Microbial diversity in bentonite, a potential buffer material for deep geological disposal of radioactive waste

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Abstract. Many countries are considering long-term disposal of nuclear waste in deep geological repositories (DGRs), encapsulated in metal containers, surrounded by a barrier, and emplaced in the host rock. Bentonite is found to be a safe barrier material because of its physical and chemical properties. The bentonite montmorillonite interlayer contains adsorbed organic matter and microorganisms, and therefore biogeochemical activity must be considered. This study focused on investigating the microbial diversity in Chinese bentonite by 16S rRNA analysis via Illumina sequencing to determine how these microorganisms can potentially influence DGR performance. The bentonite sample contained high microbial diversity, dominated by Archaea and abundant other bacteria. The community of Archaea included Nitrososphaera, Thermogymnomonas, and Methanobrevibacter, which are thought to play significant roles in nitrogen cycling, H₂ and O₂ consumption, CH₄ production, and organic matter degradation. These processes can generate excessive pressure and may compromise the safety of DGRs, and therefore the use of bentonite as a barrier material should be carefully considered.

1. Introduction
Nuclear waste generated by the production of nuclear power must be safely stored for at least 100,000 years for its radiotoxicity to decrease to levels approaching those of naturally occurring radioactive materials and their daughter products [1]. Many countries are considering long-term disposal of nuclear waste in deep geological repositories (DGRs), encapsulated in a metal container, surrounded by an engineered barrier, and emplaced in the host rock, as the safest option for the disposal of these hazardous materials [2]. However, to date, no facility for deep geological disposal of high-level radioactive waste has been completed. Several countries are at the stage of site evaluation and selection [3, 4]. In China, a concept model of DGR with a multi-barrier system, encapsulated in a corrosion-resistant metal container, surrounded by an engineered bentonite barrier, and emplaced in the host rock, has been considered. Several studies have investigated the selection and characterization of suitable backfill material for Chinese radioactive waste repositories. Gaomiaozi Na-bentonite, found in the Inner Mongolia autonomous region of China, was chosen as a reference material for safety barriers, because it is well characterized in terms of its physical and chemical properties, including its compactibility, dilatability, and geochemical properties [1].

The bentonite montmorillonite interlayer contains adsorbed organic matter and thus microorganisms [5]. Microorganisms interact with their surroundings and can greatly modify the biogeochemical environments. Such interactions may significantly influence the properties of
bentonite, as well as waste containers, and the safety of any future deep repositories placed there [6, 7]. First, the material of the containers used for waste disposal (metal, concrete) can become corroded by microbial metabolites [8]. Second, the biogeochemistry of the disposal environment can be influenced by the indigenous, introduced, or reactivated microbial activity. The biogeochemical environment could be affected the speciation, migration, and transport of radionuclides in a future deep repository [9, 10]. Third, microbial metabolites are capable of intracellular accumulation, biotransformation, and biomineralization, and microbiological processes may alter the speciation and mobility of radionuclides [11, 12]. Thus, microbial activity has the potential to compromise DGRs and must be considered. In many counties considering DGR, the relevant microbial community has been studied for more than 10 years. However, the microbial community present in bentonite in China is still not completely known. Here, we performed an analysis of the microbial diversity present in selected bentonite deposits in China, which are being considered as a safety buffer for a future Chinese DGR, using molecular approaches based on 16S rRNA gene analysis, using an Illumina sequencing platform, to better understand what types of microorganisms are present and how they could potentially influence the performance of a nuclear waste repository.

2. Materials and methods

2.1. Characteristics of bentonite samples

Bentonite samples were collected from a candidate site in Xinghe County, Inner Mongolia Autonomous Region of China in May 2014. Bentonite from these deposits is best described as Na-Bentonite, also called Gaomiaozi bentonite. It is considered to be a natural analogue of the engineered bentonite barrier for use in deep geological disposal of high-level radioactive waste, for its good compaction properties. Bentonite samples were taken from a depth of 20-30 m, using a 5-point sampling method. All samples were collected under sterile conditions, then mixed, followed by storage at −80 °C prior to DNA extraction.

2.2. DNA extraction and PCR

Total DNA was recovered from 500 mg of the mixed bentonite soil samples using an EZNA soil DNA Kit (Omega, Norcross, GA, USA). DNA yields were verified by agarose gel electrophoresis and quantified with the Qubit 2.0 Kit (Invitrogen, Carlsbad, CA, USA). DNA was frozen at −20 °C until further analysis. The genes encoding the 16S rRNA were amplified by polymerase chain reaction (PCR). The V3-V4 hypervariable region of the 16S rRNA gene was amplified using universal primers based on 16S341f and 16S805r [13, 14]. PCR reactions were performed with the specific Illumina multiplexing sequencing primers and index primers. PCR products of the correct size were extracted and recovered using the QIA quick gel extraction kit (Qiagen, Germany). Libraries were sent for Illumina Sequencing using the Miseq platform with a PE300 strategy.

2.3. Illumina sequencing and analysis

Illumina sequencing using paired-end sequencing was performed on a MiSeq System Sequencer (Illumina, USA). Sequence reads were analysed using QIIME to remove the adapter, barcode, and primer sequences, and to exclude sequences that did not meet the quality criteria. The bacterial and archaea16S rRNA genes were grouped into OTUs (97% sequence similarity) and annotated using SILVA Incremental Aligner (SINA). The sequencing coverage of the diversity indexes was calculated using the software MOTHR Ver. 1.32.2 with SILVA database using the rarefaction analysis and richness options [15]
3. Results

3.1. Metagenomic sequence data quality
The 16S rRNA genes were sequenced by high-throughput sequencing and analyzed using MiSeq after quality control; the sequence length distribution was 400–500 bp. Figure 1 shows the length distribution of the raw reads. Figure 2 shows the length distribution of the clean reads for analysis, totaling more than 5000 sequences.

![Figure 1. Length distribution of raw reads.](image1)

![Figure 2. Length distribution of clean.](image2)

Reads after normalization, 20,165 sequences were obtained from the bentonite samples, with a mean length of 400 nucleotides. Sequences were analyzed and annotated. In total, the sequences were divided into 2952 operational taxonomic units (OTUs), determined at 97% sequence identity.

3.2. Bacterial diversity in bentonite
The microbial diversity was analyzed by microbiome sequencing and the sequences were classified into 31 phyla, 58 classes, 76 orders, 172 families, and 495 genera. The bentonite samples were found to contain high bacterial diversity. Richness, evenness, and phylotype diversity were measured using conventional diversity indices. Alpha diversity analyses yielded the following results: Shannon index 6.14, Chao1 index 6536.03. Coverage was 0.914, which is close to 1, suggest a high quality of the sequencing results.

3.3. Phylum-level microbial diversity in bentonite
The results of high-throughput sequencing showed that the bacteria belonged to 31 different phyla including Thaumarchaeota, Proteobacteria, Euryarchaeota, Firmicutes, Actinobacteria, Bacteroidetes, Fusobacteria, Acidobacteria, and an unclassified phylum. Figure 3 shows the phylum-level distribution of the bacteria in the bentonite; 99% of the sequences belonged to 8 phyla. The dominant kingdom was Archaea (60%), which includes Thaumarchaeota (50%) and Euryarchaeota (10%). The second most abundant phylum was Proteobacteria (16%). The phylum of Thaumarchaeota belonging to Archaea was proposed in 2008 based on 16s rRNA data, to distinguish mesophilic ammonia-oxidizing archaeal (AOA) lineages from hyperthermophilic Crenarchaeota lineages. In 2010, this distinction was confirmed by genomic information. And the Thaumarchaeota is widely distributed in marine, soil, and aquatic environments, and members of this phylum utilize inorganic carbon as an energy source [16, 17].
3.4. **Class-level microbial diversity in bentonite**

The sequences were found to belong to 58 different classes, including β-Proteobacteria, Actinobacteria, Thermoplasmata, Bacilli, α-Proteobacteria, γ-Proteobacteria, Fusobacteria, and Methanobacteria. Figure 4 shows the distribution of the microbes by class. The dominant phylum, Proteobacteria, was represented by α-, β-, γ-, δ-, and ε-Proteobacteria. The α-, β-, and γ-Proteobacteria were dominant, representing 6.3%, 4.4%, and 4.0% of all sequences, respectively. The abundance of δ- and ε-Proteobacteria sequences was lower (0.5% and 0.3% of the total). Interestingly, the commercial clay products, including Wyoming MX-80 bentonite - the reference clay for DGR concepts in Canada, Finland and Sweden have been demonstrated to harbour a small set of bacteria. In addition, MX-80 bentonite was shown the presence of bacterial classes Gammaproteobacteria, Deltaproteobacteria, Actinobacteria and Sphingobacteria,which was also showed in Chinese Gaomiaozi bentonite. In addition, the MX-80 bentonite was detected sulfate and sulfur-reducing bacteria presumably contributed to sulfide accumulation in the different microcosm systems [18]. Almost 50% of the sequences belonged to an unclassified class. Nitrososphaerales are unclassified at the class level.

![Figure 3. Phylum-level abundance of microbes in bentonite samples.](image)

![Figure 4. Class-level abundance of microbes in bentonite samples.](image)

3.5. **Order-level microbial diversity in bentonite**

The sequences were found to belong to 76 different orders, as shown in Figure 5. The dominant order was Nitrososphaerales (52.2%), followed by an unclassified order (6.0%), Thermoplasmatales (5.7%), Actinomycetales (4.8%), Lactobacillales (4.2%), Fusobacteria (3.3%), and Burkholderiales (3.2%). Samples were also classified as Neisseriales, Sphingomonadales, Pasteurellales, Methanobacteriales, Streptococces, and Bacillales (1% each). The dominant order, Nitrososphaerales, includes Nitrosopumilaceae, unclassified Nitrosopumilales, and environmental samples.
3.6. Family-level microbial diversity in bentonite

The sequences were found to belong to 172 different families, as shown in Figure 6. The dominant family was Nitrosopumilaceae (52.2%), followed by an unclassified family (6.7%), Thermoplasmales, Streptococaceae, Neisseriaceae, Leptotrichiaceae, Sphingomonadaceae, Actinomycetaceae, Pasteurellaceae, Corynebacteriaceae, Methanobacteriaceae, Comamonadaceae, Veillonellaceae, Enterobacteriaceae, and Micrococaceae. More than 50% of samples were classified as Archaea, including Nitrososphaerales and Methanobacteriaceae, which play a major role in nitrification, gas consumption, and consumption of organic matter, and could therefore affect deep geological repositories for storage of high-level radioactive waste. The dominant family, Nitrosopumilaceae, includes Candidatus, Nitrososphaera, and unclassified Nitrososphaeraceae.

3.7. Genus-level microbial diversity in bentonite

The sequences were found to belong to 495 different genera including Nitrososphaera, Thermogymnomonas, Streptococcus, Leptotrichia, Neisseria, Sphingomonas, Actinomycetes, Corynebacterium, Haemophilus, Methanobrevibacter, Lactobacillus, and unclassified genera. Figure 7 shows that the dominant genus was Nitrososphaera. The second-most abundant genera were Thermogynomonomas. These were shown the difference from the commercial Wyoming MX-80 bentonite.
4. Discussion

Microbial activity may significantly influence the function of future deep repositories for nuclear waste, mainly because of microbe-induced corrosion (MIC) of metal or concrete containers in bentonite, and microbial metabolic processes and can be affected by the speciation, migration, and transport of radionuclides. Several countries are investigating the microbial populations and microbial diversity in relevant environments, including bentonite, underground water, and host rock or clay layers. Considerable data is available on the impact of microbes on corrosion, and their interactions with gas, radionuclides, and geochemical environments under simulated geological disposal conditions. For instance, Pedersen et al. (2010) investigated the numbers of microbes in Åspö Hard Rock, Olkiluoto and ONKALO groundwater, as well as microbial incidence on copper and titanium embedded in compacted bentonite clay [19,20]. Schütz et al. (2015) studied the effect of iron-reducing bacteria on the corrosion rate of carbon steel under geological disposal conditions in France [21]. López-Fernández et al. (2014) investigated the microbial diversity in bentonite and studied the interactions with uranium [22]. However, no data are available on the number or diversity of microbes in Chinese bentonite, to assess the function of the microbial community and assure functional and safe waste storage. This study aimed to investigate the microbial community present in bentonite in China.

We analyzed the microbial diversity of selected bentonite samples collected in China using a molecular approach based on analysis of 16S rRNA gene sequences obtained using the Illumina sequencing platform. The analysis identified 31 phyla, 58 classes, 76 orders, 172 families, and 495 genera. The results showed a high bacterial diversity in the bentonite samples. Compared with culture-dependent analysis of microbial diversity, next-generation sequencing (NGS) of 16S rRNA genes allows full characterization of the microbial community, and has been used to characterize the microbial diversity in various environmental samples, including deep mines, soil, and seawater, which is difficult to study using traditional culture enrichment techniques. In addition, a previous analysis of the microbial diversity of bentonite used both techniques, traditional clone libraries and Illumina sequencing, and the two methods showed similar results. The high microbial diversity of these Spanish clays was shown by means of culture dependent and High-throughput sequence techniques [23]. However, this rapid and high-throughput technology is limited by the short length of the reads. Therefore, to identify samples to species, analysis of 16S rRNA gene clone libraries might be applied, because it can provide almost full-length reads.

The microbial community in the bentonite samples was dominated by Archaea, including Nitrososphaera, Thermogymnomonas, and Methanobrevibacter, which represented 52.2%, 5.7%, and 4.0% of all sequences. Nitrososphaera were dominant, and are known to play a major role in the global nitrogen cycle, gaining energy by aerobically oxidizing ammonia to nitrite, thereby fixing CO2, but their growth depends on the addition of small amounts of organic acids [24]. Methanobrevibacter can use H2 and produce CH4 in anaerobic conditions, and considerable data are available on their

![Figure 7. Genus-level abundance of microbes in bentonite samples.](image-url)
competition with sulfate-reducing bacteria [25]. This archaea community has been suggested to play a significant role in nitrogen cycling, H2 and O2 consumption, CH4 production, and organic degradation, which can generate excessive pressure and may form micro fractures.

The bacterial community in the bentonite samples was diverse, consisting of Proteobacteria, Firmicutes, Actinobacteria, Bacteroidetes, Fusobacteria, and Acidobacteria. Proteobacteria was clearly dominant, represented by α-, β-, γ-, δ-, and ε-Proteobacteria. The α-, β-, and γ-Proteobacteria were preponderant, and the δ- and ε-Proteobacteria showed less than 1% abundance, but included Desulfovibacca, Syntropobacter, and Geobacter, which perform sulfate and iron metabolism. Interestingly, the Chinese Gaomiaozi bentonite sample was shown the same class-level abundance of microbes to the commercial MX-80 bentonite - the reference clay for DGR concepts in Canada, Finland and Sweden have been demonstrated to harbour a small set of bacteria. And the MX-80 bentonite was detected sulfate- and sulfur-reducing bacteria presumably contributed to sulfide accumulation in the different microcosm systems [18]. In addition, the abundant Pseudolabrys, Sphingomonas, and Haemophilus can affect bentonite properties by processes including microbial degradation of adsorbed organic matter in smectite [26]. The bacterial community identified is known to perform microbial degradation of organic compounds, which could affect the bentonite, the container material, or the radioactive waste, potentially influencing the performance of the nuclear waste repository.

5. Conclusions
Microorganisms are capable of vigorously modifying their environments through their growth and metabolite production. It implications on the performance of the DGRs have been the subject of considerable study. Especially, the dominant populations in indigenous surroundings within deep groundwater and host rock, along with microorganisms introduced with fluid seepage and an engineered bentonite barrier during construction and maintenance of a DGR, will be an unavoidable source of microbial contamination [27].

The microbial communities of the selected artificial barriers can affect the safety and performance of future DGRs at 3 different levels by: i) transformation of bentonite; ii) corrosion of metal canisters; and iii) mobilization of radionuclides through biofilm adsorption, biomineralization and redox processes [28-29].

The current work describes the microbial diversity of Chinese bentonite formations based on analysis of 16S rRNA gene sequences. Microbial diversity in Chinese bentonite samples was found to be high, dominated by Archaea and abundant bacteria. The archaea community included Nitrososphaera, Thermogymnomonas, and Methanobrevibacter, which have been suggested to play significant roles in nitrogen cycling, H2 and O2 consumption, CH4 production, and organic degradation; all of these processes can generate an excess of pressure and cause fractures. The bacterial community was also diverse, consisting of Proteobacteria, Firmicutes, Actinobacteria, Bacteroidetes, Fusobacteria, and Acidobacteria. This bacterial community was dominated by 5 groups by Proteobacteria; only the abundance of δ-Proteobacteria, which are related to sulfate-reducing bacteria, was low. The most abundant groups including Pseudolabrys, Sphingomonas, and Haemophilus, are involved in microbial degradation of organic matter.

However, further analyses are required to obtain a larger number of clones to obtain sufficient reads to gain deeper knowledge of the most abundant representatives of the microbial community in conditions relevant to siting of repositories of nuclear waste.

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