Microbial growth inhibition by compacted bentonite after an 8.5-year *in-situ* incubation

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Abstract

Compacted bentonite in future deep geological repositories for the disposal of nuclear waste will create an extreme, energy-limited environment for microorganisms, yet the long-term implications for microbial community structure and the potential emergence of sulfate-reducing bacteria (SRB) remain uncertain. Here, we use an 8.5-year *in-situ* incubation experiment in an anoxic Opalinus Clay borehole, integrating observations from successive retrieval phases after 1.5, 5.5, and 8.5 years, to examine changes in microbial persistence in compacted Wyoming bentonite across three dry densities (1.25, 1.45, and 1.55 g/cm³). 16S rRNA gene quantification and sequencing showed that microbial cell numbers remained low and declined significantly over time at all densities, with density as the primary driver and time as the dominant secondary driver of community change. After 8.5 years, SRB were detected only in the rare biosphere (<0.1% relative abundance), although they were recoverable by cultivation, indicating survival rather than growth. Altogether, these results support limited concern about the

progressive enrichment of corrosion-inducing microorganisms following oxygen depletion and demonstrate that bentonite compaction at 1.25 g/cm^3 is sufficient to inhibit microbial growth, providing an effective biological barrier.

Key words: Bentonite, deep geological repository, sulfate-reducing bacteria, rare biosphere, long-term incubation, microbial inhibition, radioactive waste disposal, Opalinus Clay rock

Introduction

Deep geological repositories (DGRs) will host high-level radioactive waste primarily from nuclear power plants. Bentonite clay is considered in some disposal concepts as one of the engineered barriers in the multibarrier system of DGRs [1]. It will be placed around the disposal canister in the emplacement drift, filling the gap between the canister and the host rock, in a process referred to as backfilling. In the case of the Swiss concept, bentonite ensures waste isolation by mechanically protecting the canister, and by combining sorption capacity with swelling pressure—properties that retain radionuclides after canister breach (expected after more than 10,000 years) [2–4]. It complements the radionuclide retention effect of the clay-rich host rock formation, Opalinus Clay.

However, bentonite backfill introduces microorganisms, in addition to those already present in the host rock and those introduced by operations [5–8]. These microorganisms possess diverse metabolic traits that can become active as repository conditions evolve from partly saturated and oxic to fully saturated and anoxic. Sulfate (SO_4^{2-}) reduction is of particular concern, as hydrogen sulfide (HS^-) produced by sulfate-reducing bacteria (SRB) may accelerate canister corrosion once oxygen (O_2) is depleted. In addition, microbial activity shapes gas turnover, particularly hydrogen (H_2) and methane (CH_4), with implications for gas buildup that must be reliably predicted to ensure repository safety [9]. To restrict microbial activity, strategies like compacting bentonite clay to high densities (for example, 1.45 g/cm³ for Wyoming bentonite) have been suggested. This approach controls pore space, pressure, water activity, and substrate diffusion rates, all of which are critical controls for the

66 proliferation of microbes [10, 11]. Yet, predicting microbial survival and activity over of
67 thousands to hundreds of thousands of years remains a major challenge.
68 Long-term laboratory and *in-situ* incubation studies on compacted bentonite have
69 therefore become key to understanding microbial fate. Results from previous studies
70 conducted after 1–8 years of incubation [12–17] indicate that: (i) microbial cell
71 numbers remain stable or decline is minimal, (ii) colonization from host rock and/or
72 porewater is limited and bentonite communities alone show little temporal change, (iii)
73 compaction is the dominant control on abundance and diversity, and (iv) irrespective
74 of incubation length or density, part of the indigenous microbiota remains alive and
75 can be revived by cultivation. Strikingly, across all studies, viable aerobic heterotrophs
76 consistently outnumber culturable anaerobes, and (facultatively) aerobic bacteria like
77 *Streptomyces* spp. and *Pseudomonas* spp. are frequently found in high abundance in
78 the sequencing datasets. Burzan et al. (2022) proposed that this paradox can be
79 explained by slow desorption and dissolution of O_2 from bentonite, which sustains
80 aerobic metabolism while suppressing anaerobic activity [12]. Whether aerobes
81 eventually decline once O_2 is consumed, allowing anaerobes such as SRB to expand,
82 or whether bentonite compaction continues to inhibit the entire community remains
83 unresolved.

84 Here, we provide a unique continuous *in-situ* dataset from Wyoming bentonite
85 incubation in the same Opalinus Clay borehole for 1.5, 5.5, and 8.5 years, providing
86 robust temporal comparisons over long timescales. By combining cultivation-based
87 enumerations with cultivation-independent 16S rRNA sequencing, we focus on
88 patterns of aerobe persistence and the potential emergence of anaerobic metabolism
89 under repository-like conditions. We further test how time, bentonite density (1.25,

1.45, 1.55 g/cm³) and formulation (bentonite blocks vs. pellets) shape microbial community structure and fate. Together, these data help refine long-term predictions of microbial impact on barrier performance in DGRs.

Materials and Methods

Experimental design and module assembly. Experimental modules consisted of stainless-steel cylinders (25 cm height, 12.6 cm outer diameter) lined with a sintered porous stainless-steel filter and filled with Wyoming bentonite at one of three dry densities and one of two bentonite formulations: 1.25 g/cm³ (pre-compacted blocks), 1.45 g/cm³ (pellets), and 1.55 g/cm³ (pre-compacted blocks). Modules containing pellets were saturated with anoxic deionized water and placed under vacuum during compaction to minimize residual oxygen. Blocks were handled and stored under 100% N_2 until assembly, after which they were similarly saturated. In addition, all modules contained metallic coupons embedded within bentonite for corrosion analysis (not covered here). Modules were deployed for 8.5 years in an anoxic, porewater-filled borehole in the Opalinus Clay at the Mont Terri Rock Laboratory (St-Ursanne, Switzerland). Results from this phase are compared with previously published data from the same borehole, including as-received bentonite and bentonite incubated for 1.5 or 5.5 years. The analysis focuses on the two most recent time points (5.5 and 8.5 years), while earlier data are included where relevant for context. Detailed analyses of earlier time points are available in the published literature [12, 18].

Bentonite sampling. After removal from the borehole, the modules were immediately packed under anoxic conditions (100% N_2), transported to the laboratory, and stored at 4 °C. In an anoxic glovebox (100% N_2), the porous filters were removed from the modules and cut along the module axis to access bentonite. Bentonite was then

sectioned using sterile tools following established protocols used in the previous phases of the Iron Corrosion (IC-A) Experiment [12]. Sections were separated and preserved at 100% Ar and 4 °C for analysis of water content, clay density, microbial community and for microbial cultivation. Later, the samples were further subsampled to isolate the outer (at the interface with the borehole surface) and inner core fractions. After subsampling, the outer and inner parts were either used for cultivation studies, in which case they were stored anoxically at 4 °C, or for gDNA enumeration and sequencing, with preservation at -20°C.

Microbial cultivation. Heterotrophic aerobes and anaerobes were enumerated in triplicate from anoxically prepared bentonite suspensions using a consistent approach across all time points. The semi-solid pour plate method was used with R2A medium [21]: liquid agar medium was cooled down to 45–50°C and grown under oxic (3 days) or anoxic (30 days) conditions at 30°C. The enumeration of SRB was performed with the most probable number (MPN) method [22]. Hungate tubes were filled with 9 ml of sterile, anoxic Postgate's Medium B (Medium 63: Desulfovibrio (Postgate) Medium, 2022) and serial dilutions from 10^{-1} to 10^{-5} were made (in triplicates) using the same inoculum as for heterotrophic anaerobes and aerobes. The tubes were sealed to maintain anoxic conditions during 7 weeks of incubation at 30°C.

DNA extractions and sequencing. DNA was extracted following methods described by [24] utilizing a modified protocol of DNeasy PowerMax Soil Kit (QIAGEN NV, Venlo, The Netherlands). Bentonite DNA was additionally purified by using 10 µL Invitrogen™Linear Acrylamide (5 mg/mL), 0.1 volume of 5 M NaCl and 2 volumes of isopropanol. The air-dried DNA pellets were resuspended in 40 µL elution buffer provided in the DNeasy® PowerMax® Soil Kit and stored at -20°C. Extracted DNA was quantified using an Invitrogen Qubit 2.0 fluorometer with 2 µL of extracted DNA and 198 µL of working solution prepared by mixing Qubit™ buffer solution and fluorescent Qubit™ reagent following the standard protocol provided by the manufacturer. Sequencing was performed targeting 16S rRNA gene V4 region with library prepared using Quick-16S Plus NGS Library Prep Kit (V4) from Zymo (DS6430) at using Miseq v2 PE150 platform.

16S rRNA gene copy quantification. Quantitative PCR (qPCR) analysis of the bacterial and archaean 16S rRNA gene was performed using the MYRA robotic system and a MIC qPCR Cycler (both BioMolecular Systems, Australia). Analyses were carried out in triplicate in 10 µL reactions. For quantification of the gene abundance, the following volumes of substrates were used: 2.5 µL template DNA, 2.1 µL water, 0.2 µL of each primer (100 mM stock) and 5 μL of 2× SensiFAST SYBR ® No-ROX Kit (Meridian Bioscience, UK). Samples were cycled (40 cycles) at 95 °C for 5 s, followed by an extension at 62 °C for 10 s and the acquisition at 72 °C for 5 s. The final melting step was carried out from 72 °C to 95 °C, at a rate of 0.1 °C/s. Analysis of the results was performed using the built-in analytical software (micPCR, BioMolecular Systems, ver. 2.12.6). Average efficiency (0.943 – 1.003) and r^2 values (> 0.99) were determined from seven points of the serial dilutions (10^7 –10^1 copies) for bacterial 16S rRNA gene. Based on calibration curves obtained using *E. coli* DNA, C_q values were used to calculate the gene copy numbers which were normalized against mass (ng) of the extracted DNA. The primer pair used for the reaction to quantify bacterial 16S rRNA gene consisted of 338f: 5'-ACT CCT ACG GGA GGC AGC AG-3' and 534r: 5'-ATT ACC GCG GCT GCT GGC A-3'.

Bioinformatic analysis. The multiplexing barcodes were removed and raw sequence read quality was assessed using FastQC [25]. The reads were then processed in the R (version 4.5.2) [26] package dada2 [27]. Forward 'AGRGTTYGATYMTGGCTCAG' and reverse 'RGYTACCTTGTTACGACTT' primers were removed. After filtering and dereplication, error rates were learned to denoise the reads and obtain Amplicon Sequence Variants (ASVs). Taxonomy of ASVs was determined using the SILVA database (released 138.1) formatted to be compatible with dada2 and the RDP Naive

Bayesian Classifier algorithm [28, 29]. Chimeras were detected and removed for subsequent analysis. PCoA was based on Bray-Curtis dissimilarity (*vegdist, cmdscale; vegan*), and RDA was performed using rda with significance assessed by permutation tests (*anova.cca*) [30]. The R package *ulrb* was used to define the rare biosphere in the samples by comparing ASV abundances and clustering them into three relative categories: 'rare', 'abundant', and 'undetermined', which avoids using arbitrary abundance thresholds [31]. Functional profiles were predicted from 16S rRNA gene data using PICRUSt2 [32]. Data processing was performed using *dplyr* and *tidyr*, and figures were generated with *ggplot2* [33, 34].

Bentonite water content and dry density determination. Water content was calculated by obtaining the bentonite wet and dry mass after 24h in an oven at 105°C and reported in Figure S1 and **Table S1**.

Statistical analysis. Analysis of Variance (ANOVA) at a significance level (alpha) of 0.05, was used to discern significant differences between the treatments. In instances for which the ANOVA indicated that significant differences existed within data sets, pairwise comparisons were conducted using the t-Student test for each pair at a significance level (alpha), adjusted for multiple comparisons using the Bonferroni correction method.

Results and Discussion

Microorganisms remain viable and cultivable after 8.5 years in compacted bentonite

After 8.5 years of *in-situ* incubation, microorganisms remained detectable and, in part, viable across all bentonite densities. Quantitative PCR confirmed the presence of bacterial and archaeal 16S rRNA genes in all samples, with abundances overall

191 decreasing systematically and significantly with increasing dry density (ANOVA,
192 p=0.05) (**Figures 1A, S2**). This inverse relationship confirms that compaction is a
193 strong constraint on microbial persistence. Significant differences in bacterial cell
194 numbers (pairwise t-student, p=0.05) between the outer layer and the inner part of
195 bentonite were observed only for the bentonite at the lowest density (1.25 g/cm^3).
196 Despite long-term energy-limiting conditions, cultivable anaerobic and aerobic
197 heterotrophic microorganisms were still recoverable. In general, the number of colony-
198 forming units (CFU) followed the same trend as 16S rRNA gene copy numbers: the
199 lower the dry density, the higher the number of CFU. No statistical difference was
200 shown between the outer and inner parts of the core at any of the densities (pairwise
201 t-student tests, p=0.05). CFU numbers of anaerobes were on average two orders of
202 magnitude lower than those of aerobes. Similarly to aerobes, there was no statistical
203 difference between the outer parts of the core and inner parts in terms of CFU
204 numbers. The average numbers, together with standard deviations for both aerobes
205 and anaerobes (CFUs) are presented in **Table S2.**
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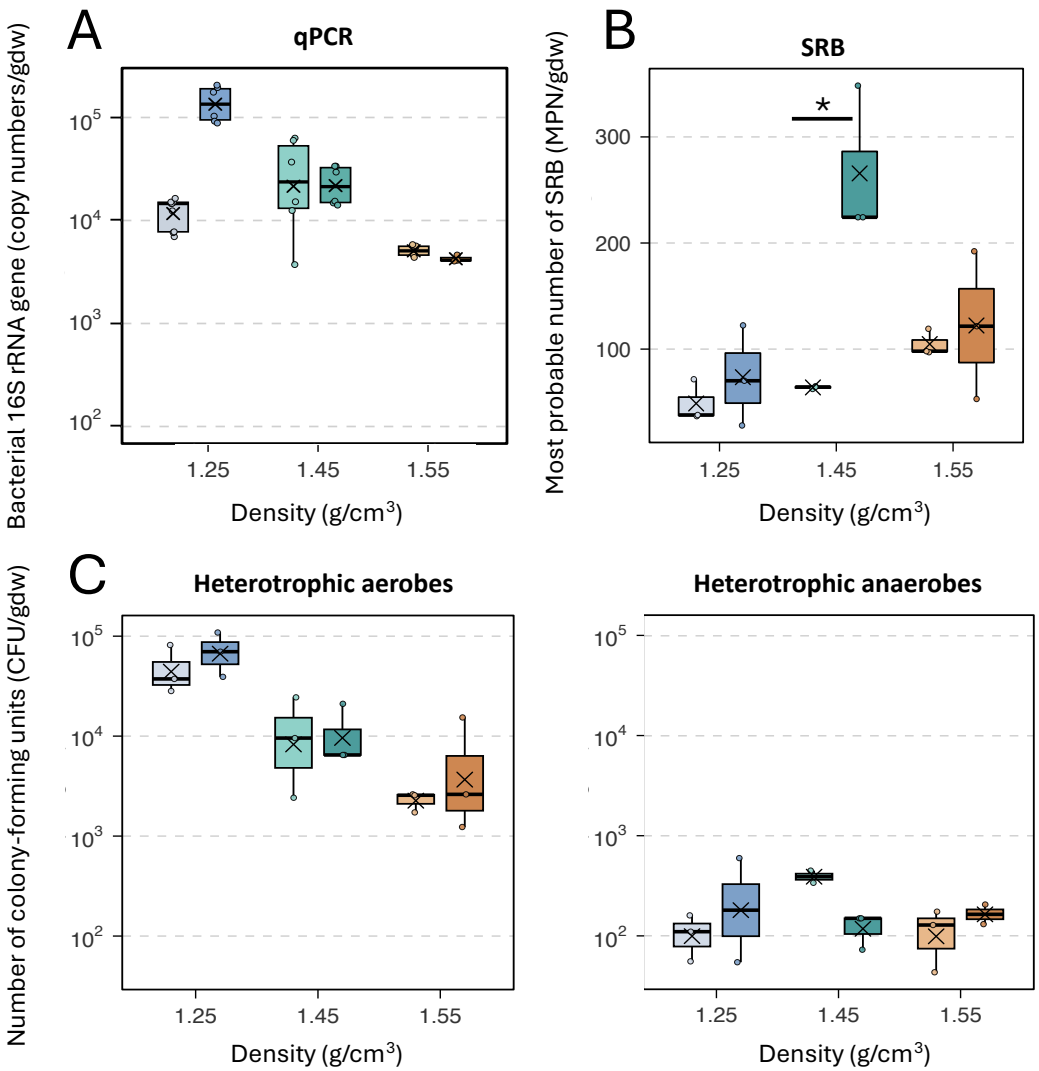

Figure 1. Microbial abundance and viability in bentonite after 8.5 years of incubation. For each dry density, inner core samples are shown in the left box and outer core samples in the right box. **(A)** 16S rRNA gene copy numbers per gram dry weight (gdw) determined by qPCR after 8.5 years of borehole incubation**. (B)** Sulfate-reducing bacteria viability determined by MPN counts (linear scale). **(C)** Number of colony-forming units (CFU) of heterotrophic aerobes and anaerobes ($\log_{10}$ scale). Colors indicate bentonite density (1.25, 1.45, and 1.55 g/cm) and sample position. Boxes show the interquartile range (IQR), the line indicates the median, whiskers extend to 1.5× IQR, crosses show the mean, and points represent individual samples. Asterisks indicate significant differences (*$p < 0.05$).

The large difference in cultivable aerobes and anaerobes has been previously reported in other long-term *in-situ* incubation experiments [12, 17] and suggests that at least some of the aerobes found in bentonite incubated under anoxic conditions for 8.5 years are obligate aerobes. If this were not the case, the cultivation on the anoxic medium should result in higher CFU counts, supporting both obligate and facultative

anaerobes. Further, this suggests that either the viable aerobes persist in compacted bentonite in a dormant state, without active growth, or that even after 8.5 years, they are still at least partially supported by residual oxygen as previously described [12, 35].

Cultivable sulfate-reducing bacteria (SRB) were detected in all bentonite cores after 8.5 years of incubation. No consistent relationship was observed between SRB abundance and bentonite density. In the outer core, the difference between the lowest (1.25 g/cm³; 73.5 ± 47.3 MPN g^{-1}dw) and highest density (1.55 g/cm³; 122.3 ± 69.6 MPN g^{-1}dw) was not significant, whereas the 1.45 g/cm³ module showed significantly higher values (265.6 ± 71.7 MPN g^{-1}dw). This indicates that bentonite formulation (blocks vs. pellets), rather than density alone, might play a more important role in SRB presence and survival. Except for the 1.45 g/cm³ case, no significant differences were observed between outer and inner core samples.

Across both cultivation-dependent and -independent assays, slightly higher abundances were often detected in the outer parts, but these differences were generally not significant. This suggests that small-scale spatial gradients within the cores are negligible. Therefore, for consistency with previous studies, outer and inner samples are averaged in the rest of the manuscript when compared with previous time points.

Time is the dominant driver of long-term microbial community change

Ordination analyses based on 16S rRNA gene sequencing data indicated that incubation time was the primary driver of bentonite community composition. PCoA demonstrated a strong separation between 5.5-year and 8.5-year samples (**Figure 2A**). An RDA showed that time explained approximately 78% of the total variance for

bentonite samples (**Figure 2A, B**), while borehole porewater communities remained similar (**Figure 2B**). This temporal change was far more explanatory than density or formulation (**Figure 2B**). These results suggest that microbial communities in compacted bentonite change slowly but persistently over nearly a decade, even under, and perhaps in response to, extreme nutrient and physical limitations. After accounting for the strong temporal effect, density was shown to be the main factor influencing community composition at each time point (**Figure 2B**). The impact of formulation (block vs. pellet) could not be fully determined due to limited replication, as only one density was tested in the block form. Overall, these findings suggest that bentonite density initially structures microbial community composition, after which temporal effects become increasingly dominant, driving stronger divergence over time (5.5 and 8.5 years).

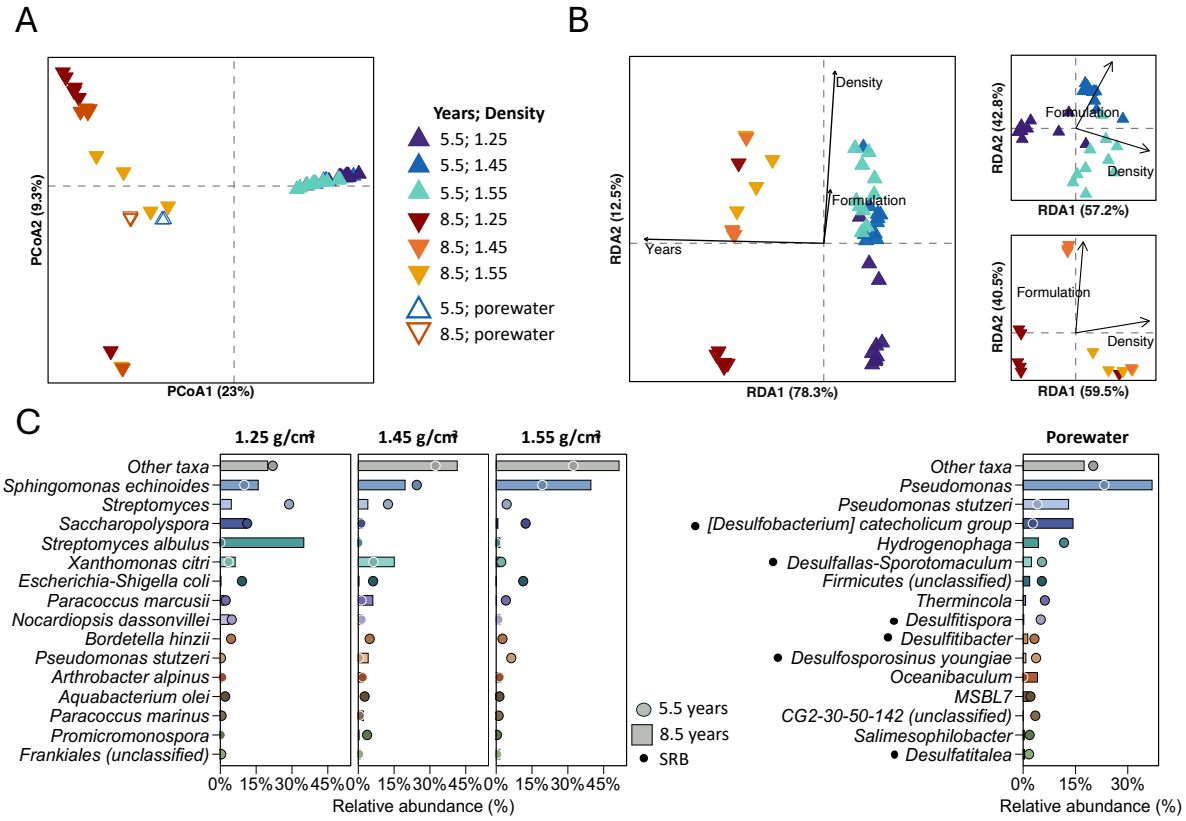

Figure 2. Microbial community structure and dynamics in compacted bentonite and porewater. **(A)** PCoA of microbial community composition based on 16S rRNA gene sequencing data. **(B)** RDA of bentonite samples illustrating the influence of dry density and incubation time on community structure. The three subpanels correspond to all samples (left), 5.5 years (top right) and 8.5 years (bottom right). **(C)** Relative abundance of the 15 most abundant bacterial taxa across bentonite densities (1.25, 1.45, and 1.55 g/cm³) and porewater; dots indicate 5.5-year samples and bars indicate 8.5-year samples.

Bentonite and borehole porewater host distinct microbial communities

To identify which microbial species are changing in abundance over time, the communities were sorted by the 15 most abundant microorganisms in bentonite and in porewater, all bacteria. Among all bentonite samples, a small set of taxa dominated the bentonite microbiome, including *Sphingomonas*, *Streptomyces*, *Saccharopolyspora*, *Xanthomonas, and Nocardiopsis spp.*, previously identified as the core bentonite microbiome [8] (**Figure 2C**). These taxa are characterized by adaptation to extreme environments via metabolic versatility, stress tolerance, and in some cases, spore formation, and oligotrophy [36–42].

Relative to the earlier sampling (5.5-year time point), density-dependent trends were observed, with *Sphingomonas* and *Streptomyces albulus* increasing at 1.25 g/cm³. At 1.45 g/cm³, *Paracoccus* and *Pseudomonas stutzeri* became more dominant. At 1.55 g/cm³, most taxa declined, except for *Streptomyces echinoides*, suggesting that time selects for a narrow set of highly resilient organisms. In terms of absolute abundance, consistent with the overall decline in total 16S rRNA gene copy numbers, all taxa decreased after 8.5 years of incubation compared to 5.5 years, regardless of bentonite density. The only exception was *Streptomyces albulus*, which increased to 2.26×10^4 16S rRNA gene copies at a density of 1.25 g/cm³, making it the most abundant microorganism in all bentonite samples after 8.5 years (**Figure S3**; see SI for further discussion). Archaea were also detected in bentonite samples after 8.5 years but did

not exceed 0.3% of the total relative abundance, except in samples collected from the interior of module M8 (1.55 g/cm³, blocks), where they accounted for 3.8% of the overall microbial community. Details and discussion of archaeal community composition are provided in the SI.

The porewater showed a distinct taxonomic composition to that of the bentonite-associated communities. It was dominated by two *Pseudomonas* spp. and by taxa commonly classified as sulfate-reducing bacteria (SRB), which accounted for 21.6 ± 0.77% of the community at the time of module retrieval after 8.5 years of incubation (**Figure 2C**). Some SRB lineages, such as the *Desulfobacterium catecholicum* group, increased over time (from 2.78 ± 0.72 to 14.31 ± 1.39). Other SRB among the 15 most abundant porewater taxa included members of the *Desulfallas–Sporotomaculum* group, *Desulfosporosinus youngiae*, and *Desulfatitalea* spp.. Other sulfidogens, the sulfite reducers *Desulfitispora* and *Desulfitibacter*, were also prominent in the community.

Despite the long incubation time of the bentonite in the borehole, there was remarkably little taxonomic overlap between the borehole porewater and the bentonite communities. This indicates a very limited capacity of borehole microorganisms to colonize the compacted bentonite matrix, even after nearly a decade of physical contact. This observation is consistent with previous long-term *in-situ* studies in the same borehole [12] and with recent work showing that porewater-derived microorganisms penetrate only the outermost ~1 cm of the compacted bentonite [35].

Sulfate reducers persist as a rare biosphere in bentonite

Because the bentonite community is dominated by low-abundance taxa, we analyzed community structure using rank abundance. Taxa were classified into categories

based on their relative abundance across samples, defining abundant, intermediate (undetermined), and rare fractions (see Methods). This classification allowed us to assess whether sulfate-reducing bacteria (SRB) preferentially occur within specific abundance fractions and how this distribution may change over time and across densities.

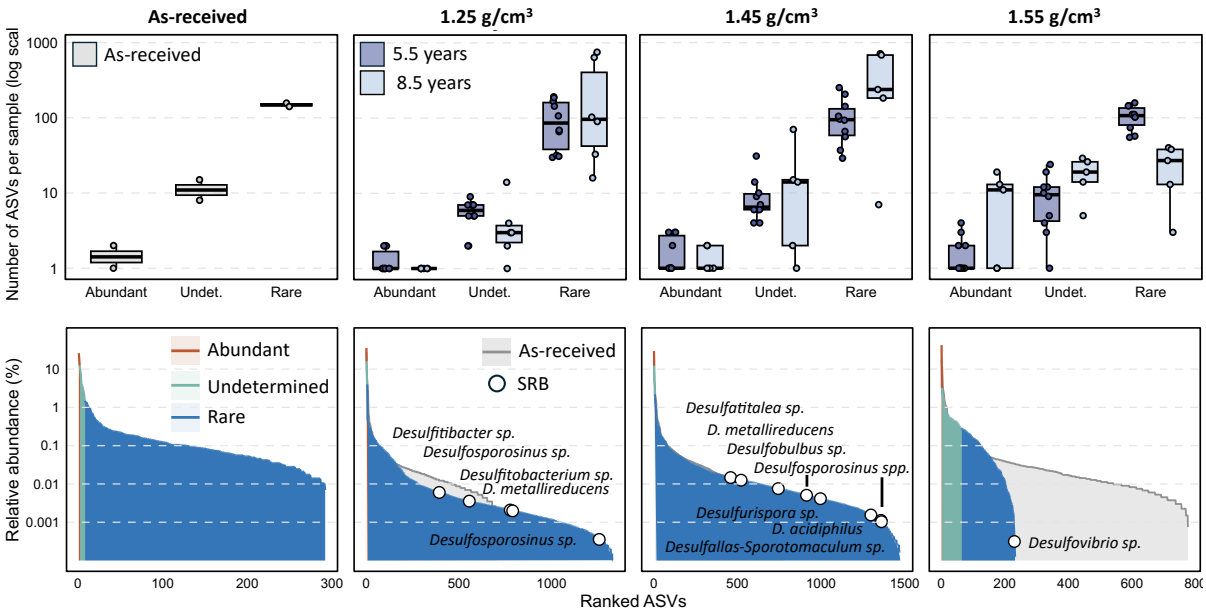

Figure 3. Unsupervised learning-based classification of the bentonite microbiome into abundant, rare, and undetermined populations. Top three panels: boxplots showing the number of abundant, rare and undetermined ASVs for each incubation duration (5.5 or 8.5 years) and across dry densities: boxes show the interquartile range (IQR), the line indicates the median, whiskers extend to 1.5× IQR, crosses show the mean, and points represent outliers. Bottom three panels: rank abundance plots comparing as-received bentonite to community distribution after 8.5 years of incubation. SRB that were only detected at 8.5 years are highlighted with white circles.

At low and intermediate densities (1.25 and 1.45 g/cm³), the relative contribution of rare taxa increased over time, while at high density (1.55 g/cm³), the rare fraction declined (**Figure 3**). One possible explanation for the apparent increase in the rare fraction is the gradual loss of previously abundant or undetermined taxa, which would cause them to fall into the rare category. However, this occurrence alone cannot fully explain the observed patterns, because abundant and undetermined taxa together

represent only a small fraction of the total community (approximately 10–100 out of ~1000 ASVs). Instead, the pattern could be explained by the emergence of slow growing, highly specialized microorganisms that were initially below detection. Conversely, the sharp decline of rare taxa at 1.55 g/cm³ indicates that even these specialists are unable to persist at high compaction. This suggests that a critical density threshold between 1.45 and 1.55 g/cm³ strongly limits microbial survival and leads to the decline of the rare biosphere.

At all three densities, taxonomically identified SRB-related taxa were exclusively found within the rare biosphere (≤0.01% relative abundance), with the highest **(Figure S4)** in the 1.45 g/cm³ case. At 1.55 g/cm³, only a single SRB-related species, *Desulfovibrio sp.,* was identified. Notably, SRB-related taxa were observed only in bentonite that had been incubated in the borehole and were absent from the as-received bentonite prior to incubation. This suggests that these SRB are either introduced from the surrounding porewater and subsequently migrate into the bentonite, though their migration is spatially limited [35], or more likely, are initially present in the bentonite at low abundances and become detectable only under borehole incubation conditions. However, their consistently low relative abundance, particularly at high dry density, indicates that while borehole conditions may support their persistence, the compacted bentonite matrix strongly suppresses their growth.

To assess how the sulfate reduction potential evolves over time, we used metabolic predictions derived from taxonomic data. PICRUSt2 analysis revealed no significant changes in the predicted abundance of genes related to sulfate reduction (**Figure 4A**) between 5.5 and 8.5 years. Consistent with this result, SRB MPN cultivation results (**Figure 4B**) showed no statistically significant temporal changes at 1.25 g/cm³ across

the compared timepoints (1.5–5.5–8.5 years). In contrast, at 1.55 g/cm³ (that has the same block formulation), a significant increase in SRB MPN counts was observed over time, indicating a selective advantage for the small subset of microorganisms capable of sulfate reduction, that persist and grow under conducive cultivation conditions. Note that the Postgate medium used for MPN was originally optimized for *Desulfovibrio desulfuricans* as a model SRB species [43], and because *Desulfovibrio sp.* were the only SRB detected at density of 1.55 g/cm³, they likely benefited both from reduced competition and from growth conditions designed for this group. None of the other metabolic groups tested in cultivation, including heterotrophic anaerobes and aerobes, showed a similar response at 1.55 g/cm³, with revived populations remaining stable (aerobes) or declining (anaerobes) (**Figure 4B**). This decoupling between abundance, diversity, and cultivability highlights the importance of monitoring rare taxa, which may carry the potential for critical processes such as sulfate reduction.

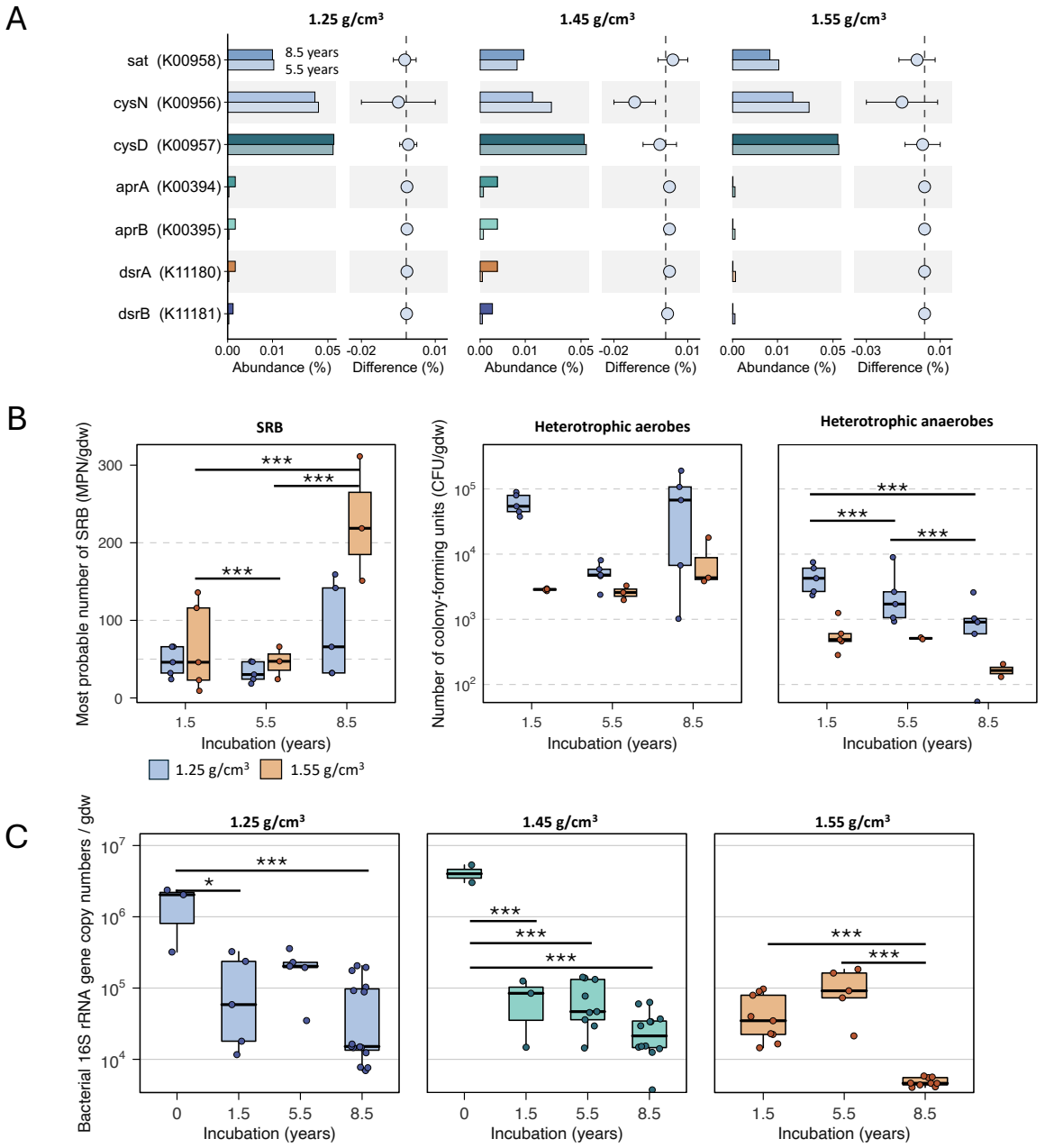

Figure 4. Temporal changes in sulfate reduction potential, microbial viability, and microbial abundance in compacted bentonite.

(A) PICRUSt2 predictions of genes associated with sulfate reduction in bentonite samples after 5.5 (lower bar) and 8.5 (upper bar) years of incubation for the three densities. **(B)** Cultivation-based most probable number (MPN) estimates of sulfate-reducing bacteria (SRB), aerobic heterotrophs, and anaerobic heterotrophs at 5.5 and 8.5 years. Note that for the density of 1.45 g/cm³, complete cultivation data from earlier time points are unavailable and therefore not included in the comparison. **(C)** Bacterial 16S rRNA gene copy numbers determined by qPCR for as-received bentonite and after 1.5, 5.5, and 8.5 years of *in-situ* incubation.

Overall microbial abundance and activity decline over time

A comparison of 16S rRNA gene copy numbers throughout all experimental phases (**Figure 4C**) revealed that gene copies quantified after 8.5 years of incubation are significantly lower (pairwise t-student, <0.05; **Table S3**) when compared to available reference materials before the deployment into the borehole and, in case of the most compacted bentonite, the 1.55 g/cm^3 case, also are significantly lower when compared to the last sampling point after 5.5 years of incubation. The decrease indicates bacterial death, but also the possible degradation of extracellular DNA (eDNA) that was initially present in the as-received bentonite but did not correspond to living cells. A decrease in the cultivability of anaerobic heterotrophs relative to previous time points further supports bacterial death over the years of incubation. Together, these results indicate a progressive loss of viable microbial biomass under bentonite confinement, particularly under the highest density compaction.

The microbial population data presented here, together with earlier time points from the same Opalinus Clay borehole at the Mont Terri Rock Laboratory [12], show a different pattern from that observed in a parallel experiment in granitic rock at the Grimsel Test Site. In an Opalinus Clay borehole, microbial abundance in bentonite declined after 1 year and continued to decrease over 5.5 and 8.5 years, whereas the microbial populations in bentonite in a Grimsel Test Site borehole remained stable over 1, 5 and 7 years [13, 14, 16]. This contrast may reflect differences in porewater chemistry. Opalinus Clay borehole water has circumneutral pH, high sulfate concentration but also high ionic strength (pH ~7.2–7.6, ~0.3–0.4 mol L^{-1} ionic strength, SO_4^{2-} ~10–25 mmol L^{-1})[44], whereas Grimsel borehole water has higher pH (~9–9.5), low sulfate (~0.06 mmol L^{-1}) and low ionic strength (~10^{-3} mol L^{-1})[45]. The

higher ionic strength corresponding to higher salinity may create stronger osmotic stress, potentially causing the observed decline. Except for the differences in abundance, both *in-situ* experiments show similar results: taxa typically classified as aerobic remain dominant despite anoxic conditions, while SRB can be cultivated after 7–8.5 years but remain a minor component of the community.

Conclusion and implications for the long-term performance of geological repositories

Our 8.5-year-long incubation experiment demonstrated that the microbial inhibition in underground repositories using bentonite as a backfill material is effective. For all tested bentonite buffer densities , microbial cell numbers remain low and generally decline over time, with dry density emerging as the dominant control on microbial persistence.

At the lowest tested dry density (1.25 g/cm³), microbial abundances were initially higher than at the more compacted densities. Nevertheless, no overall population increase was observed; instead, microbial numbers steadily declined throughout the experiment. These findings indicate that Wyoming bentonite compacted to 1.25 g/cm³, which is below the often-proposed target repository density of 1.45 g/cm³, is sufficient to prevent sustained microbial growth and serves as an effective biological barrier. At higher densities (≥1.55 g/cm³), both microbial abundance and diversity were strongly limited, including within the rare biosphere, suggesting the existence of a density threshold above which even highly specialized and slow-growing microorganisms cannot persist.

Importantly, sulfate-reducing bacteria (SRB), although recoverable in cultivation assays, did not increase in relative abundance in the community over time and

remained confined to the rare biosphere. This decoupling between cultivability and *in-situ* abundance indicates that SRB persistence reflects survival rather than actual growth, limiting concerns about the progressive enrichment of corrosion-inducing microorganisms within the bentonite due to developing anoxic conditions. In addition, the bentonite barrier effectively limited the colonization of borehole microorganisms, which is particularly relevant given that the surrounding porewater community contains a substantial fraction of SRB (up to 22%). This confirms that bentonite acts not only as a physical and geochemical barrier but also as an effective biological isolation barrier.

The detection of methanogenic archaea reaching up to 3.8% relative abundance highlights that certain anaerobic metabolic pathways can persist and even slowly emerge under repository-relevant conditions, suggesting that methane production cannot be excluded over very long timescales. However, the low absolute abundances and strong density dependence indicate that such processes are likely to remain spatially constrained.

Finally, the persistence of aerobic heterotrophs, despite long-term anoxic conditions, shows the importance of dormancy, metabolic flexibility, and maintenance energy strategies for microbial survival in compacted bentonite, and confirms that residual oxygen and redox niches may persist over longer timescales than often assumed.

Overall, our findings indicate that density is the primary factor shaping microbial communities in bentonite, while time becomes important at long temporal scales, driving further gradual changes. The communities that persist are characterized by low abundance, high specialization, and restricted function. Together, these processes lead to a progressive decline in microbial abundance and activity,

supporting the long-term effectiveness of Wyoming bentonite as a biological barrier in deep geological repositories.

Data availability statement

Raw DNA sequence data underlying this article are available in Zenodo repository under DOI https://doi.org/10.5281/zenodo.4883717 for the 1.5- and 5.5-year time points and under DOI https://doi.org/10.5281/zenodo.20964472 for the 8.5-year time point.

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Supplementary information

Microbial growth inhibition by compacted bentonite after an 8.5-year of *in-situ* incubation

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Supplementary Methods

Alpha diversity. Shannon diversity, Simpson diversity, and observed richness (ASV richness) metrics were calculated in R (version 4.5.2) [1] using *vegan* from the filtered ASV count table. Shannon diversity, Simpson diversity, and observed richness (ASV count) were computed per sample. Metadata were matched by sample ID, with starting material treated as a separate density category (Years = 0). Data processing was performed using *readr, dplyr, tidyr*, and *tibble*, and visualizations were generated in *ggplot2*, with means ± standard error and individual data points shown.

Supplementary Results and Discussion

Bentonite water content and density

The average observed dry density of the bentonite varied from the theoretical dry density for all modules and yielded 1.34 ± 0.16 g/cm^3 (n=3) for M7 (theoretical value 1.25 g/cm^3), 1.63 ± 016 g/cm^3 (n=3) for M8 (1.55 g/cm^3) and a lower value for than expected for M9 **(**1.45 g/cm^3): 1.40 ± 0.01 (n=3). It is important to note that density directly impacts microbial activity, which is why the obtained data were compared with values measured in previous retrieval phases to ensure comparability of microbial data between the phases. The data collected during phase 1 (1.5 years) did not follow the expected values, whereas those from phase 2 (5.5 years) and phase 3 (8.5 years) were closer to the expected value. The most discrepancy was observed for the 1.45 g/cm^3 samples, which is most likely related to the different formulations of the materials: pellets in phase 2 (marked with 'p' for pellets on **Figure S1**) and blocks in phase 3. Note that the method for observed dry density measurements has been improved between phase 1 and phase 2, which may explain the lack of consistency in the data from phase 1. Water content for the deployed bentonite differed depending on the density, with the highest water contents in blocks of lower density. Specific values on the observed dry density and water content collected from bentonite after 8.5 years are in the table (**Table S1**).

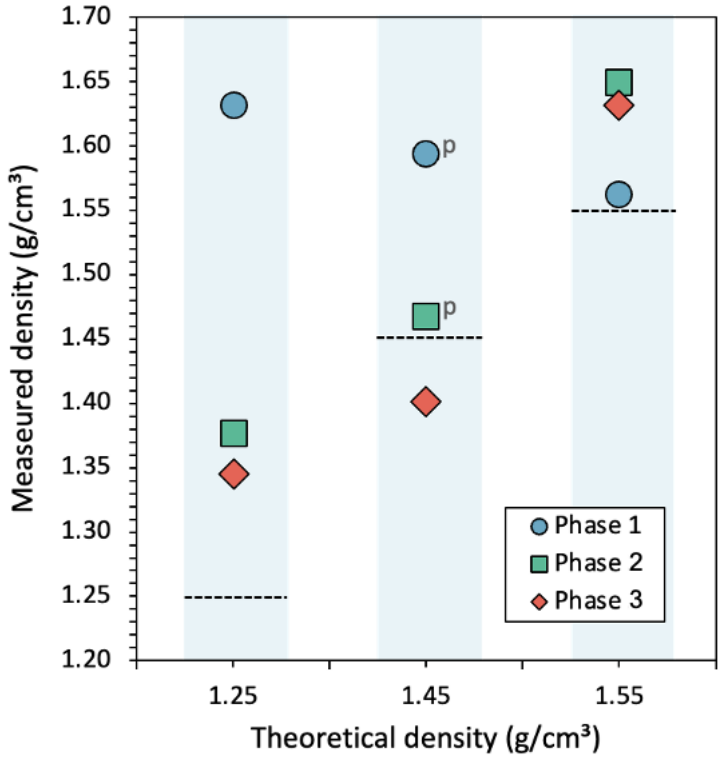

Figure S1. Comparison of observed dry densities measured after the retrieval of modules at three different targeted densities (1.25, 1.45 and 1.55 g/cm^3) density across three phases (1 (blue circles), 2 (green squares), 3 (orange diamonds)) corresponding to *in-situ* incubation time of 1.5, 5.5 and 8.5 years, respectively. The letter 'p' next to the marker points indicates bentonite in the form of pellets. For all other modules, bentonite in blocks was used. Targeted dry density is indicated by a horizontal dashed line.

Archaeal abundance (qPCR), identity and abundance after 8.5 years of incubation

Archaea 16S rRNA gene copy numbers were, on average, 4- to 3-orders of magnitude lower than bacterial copy numbers, showing that these microorganisms account for only a fraction of the microbial population. Unlike bacteria, the average number of gene copies was not related to density; there was no statistical difference between the different modules nor the external and internal parts of bentonite at any of the tested densities (post-hoc pairwise t-student, p=0.05). On average (bulk bentonite, both outer and inner layer combined), the highest numbers were found in pelletized bentonite (1.45 g/cm^3; $2.05\times10^1 \pm 1.87\times10^1$) and the lowest in highly compacted bentonite (1.55 g/cm^3; $^7.31 \pm 1.17\times10^1$). Archaea were identified in bentonite samples (and not porewater) but did not exceed 0.3% of relative abundance except for samples collected from the interior of module M8 (1.55 g/cm^3, blocks), where they reached 3.8% of relative abundance in the overall microbial community. Interestingly, all detected Archaea are linked to methane or ammonium metabolism. All archaea identified as members of the family of *Methanobacteriaceae,* known to have the genetic potential for formate and hydrogen/carbon dioxide utilization for methanogenesis [2, 3] were related to *Methanobacterium beijingense*, *M. marisnigri* and two unidentified species. Next, *Nitrosopumilaceae* were identified with close relatives to *Candidatus* Nitrosotenuis and other unidentified species, all potential ammonia-oxidizing archaea [4, 5]. Besides that, members of Order *Woesearchaeales* were found, which has been previously proposed to be anaerobic heterotrophs in a potential syntrophic relationship with methanogenic archaea [6].

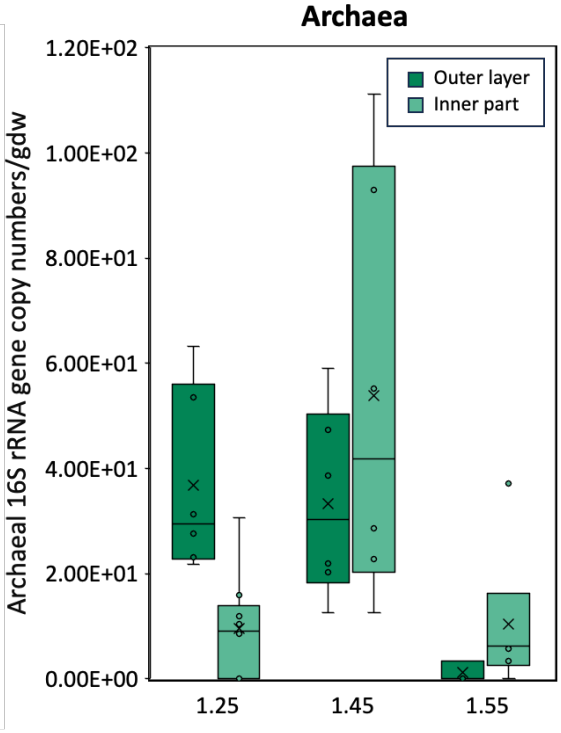

Figure S2. Archaeal abundance as 16S rRNA gene copy numbers per gram of bentonite (dry weight) at varying densities: 1.25 g/cm^3, 1.45 g/cm^3 and 1.55 g/cm^3. Data are presented in a box and whisker plot in which the median is indicated with a cross, the mean with a line, each data point with a dot, the box shows the 25th to 75th percentile and the whiskers show the maximum and minimum values of the data.

Microbial community identity and dynamics in compacted bentonite after 5.5 and 8.5 years of incubation

To evaluate absolute changes in the abundance of the top 15 taxa across all bentonite densities and complement the relative abundance analyses presented in the main text, the 16S rRNA gene copy–standardized abundances were compared between the two last time points (**Figure S4**). Consistent with the overall decline in total 16S gene copy numbers, nearly all dominant taxa showed decreased absolute abundances after 8.5 years of incubation compared to 5.5 years, regardless of density. The only exception was *Streptomyces albulus*, whose absolute abundance increased to 2.26×10^4 copies, but only at a density of 1.25 g/cm³, making it the most abundant taxon at the 8.5 time point across all densities.

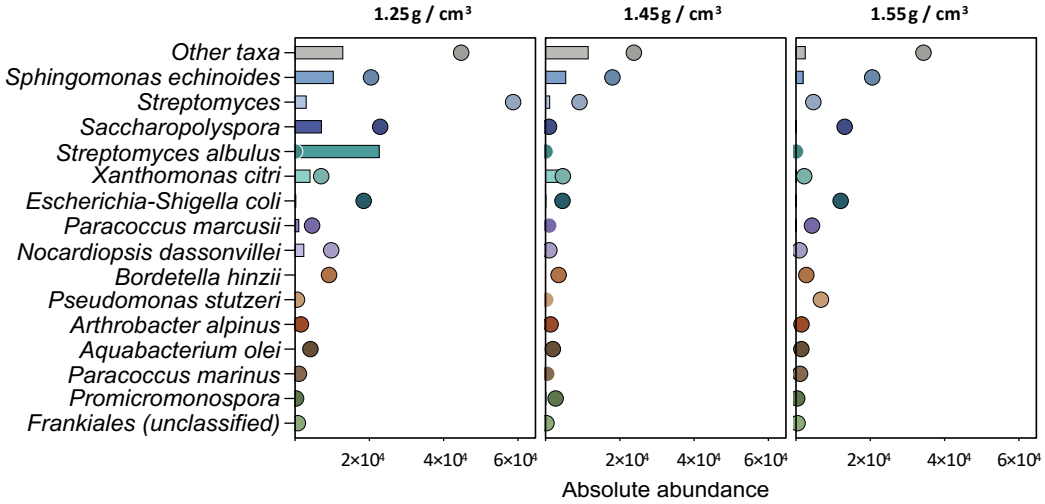

Figure S3. Absolute abundance of the 15 most abundant bacterial taxa across bentonite densities (1.25, 1.45, and 1.55 g/cm³) and porewater; dots indicate 5.5-year samples and bars indicate 8.5-year samples.

Diversity indices after 5.5 and 8.5 years of incubation

At a density of 1.25 g/cm³, richness increased over time, whereas both Shannon and Simpson diversity indices decreased. This indicates that although additional taxa became detectable by sequencing, they were predominantly very low in relative abundance, consistent with their classification as members of the rare biosphere. The decline in Shannon and Simpson diversity indices indicates increasing dominance by a small number of taxa, most likely *Streptomyces albulus* and *Sphingomonas echinoides* (**Figure 2C**). Together, these results suggest the progressive specialization of the community, with slow growth of a subset of bacteria (well-adapted to surviving and staying abundant, and very specialized). Note that there are only a few datapoints drawing the average upwards (richness) and downwards (Shannon and Simpson). Without these outliers, the community structure seems to be stable.
At a density of 1.45 g/cm^{-3}, richness increased or remained high over time, while Shannon diversity stayed high or slightly increased and Simpson diversity showed only a modest decrease. This indicates that although community composition may have changed, overall diversity and evenness were largely maintained. The 1.45 g/cm³

compaction therefore appears to represent an intermediate regime that is not permissive enough to allow some of the fast-growing taxa to dominate, as observed at lower density, but also not sufficiently restrictive to eliminate most taxa, as observed at higher density.
At 1.55 g/cm^3 density, the bentonite community decreased across all indices: richness, Shannon diversity, and Simpson diversity, indicating progressive community decline with a few persistent survivors.

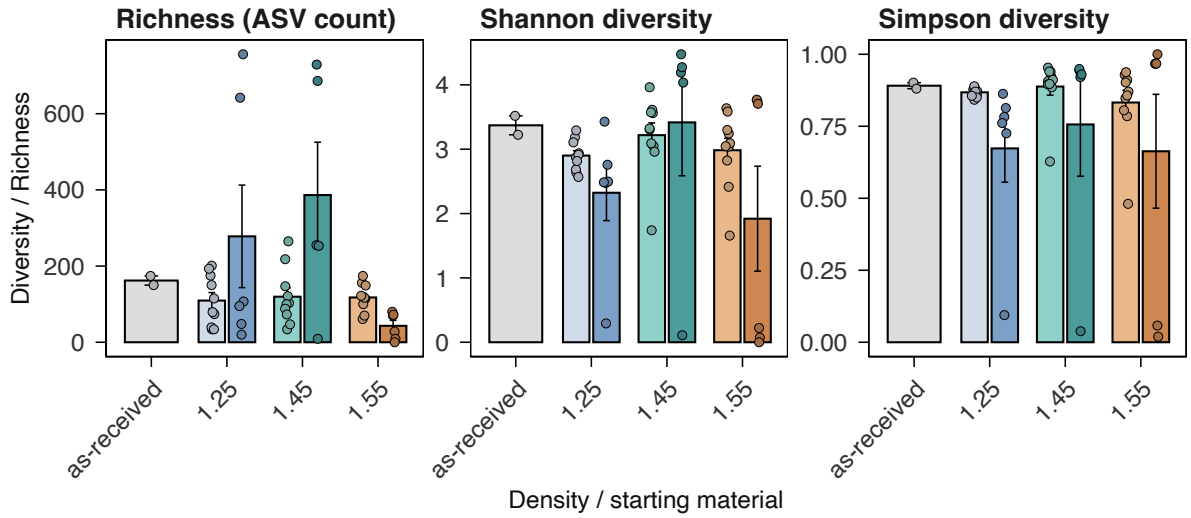

Figure S4. Richness, Shannon, and Simpson diversity indices compared between 5.5 and 8.5 years of *in-situ* incubation.

Supplementary Tables

Table S1. Bulk bentonite dry density was observed after the retrieval of modules together with water content relative to the wet mass and dry bentonite mass. All values are represented by average (n=3), while the errors indicate standard deviation (n=3).

	Theoretical dry density (g/cm^3)	Observed dry density (g/cm^3)	Water content (%$_{WT}$) *	Water content (%$_{DW}$) **
M7	1.25 (block)	1.34 ± 0.16	28.22 ± 0.22	39.92 ± 0.43
M8	1.55 (block)	1.63 ± 016	18.52 ± 0.04	22.73 ± 0.07
M9	1.45 (pellet)	1.40 ± 0.01	24.87 ± 0.26	33.11 ± 0.46

*Calculated as (weight of water x 100) divided by the weight of the original sample
**Calculated as (weight of water x 100) divided by the weight of the oven-dried (105°C; 24h) sample

Table S2. Significance values (alpha = 0.05) for comparison of cultivation results after 1.5, 5.5 and 8.5 years of the IC-A experiment, using pairwise t-test. The second column shows the compared modules with their specific module ID (e.g., M2, M5, M7) together with years of incubation in parentheses. Modules M7-8 correspond to 8.5 years. Detailed data from earlier time points, together with a detailed description of modules M2-6, can be found in [7].

		Anaerobes		Aerobes		SRB	
		Inner	Outer	Inner	Outer	Inner	Outer
1.25 g/cm^3	M2 (1.5), M5 (5.5)	0.13391185	0.42504814	0.01022871	6.27535E-06	0.73932239	0.20948083
	M5 (5.5), M7 (8.5)	0.18118355	0.42504814	0.00831548	0.003235285	0.04555373	0.03452697
	M2 (1.5), M7 (8.5)	0.00112907	0.05078041	0.29756575	0.0322084	0.05917991	0.06075886
1.55 g/cm^3	M3 (1.5), M6 (5.5)	0.88377157	0.04474357	0.34473069	0.198049924	0.12304841	0.45689076
	M6 (5.5), M8 (8.5)	0.00872301	0.01292808	0.03470127	0.165240569	1.2815E-05	0.01962877
	M3 (1.5), M8 (8.5)	0.00870298	0.11288825	0.8975601	0.26595023	0.07498819	0.05475674

Table S3. Significance values for qPCR comparisons between modules collected across different phases of the IC-A experiment. Materials include reference bentonite (as-received, Years = 0, or 1.5 years where no as-received control was available) and compacted bentonite after 5.5 and 8.5 years of in situ incubation. Modules are abbreviated as M followed by the module ID, with incubation time indicated in brackets. Detailed data from earlier time points used in these analyses are reported in [7].

	Materials compared	Pairwise t-test
Comparison to reference material (as-received)	Reference 1.25 g/cm^3 (0), M7 (8.5)	0.0002378
	Reference 1.45 g/cm^3 (0), M9 (8.5)	0.00948604
	M14 (1.5), M8 (8.5)	0.00047849
Comparison to the last sampling point (5.5 years)	M5 (5.5), M7 (8.5)	0.0002378
	M15 (5.5), M9 (8.5)	0.00948604
	M6 (5.5), M8 (8.5)	0.00047849

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