored at − 20 °C until further processing. DNA concentrations were measured in biological triplicates of compacted bentonite samples after 6 and 24 months of incubation. Triplicate extractions from each sample were performed to examine possible variations on the results.

2.2.2. *Library preparation and sequencing*

The extracted DNA was further concentrated and purified using the Genomic DNA Clean & Concentrator™ kit (Zymo research). Two consecutive PCR reactions were performed for each sample with the use of normal and bar code fusion primers for the library preparation. The primers used for amplification of the 16S rRNA gene V4 hypervariable region were 530F (Dowd et al., 2008) and 802R (Claesson et al., 2010). Moreover, the size of the amplicon was kept below 400 bp to cover the maximum microbial diversity (Němeček et al., 2017). The first PCR conditions were as follows: one cycle at 95 °C for 3 min; 15 cycles at 98 °C for 20 s, 50 °C for 15 s, and 72 °C for 45 s; followed by a final extension step at 72 °C for 1 min. The same conditions were used for the second PCR with the barcoded fusion primer with 38 cycles. Gel-electrophoresis was used to check the quality of the library product. Additionally, the PCR products were purified using the Agencourt Ampure XP system (Beckman Coulter, Brea, CA, USA). The barcode-tagged amplicons from different samples were mixed in equimolar concentrations. Templating and enrichment for sequencing was performed using the One-Touch 2 and One-Touch ES systems (Life Technologies, USA). Sequencing of the amplicons was performed on the Ion Torrent Personal Genome Machine (PGM) using the Ion PGM Hi-Q Sequencing Kit with the Ion 314 Chip v2 (Thermo Fisher Scientific) following the manufacturer's instructions.

2.2.3. *Bioinformatics and bacterial diversity analyses*

Raw reads were split into samples using Mothur software (Schloss et al., 2009) and subsequently processed by the DADA2 software package (Callahan et al., 2016). Low quality and short reads were removed along with chimeric sequences (Edgar, 2013). Sequences were classified using the SILVA database (version 132, www.arb-silva.de) by the DADA2 package. The accuracy of classification was verified and evaluated against an artificial mock community sample containing four bacterial strains (*Klebsiella pneumonia*, *Bacillus subtilis*, *Staphylococcus aureus* and *Enterococcus faecalis*) and one yeast (*Saccharomyces cerevisiae*). Cluster analysis was performed using the 'vegan' package in the R statistical software (Oksanen et al., 2019).

Clustered and annotated OTUs were analyzed using Explicet 2.10.5 (Robertson et al., 2013) for relative abundance and alpha diversity indices. Taxa with $\geq$ 1% relative abundance were visualized as a heatmap made using the 'heatmap.2' function in the R 'gplots' v.3.0.1.1 and RColorBrewer packages (Warnes et al., 2019). The data comprising the relative abundances at the genus taxonomic level were used to construct sample-similarity matrices by the Bray–Curtis algorithm, where samples were ordinated by Principal Coordinate Analysis (PCoA) using Past3 (Hammer et al., 2001).

Raw sequences for this study were deposited in the NCBI sequence read archive (SRA) under the accession number PRJNA645295.

2.3. *Chemical, mineralogical, and microscopic characterization of the compacted bentonite*

The pH of compacted bentonite samples was measured in triplicate as described in Povedano-Priego et al. (2019). Before measuring, compacted specimens were ground and introduced into 50 mL tubes containing the corresponding volume of 0.01 M $CaCl_2$ to establish 1:15 bentonite:solvent ratio. After 24 months of incubation, the profile of the compacted bentonite samples was analyzed using Variable Pressure

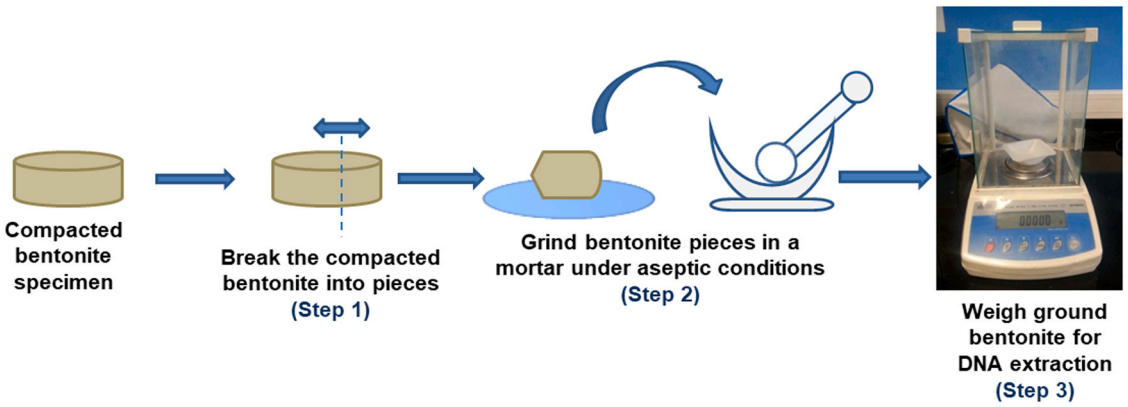

Fig. 2. Sample pre-treatment workflow (Steps 1–3).

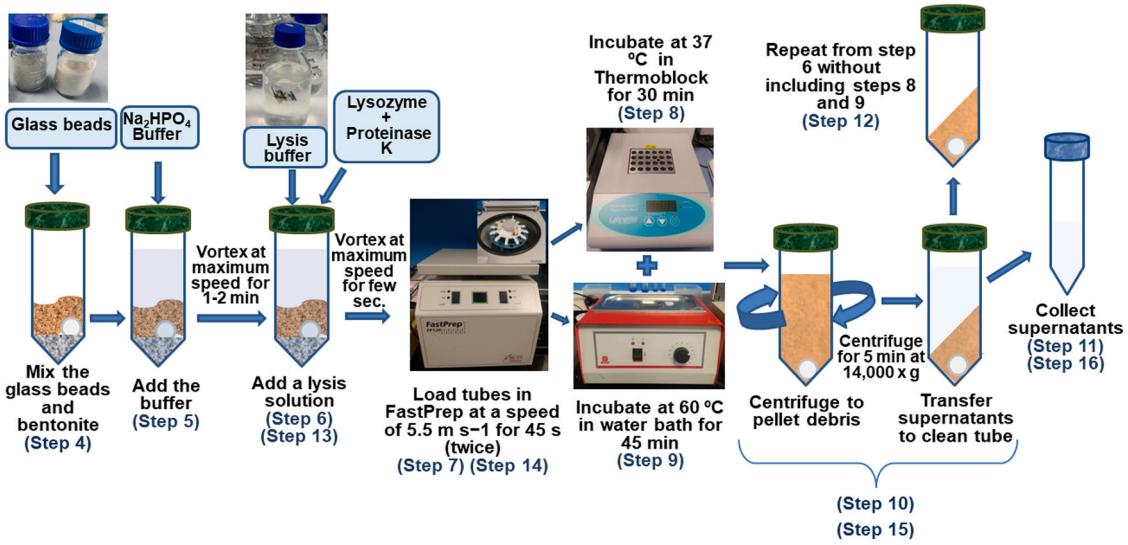

Fig. 3. Mechanical and chemical lysis workflow (Steps 4–16).

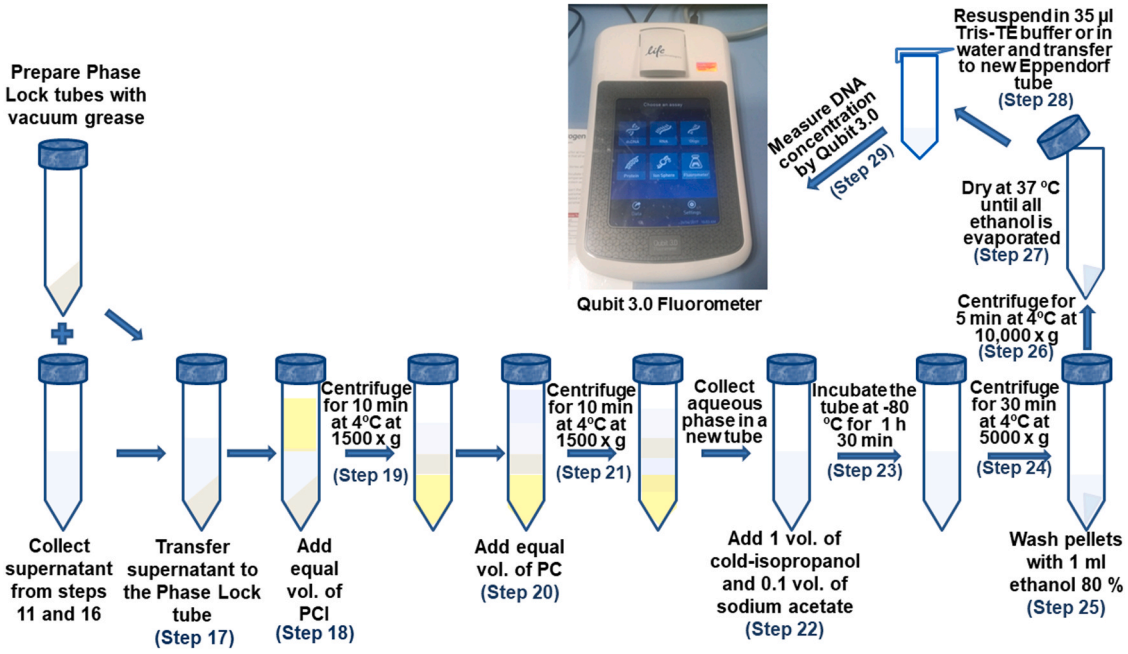

Fig. 4. DNA extraction, purification, and quantification workflow (Steps 17–29).

Field Emission Scanning Electron Microscopy (VP-FESEM) ZeissSupra 40VP equipped with SE (InLens) and BSE detectors to provide morphological and chemical characterizations. For this purpose, the compacted bentonite specimen was broken and the pieces were mounted on aluminum stubs using carbon adhesive tape.

3. Results

3.1. Geochemical, mineralogical and microscopic characterization of the compacted bentonites

Spanish bentonite treated with sodium acetate (A) and distilled water (C) was compacted at two different dry densities (1.5 and 1.7 g/cm^3) and incubated under anoxic conditions for 24 months (Table 1). Geochemical analyses of the bentonite samples prior to incubation showed dominant oxides such as SiO_2 (61.85 ± 3.59%), Al_2O_3 (15.41 ± 1.41%), and F_2O_3 (3.39 ± 0.21%) as described in Povedano-Priego et al. (2019). The pH of the compacted bentonites ranged between 7.90 and 8.06 (Table 1) with no relevant pH differences between the compacted bentonites samples.

VP-FESEM analyses of the cross-section profile of the compacted bentonites after 24 months of incubation showed several cracks, and small fissures in the bentonite aggregates in a low magnification scanning area of approximately 1 cm^2 (Fig. 5A and B). At high magnification, the typical leaf-like morphology of smectites in addition to pores and cracks were observed (Fig. 5C–F). However, bacterial cells could not be identified with this technique.

3.2. Optimization of DNA extraction protocol from compacted bentonite

Traditional phenol:chloroform based DNA extraction from compacted bentonites after 6 months of incubation yielded up to 0.6 ng/μL (Fig. 6), an insufficient quantity for PCR amplification. Therefore, optimization of the method was implemented including (1) the use of sodium phosphate buffer; (2) a strong mechanical lysis step using two cycles of FastPrep combined with enzymatic lysis with lysozyme and proteinase K; and (3) an additional DNA washing step using chloroform and centrifugation to avoid phenol contamination that will inhibit PCR amplification. The second cycle of mechanical lysis was necessary to ensure the collection of all DNA liberated from the cells that may have been trapped on the bentonite particles. Na_2HPO_4 buffer was employed for DNA desorption from the bentonite particles, which is affected by monovalent cations such as Na^+ and its use is commonly required for effective DNA extraction from soil (Lombard et al., 2011). Other minor changes in the protocol that contributed to the extraction efficiency included: (1) the use of 0.3 and 3 mm diameter glass beads to aid mixing of the solution and to disrupt the cells via mechanical lysis and (2) incubation at − 80 °C instead of − 20 °C in the DNA precipitation step. The detailed protocol is described in Figs. 2–4 and Supplementary material S1.

Using this protocol, up to eight-fold higher DNA concentrations (between 1.7 and 3.4 ng/μL) were obtained from the compacted bentonites after 6 months of incubation (Fig. 6) and similar results were obtained after the 24 month incubation (data not shown). The 24-month incubation samples were used for diversity analyses as typically slow growth rates in oligotrophic environments require long incubation times for community differences to become apparent.

Table 1
Experimental conditions of the analyzed bentonite samples and pH values of the compacted bentonite before and after 24 months of anoxic incubation measured in a calcium chloride suspension (0.01 M). C: control bentonite; 1.5A: acetate-treated bentonite at 1.5 g/cm^3 density, 1.5C: control bentonite at 1.5 g/cm^3 density, 1.7A: acetate-treated bentonite at 1.7 g/cm^3 density, and 1.7C: control bentonite at 1.7 g/cm^3 density.

Sample ID	Replicates	Compaction (g/cm^3)	Carbon source (Acetate, mM)	pH values
1.5A	3	1.5	30	8.06 ± 0.05
1.5C	3	1.5	0	8.03 ± 0.03
1.7A	3	1.7	30	7.90 ± 0.05
1.7C	3	1.7	0	8.02 ± 0.05

3.3. Bacterial diversity in compacted bentonite under anoxic conditions

Total DNA was extracted and sequenced from both the untreated (control) and the acetate-treated compacted bentonite after 24-month of incubation. Rarefaction curves (Supplementary Fig. S1) and Good's coverage index (Table 2) indicated a sufficient sequencing depth was achieved. A total of 303,053 bacterial reads were annotated giving 1383 OTUs classified into phylum (99% of phylotypes), class (99%), order (91%), family (85%), and genus (80%) taxonomic levels. Richness, evenness, and diversity of all the samples are shown in Table 2.

Bacterial diversity analyses indicated no significant differences (ANOVA test, $\alpha = 0.25$) between untreated and acetate-treated bentonites at both phylum and genus levels, or between the 1.5 g/cm^3 and 1.7 g/cm^3 compaction densities (Supplementary Tables S1 and S2). In addition, the PCoA analysis at the genus level suggested similar bacterial communities in the bentonite samples (Fig. 7). However, a heatmap plot showed the microbial communities in the 1.5A_1 and 1.7A_1 acetate-treated samples were most dissimilar to the other communities (Fig. 8). This was likely due to an increased relative abundance of 16S rRNA gene sequences aligning within the genera *Geobacillus*, *Thermicanus*, *Bacillus*, *Stenotrophomonas*, and *Delftia* that typically utilize acetate as a carbon source (Gößner et al., 1999; Jangir et al., 2016; Sajjad et al., 2016; Sánchez-Castro et al., 2017).

After 24 months of anaerobic incubation, both compacted bentonite samples were dominated by Actinobacteria (58% and 52% relative abundance) and Proteobacteria (both ~23%; Fig. 9 and Supplementary Table S3) followed by Firmicutes, Chloroflexi, Bacteroidetes, and Cyanobacteria. Interestingly, Firmicutes showed differences between acetate-treated and untreated samples, accounting for 5.8% and 9.3% in 1.5A and 1.7A, respectively, and 0.7% and 2.8% in 1.5C and 1.7C (Fig. 9 and Supplementary Table S3).

No significant differences were found between the different treatments at the genus level (ANOVA test, $\alpha = 0.25$; Supplementary Table S2). The bacterial communities were mainly composed of *Pseudarthrobacter* with 25.0–33.6% relative abundance while *Arthrobacter* represented 6% of the total community (Fig. 10 and Supplementary Table S4). *Pseudarthrobacter* and *Arthrobacter* are both within the *Micrococcaceae* family and survive in oxygen limitation conditions by fermenting organic molecules such as organic acids, sugar, amino acids, among others (Busse and Wieser, 2014; Eschbach et al., 2003). The second most abundant genus was *Nocardiodes* (7% relative abundance) that is a nitrate-reducing Fe(II)-oxidizer (Benzine et al., 2013; Nordhoff et al., 2017) followed by *Marmoricola* (3.2%), *Geobacillus* (2.7%), *Mesorhizobium* (2.7%), *Ralstonia* (2.1%), *Promicromonospora* (1.9%), and *Delftia* (1%) (Fig. 10, Supplementary Table S4). Several genera involved in biogeochemical sulfur cycling were detected after 24 months including sulfur oxidizing bacteria (SOB) (e.g., *Delftia*, *Paracoccus*, *Mesorhizobium,* and *Sulfurifustis*) and SRB (e.g., *Pseudomonas, Desulfuromonas, Desulfovibrio,* and *Desulfosporosinus*). In addition, OTUs aligning with IRB included *Geobacillus*, *Stenotrophomonas, Thermicanus,* and *Ralstonia*. Finally, *Geobacillus* (8.8% and 9.9%) and *Thermicanus* (1.9% and 2.7%) were enriched in the 1.5A_1 and 1.7A_1 acetate treated samples compared to the control samples that had relative abundances of 0.0–5.1% and 0.0–0.25%, respectively (Supplementary Table S4). Therefore, acetate affected the bacterial diversities by enhancing the presence of several iron-reducing bacteria including those capable of using acetate as a carbon and energy source.

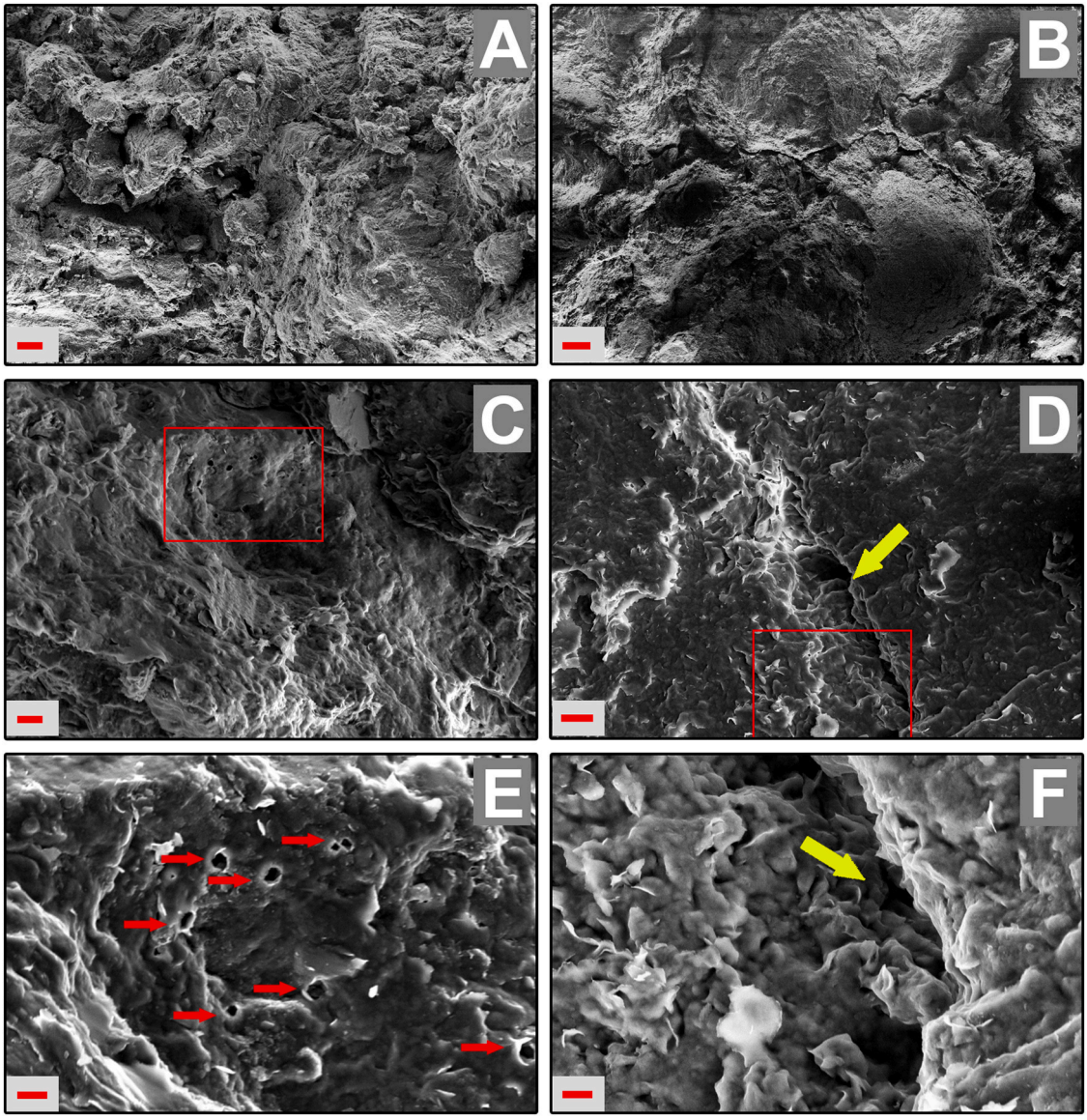

Fig. 5. VP-FESEM images of compacted bentonite at 1.5 g/cm^3 density (A, C, and E) and 1.7 g/cm^3 density (B, D, and F). Images are shown in secondary electrons with the InLens detector of the bentonites (D, E, and F) and in secondary-backscattered electron mixing mode (A, B, C). Arrows indicate pores (red) and cracks (yellow) in D, E, and F. Scale bar represents 10 μm in A and B, 1 μm in C and D, and 200 nm in E and F. Red squares in C and D were amplified in E and F, respectively. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

4. Discussion

In the DGR system, the compacted bentonites will contribute to very harsh conditions for the activity and survival of both indigenous and allochthonous microorganisms (Lopez-Fernandez et al., 2018d). However, the bentonite buffer may only repress bacterial activity rather than killing them. In addition, because of the barrier design and installation procedures, gaps may develop between the bentonite and canisters, at bentonite-rock interfaces, and between the bentonite blocks. These voids could be filled with groundwater from rock fractures promoting the growth of microorganisms (Pedersen et al., 2000a) that have the potential to adversely affect waste container corrosion rates and properties of the bentonite itself (Haynes et al., 2018). In a study using mercury intrusion porosimetry (MIP), FEBEX bentonite samples compacted to different dry densities with hygroscopic water content showed that the proportion of micropores (< 2 nm) and mesopores (< 0.006 μm) increased as the dry density was raised while macropores (< 600 μm) decreased (Villar et al., 2012). In the present study, compacted bentonites specimens at both dry densities developed pores (Fig. 5) that may host small-sized bacteria and allow a microbial activity (Pedersen et al., 2000a). Under energy limitation and physicochemical stress conditions, microorganisms may considerably decrease their size and morphology to increase their survival (Chien et al., 2012; Ghuneim et al., 2018). These include *Sphingomonas alaskensis* (Ghuneim et al., 2018) and *Pseudomonas syringae* (Monier and Lindow, 2003) that were similar to sequenced 16S rRNA gene sequences in the compacted bentonite before and after 24 months of anoxic incubation. In addition, nano-sized active microbes that pass through a 0.2 μm filter are present in the deep biosphere (Lopez-Fernandez et al., 2018c; Wu et al., 2016) that highlights the need for studying compacted bentonites in relation to the DGR concept.

DNA extraction from bentonite is the first and most crucial step to explore the microbiology of such an extreme environment. However, it is rarely possible to efficiently extract genomic DNA from compacted samples due to bentonite swelling and DNA absorption that protects against chemical or enzymatic degradation (Perdrial et al., 2009). It is

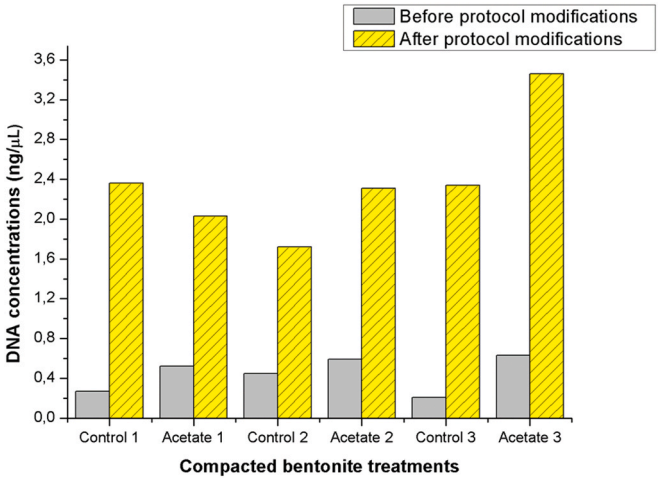

Fig. 6. DNA concentrations (ng/µL) of each control and acetate-treated compacted bentonite at 1.5 g/cm^3 after 6 months of incubation.

Table 2
Alpha-diversity indices of compacted bentonite samples after 24 months of incubation (1.5C: control bentonite at 1.5 g/cm^3 density, 1.5A: acetate-treated bentonite at 1.5 g/cm^3 density, 1.7C: control bentonite at 1.7 g/cm^3 density, and 1.7A: acetate-treated bentonite at 1.7 g/cm^3 density). Richness index (S), diversity indices (ShannonD, and SimpsonD), evenness index (ShannonE), and Good's coverage values are shown.

	S	ShannonD	ShannonE	SimpsonD	Good's coverage
1.5A[1]	361	5.11	0.60	0.87	0.999
1.5C[1]	323	5.13	0.62	0.89	0.999
1.7A[1]	283	5.16	0.63	0.90	0.999
1.7C[1]	306	5.36	0.65	0.92	0.999

[a] Cut-off size: 53,926.

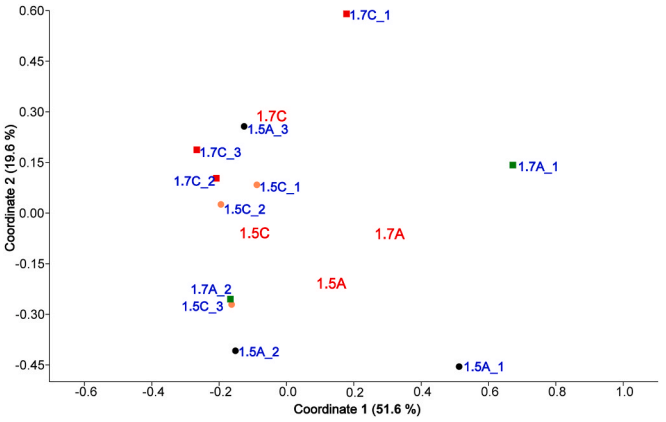

Fig. 7. Principal Coordinate Analysis (PCoA) plot comparing the bacterial community structure at genus level of the different acetate-treated and control compacted bentonite samples after 24 months of incubation showing triplicates (except for 1.7A). 1.5C: control bentonite at 1.5 g/cm^3 density, 1.5A: acetate-treated bentonite at 1.5 g/cm^3 density, 1.7C: control bentonite at 1.7 g/cm^3 density, and 1.7A: acetate-treated bentonite at 1.7 g/cm^3 density. The percentages of variation explained by Coordinate 1 and Coordinate 2 are indicated on the axes.

suggested that the interaction between DNA and clay is influenced by several factors such as ionic strength, mineralogy of the sorbent, length of DNA polymer, and environment pH (Paget et al., 1992). Recently, Engel et al. (2019) extracted DNA from compacted bentonite (1.25 and 1.5 g/cm^3) using the PowerMax DNA isolation kit (MO BIO Laboratories) but the DNA yield was very low (up to 0.15 ng/µL) or below detection limit as determined by the Qubit dsDNA High Sensitivity assay kit. The optimized protocol described here shows the steps to isolate high quality and high-yield DNA (from 1.7 to 3.4 ng/µL) from compacted bentonite. Additionally, this method could be adapted for other environments such as marine sediments and uranium contaminated groundwater as was reported by Jroundi et al. (2020a, 2020b). To the best of our knowledge, this is the first study describing the structure and composition of native bacterial populations in highly compacted bentonite treated with acetate. The acetate was amended as electron donor to stimulate the growth of indigenous bacteria and to assess its effect on the diversity of in particular IRB and SRB and their impact on the bentonite mineralogy.

A high bacterial diversity was identified in samples compacted up to 1.7 g/cm^3 of dry density. In contrast, Engel et al. (2019) found lower bacterial diversity in compacted bentonite using an isolation kit with *Streptomyces*, *Xhanthomonas*, and *Pseudomonas* among the detected genera. On other hand, the lack of significant differences in the bacterial community structures between 1.5 and 1.7 g/cm^3 dry densities indicated that both showed the same effect on the diversity of indigenous bacteria. In contrast, compaction has an influence on the bacterial behavior, concretely on cultivability, which decreases with higher compaction density as was reported by Stroes-Gascoyne et al. (2010, 2011).

SRB populations are of concern in the DGR concept as they produce sulfide leading to the corrosion of the containers (Grigoryan et al., 2018; Loka Bharathi, 2008; Pedersen, 2010). Acetate is a common electron donor used by SRB (van den Brand et al., 2014); therefore its amendment could enhance the presence of these potentially detrimental bacteria. Although not statistically supported, acetate treatment selected for a minor increase of the relative abundance of 16S rRNA sequences aligning with acetate utilizing taxa. These included *Delftia*, *Paracoccus*, *Stenotrophomonas*, *Thermicanus, Desulfuromonas*, and *Pseudomonas* (Finster et al., 1994; Gößner et al., 1999; Jangir et al., 2016; Sajjad et al., 2016; Sánchez-Castro et al., 2017; Yang et al., 2019). At a higher taxonomic resolution, low levels of 16S rRNA sequences similar to sulfur and sulfate-reducing populations belonging to *Desulfuromonas* and *Desulfosporosinus* were identified in the compacted bentonite samples. Sequences belonging to *Desulfosporosinus* are also detected in compacted bentonite saturated with groundwater and amended with lactate (Chi Fru and Athar, 2008). However, a recent study concluded that the corrosion of copper material by SRB was inhibited to some extent by *P. aeruginosa* probably due to the production of extracellular polymeric substances that change the properties of the substrate and negatively affect SRB activity (Xiaodong et al., 2019). In contrast to SRB, SOB couple inorganic sulfur metabolism to nitrate as the electron acceptor to produce sulfite or sulfate (Poser et al., 2014). In this study, several SOB were found such as *Delftia*, *Paracoccus*, *Mesorhizobium*, *Thiobacillus*, and *Sulfurifustis* (Huber et al., 2016; Poser et al., 2014; Quentmeier et al., 2003). Together with the SRB, these populations likely create a cryptic sulfur cycle that supports the recent suggestion of faster sulfur dependent microbial growth rates than previously anticipated in DGRs (Bell et al., 2020).

The oxidation of acetate coupled to Fe(III) reduction by IRB (Rickard, 2012) is also a concern in the DGR environment. This is because the reduction of structural Fe(III) in the bentonite smectite octahedral layer destabilizes its structure (Esnault et al., 2010; Rickard, 2012) by illitization process that lead to: (1) an increase of the layer charge; (2) prevention of clay expansion upon hydration; (3) water release; and (4) changes in the hydraulic conductivity (Dong, 2012; Dong et al., 2009). Kostka et al. (2002) reported the growth of *Shewanella oneidensis* using the iron bound in smectite as the sole electron acceptor. In addition, TEM and XRD were applied to report its ability to promote the dissolution of smectite through reduction of structural Fe(III) (Kim et al., 2004). In the present study, 16S rRNA gene sequences in the acetate-treated compacted bentonite most similar to IRB included those

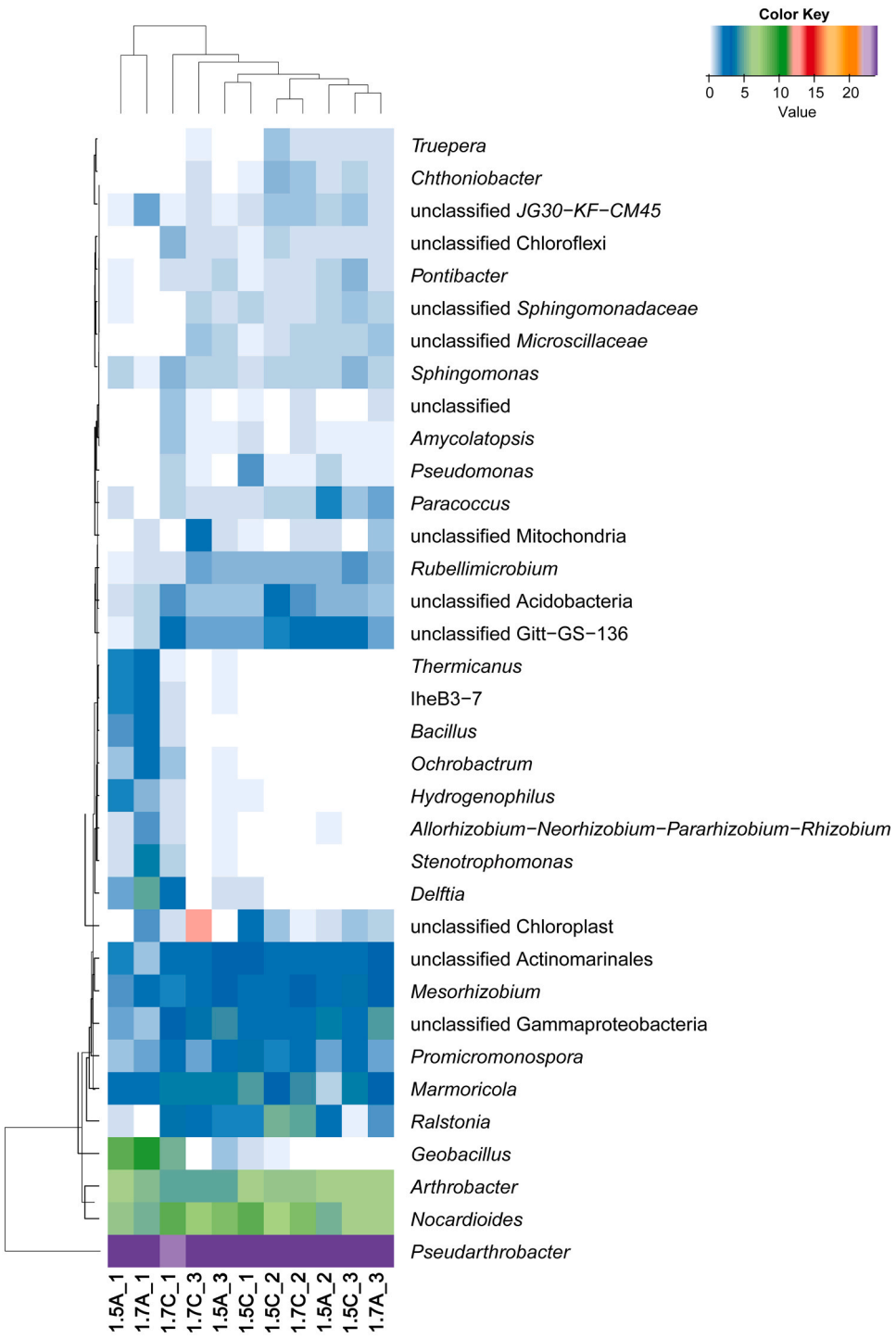

Fig. 8. Heatmap of the relative abundance at the genus level in the compacted bentonites (1.5C, 1.5A, and 1.7C in triplicates plus 1.7A in duplicates) with clustering based on Manhattan distance and average linkage for both columns and rows throughout the sample set. The relative abundance of each genus was shown by different color indicated in the color bar. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

aligning with *Geobacillus*, *Pseudomonas*, *Stenotrophomonas*, *Bacillus*, and *Thermicanus* (Brooke, 2012; Gößner et al., 1999; Valencia-Cantero and Peña-Cabriales, 2014). Examples of *Bacillus* capable of ferric iron reduction include *B. subterraneus* and *B. infernus* (Kanso et al., 2002) while *Pseudomonas* spp. reduce Fe(III) through respiratory metabolisms (Kooli et al., 2018). Moreover, *Pseudomonas* spp. form a biofilm on carbon steel surfaces where they utilize the Fe^0 as electron donor causing corrosion (Jia et al., 2017) that could adversely alter the carbon steel containers suggested to be used in radioactive waste DGRs. On the other hand, *Stenotrophomonas* and *Acidiferrobacter* promote iron reduction under anoxic conditions that could be detrimental for the structural stability of smectites (Hallberg et al., 2011; Ivanov et al., 2005; Valencia-Cantero et al., 2007).

5. Conclusions

Bentonites are being considered as sealing and backfilling material for the disposal of radioactive wastes with compaction at a suitable dry

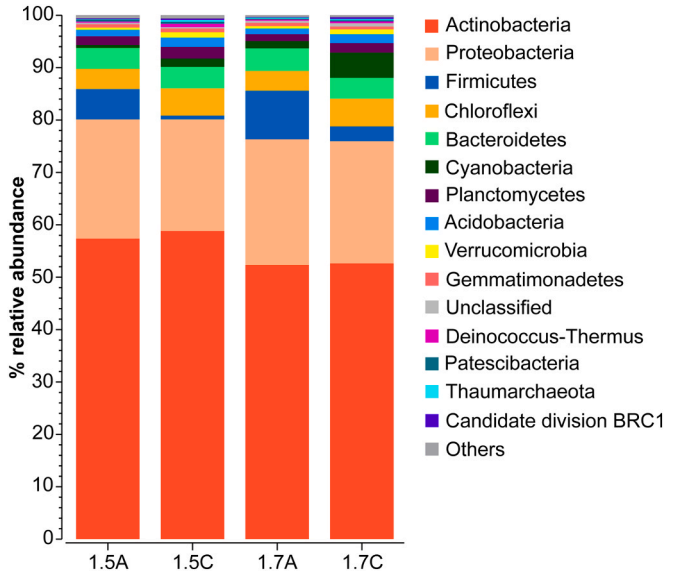

Fig. 9. Relative abundance of phyla in bentonite samples after 24 months of anoxic incubation showing averages of biological triplicates (duplicates in 1.7A sample). C: control bentonite, A: acetate-treated bentonite; 1.5C: control bentonite at 1.5 g/cm^3 density, 1.5A: acetate-treated bentonite at 1.5 g/cm^3 density, 1.7C: control bentonite at 1.7 g/cm^3 density, and 1.7A: acetate-treated bentonite at 1.7 g/cm^3 density.

density ensuring low hydraulic conductivity and adequate swelling to close construction gaps. However, it is rarely possible to efficiently extract genomic DNA from compacted samples due to its swelling characteristic and adsorption of cells, debris, and nucleic acids to smectite and other particles of these clays. The optimized protocol described here enables the extraction of high DNA-yields from compacted bentonites for e.g. metagenomic sequencing to elucidate the complete microbial community from this challenging environment. This new methodology has enabled a significant advance in studies relating to nuclear waste disposal. Furthermore, despite being a demanding environment for the existence of microorganisms, a complex bacterial community was found where both potentially detrimental and neutral bacteria for the safety of DGR coexist. 16S rRNA gene sequencing revealed populations mediating the sulfur and iron biogeochemical cycles that were enriched by the addition of acetate. However, no statistically significant differences in bacterial composition of the community were observed between acetate-treated and untreated compacted bentonites. This could be explained by the short incubation time (2 years), which may be insufficient to detect an enhancement of the IRB and SRB communities, likely as the growth rate in these conditions was too low to observe shifts in the bacterial diversity. Long-term incubation under anoxic conditions of compacted bentonite could lead to the enrichment of these groups of bacteria, altering the stability of bentonites (by structural Fe(III) reduction) and inducing metal canister corrosion (by sulfide production). Therefore, further studies performing long-term incubation (e.g., at least 10 years) and providing efficient electron donors (e.g., lactate and acetate) and terminal electron acceptors, such as sulfate and Fe(III), are required for a better understanding of the implications of SRB and IRB in the DGR concept.

CRediT authorship contribution statement

Cristina Povedano-Priego: Methodology, Investigation, Formal analysis, Resources, Visualization, Writing - original draft, Writing - review & editing. **Fadwa Jroundi**: Methodology, Investigation, Writing - original draft, Writing - review & editing. **Margarita Lopez-Fernandez**: Methodology, Writing - review & editing. **Rojina Shrestha**: Investigation, Writing - review & editing. **Roman Spanek**: Data curation, Visualization, Writing - review & editing. **Inés Martin-Sánchez**: Writing - review & editing. **María V. Villar**: Methodology, Writing - review & editing. **Alena Ševců**: Writing - review & editing. **Mark Dopson**: Supervision, Writing - review & editing. **Mohamed L. Merroun**: Conceptualization, Supervision, Project administration, Funding

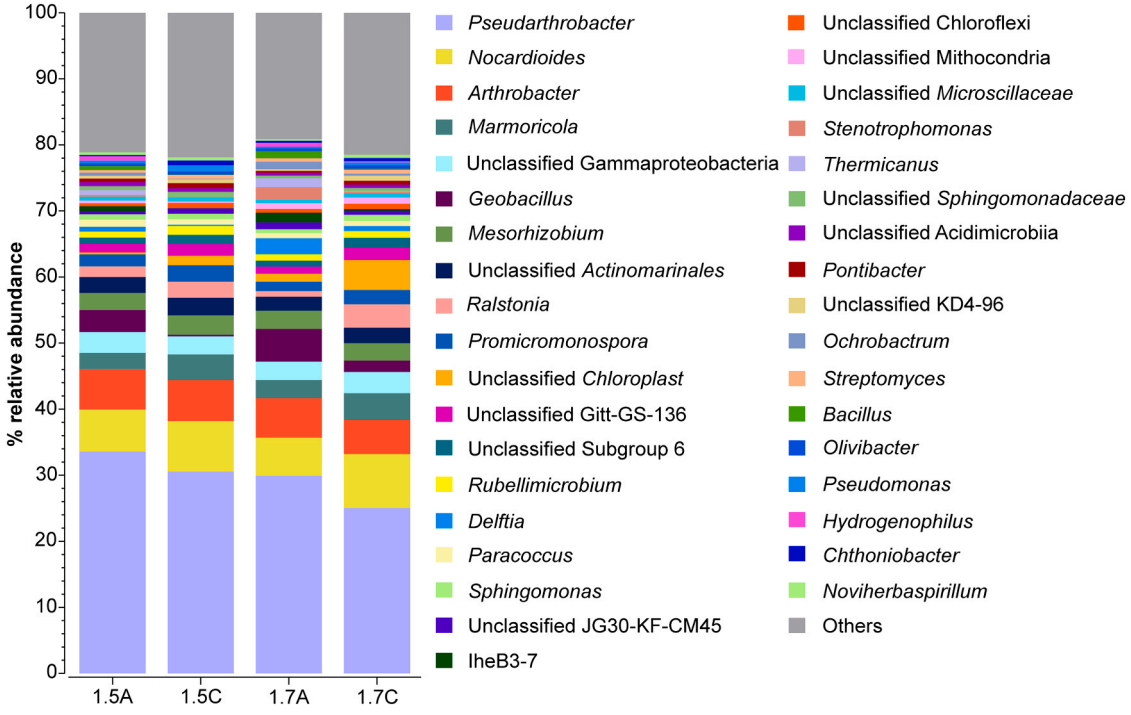

Fig. 10. Relative abundance of genera in bentonite samples after 24 months of anoxic incubation showing averages of biological triplicates (duplicates in 1.7A sample). C: control bentonite, A: acetate-treated bentonite; 1.5C: control bentonite at 1.5 g/cm^3 density, 1.5A: acetate-treated bentonite at 1.5 g/cm^3 density, 1.7C: control bentonite at 1.7 g/cm^3 density, and 1.7A: acetate-treated bentonite at 1.7 g/cm^3 density.

acquisition, Writing - review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jhazmat.2020.124600.

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