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Microbial community associated with the corrosion of steel in a bentonite matrix

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Publishable Summary

A long-term *in situ* corrosion experiment is ongoing in the Mont Terri Underground Research Laboratory in Switzerland to (i) measure the *in situ* corrosion behavior of carbon steel in compacted bentonite under simulated repository conditions and (ii) study the effect of the bentonite buffer density on microbial activity and microbially-influenced corrosion. Here, we investigate the composition of the microbial community in the bentonite in the vicinity of steel coupons and in other locations. After 1, ~2.5, or 5.5 years of exposure to the porewater, the bentonite was sampled and the microbial community characterized using molecular tools. A pattern emerges from the results as a function of space. In the bulk bentonite and near the corrosion coupons, there is no evidence of abundant sulfate-reducing bacteria (SRB). In contrast, on the surface of the canister, and on the surface of the bentonite, in black spots, SRB are clearly present and growing. The bulk bentonite harbors primarily aerobic microorganisms, suggesting the persistence of adsorbed oxygen in the bentonite for extended periods of time. Questions remain about the further evolution of the system as to whether SRB will progressively colonize an increasingly thicker layer of the bentonite. Further time points in this 10-year experiment will be able to address this question.

Contents

1 Introduction

Nagra (National Cooperative for the Disposal of Radioactive Waste, Switzerland) is considering using carbon steel as a potential canister material for the disposal of high-level waste and spent fuel in a deep geological repository in Opalinus Clay rock. Bentonite clay with a dry density of 1,450 kg m^{-3} is proposed to backfill the emplacement tunnels and will be placed around and between the disposal canisters.

Here, we report on the corrosion experiment IC-A (iron corrosion) in the Underground Rock Laboratory (URL) Mont Terri, St.-Ursanne, Switzerland. The IC-A experiment is tasked with investigating the *in-situ* corrosion rates of steel with the overall long-term objective of providing measurements of the corrosion rate of carbon steel in compacted bentonite under simulated repository conditions. The results have been published by Smart *et al.* [1] and some are presented in this document, along with updated microbial results.

The potential role of the microbial community on the rate of corrosion is another goal of this experiment. The major goal of the work presented here is characterize the microbial community associated with steel, in the bentonite and in the surrounding borehole.

This report describes the set-up of the experiments and summarizes the results of the microbial community for three time points (1 year, 2.5 years, and 5.5 years).

1.1 Experimental layout

The experiment was installed in a vertical descending borehole in Opalinus Clay rock. Stainless steel modules, containing the carbon steel corrosion coupons (20 mm of diameter, thickness of 10 mm) embedded in compacted bentonite or in a pellet-powder mix of bentonite, with a range of controlled dry-densities, were prepared under anoxic conditions and inserted into the borehole, which contained natural Opalinus Clay porewater, and then sealed to maintain the long-term, low-temperature, anoxic conditions representative of those expected in a deep geological repository. The modules permitted the free exchange of water with the host rock. A set of 12 modules (Modules 1 to 12) was initially installed in the borehole; they will be removed and analyzed, and some will be replaced with newly prepared modules, according to a planned schedule over a 10-year period. In particular, Modules 13-15 were deployed 1.5 years into the experiment and Modules 16-18, 4 years into the experiment.

1.1.1 Bentonite preparation

Volclay MX80 bentonite from Wyoming, U.S.A. was used throughout the experiment. Four different conditions of bentonite in two different formulations were used, as follows:

- 1250 kg m^{-3} , 1450 kg m^{-3} and 1550 kg m^{-3} compacted blocks: the compacted bentonite was provided by Clay Technology, Sweden. A 100 mm diameter mold that incorporated recesses for the corrosion coupons was manufactured. The bentonite was prepared to give a 95– 99% degree of saturation using deionized water. It was placed in the mold, flushed with nitrogen and then placed under vacuum during compaction, in order to minimize the amount of residual oxygen present in the bentonite at the start of the experiment. The blocks of bentonite were then handled and stored in a nitrogen atmosphere until the modules were assembled.
- 1450 kg m^{-3}, using a mixture of pellets and powder: this granular material was provided in the required density.

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1.1.2 Assembly of corrosion test modules

The test modules were fabricated from stainless steel and were 250 mm long with an external diameter of 126 mm. The sintered stainless steel filters had an external diameter of 106 mm, a wall thickness of 3 mm, a porosity of 30% and an average pore size of 18 µm. The housings and filters for each module were cleaned in acetone and deionized water and then the modules were assembled in an argon-filled glove box (<0.1 ppm oxygen) to ensure that the starting conditions for the experiments were anoxic. Four layers of coupons, with three specimens in each layer, positioned at 120° intervals were installed in each module (Figure 1). The outer edge of the coupons was located 5 mm from the outer circumference of the bentonite. The modules were then pre-saturated with anoxic artificial Opalinus Clay porewater by placing them in water-filled plastic containers for 2 days in the case of modules containing bentonite blocks, while the powder-pellet bentonite modules were soaked for 10 days. After soaking, the modules were heat-sealed into three layers of low permeability plastic (Mylar™) bags to retain an anoxic argon atmosphere during transport.

Figure 1: *Schematic cross-section through a test module showing the arrangement of corrosion coupons. Figure obtained from Smart et al.* [1]

1.1.3 Exposure

A 15 m deep vertical descending borehole was drilled in Opalinus Clay at the Mont Terri Underground Research Laboratory St. Ursanne, Switzerland on 20 March 2012. After drilling, the borehole was flushed with argon four times, pressurized at 3.5 bar and sealed with a 2.5 m long hydraulic packer to maintain anoxic conditions. After sealing, there was an inflow of natural Opalinus Clay porewater at a rate of 44 mL.day^{-1}. The modules were installed in the Mont Terri facility on 10 January 2013. At that time, samples from the porewater that had accumulated in the borehole were taken for analysis and an additional 9 L of anoxic synthetic Opalinus Clay porewater were added in the borehole. During exposure, the *in situ* borehole temperature was ~ 14°C.

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1.2 Removal and analysis

1.2.1 Removal of test modules

The first set of three of the test modules (Modules 1-3) was removed for analysis on 30 September 2014 after 20 (~1.5 y) months of exposure. The second set of three modules (Modules 13-15) was removed for analysis on 4 July 2017 (after 33 months (~2.5 y)). The third set of six modules (Modules 16-18 and Modules 4-6) was removed in July 2018 after respectively 12 and 66 months (1 y and 5.5 y) of exposure (Figure 2). During removal, porewater samples were taken from the borehole for microbial analyses. The modules were removed from the borehole while purging with argon and put into purpose-built stainless steel transfer flasks that were filled with water from the borehole and purged with argon before transport to the UK for analysis, where they were placed into a pre-cleaned and sterilized (wiped with a 70% isopropyl alcohol solution) argon-purged glove box for dismantling. The microbial community analysis was carried out for Modules 4-6 (5.5 years of deployment), Modules 13-15 (2.5 y of deployment), and Modules 16-18 (1 year of deployment). We are presenting the data available to date.

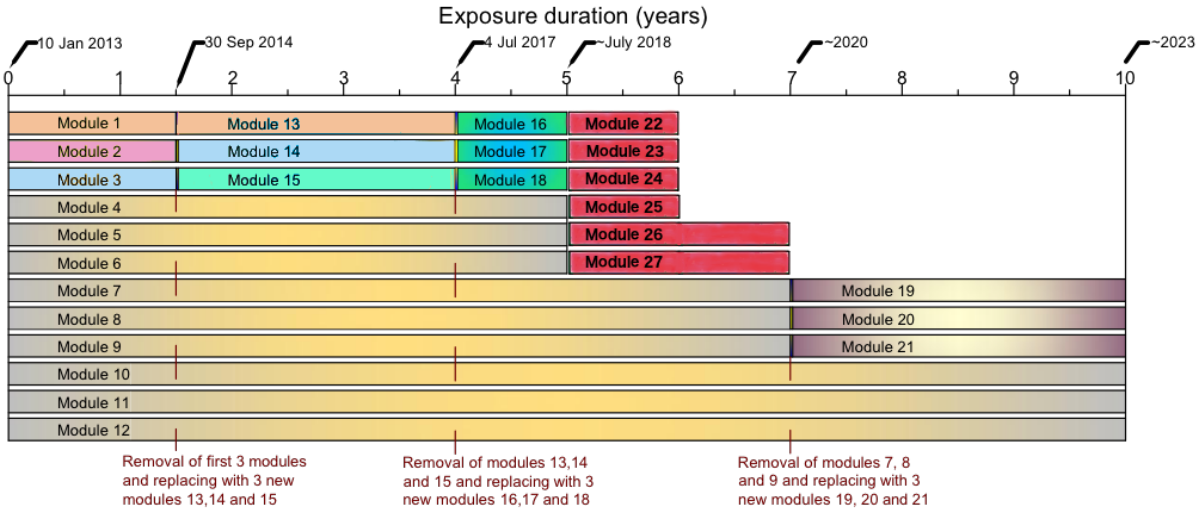

Figure 2: *Schematic of the deployment and retrieval schedule for the modules. In this report, modules 1-6, 13-15, and 16-18 are discussed.*

1.2.2 Microbial community analysis

The modules were shipped to the laboratories of Wood located at the Harwell Science Campus, Oxfordshire, United Kingdom. The external surface of the transport canisters was cleaned with 70% isopropanol and they were opened under anoxic conditions in a MBraun glove box filled with argon gas (MBraun, Garchingen, Germany). The modules were retrieved from the transport canister. Swab samples were taken from each transport canister to track any changes in the microbial community during the transport. The sintered filter was cut in half (longitudinally) with a metal saw (Dremel DSM20, Mount Prospect, IL, USA). Due to the resulting metal dust, the whole glove box was cleaned with 70% isopropanol before microbial sampling of bentonite. The upper half of the filter was removed, exposing the bentonite core. The lower half was left in place and placed in a vice for processing. The bentonite surface and the filter's interior were sampled with swabs. For each type of surface (bentonite or filter), swabs were used to sample the entire surface as well as black spots and other marks. Additionally, for some black spots, a bentonite sample was collected for DNA isolation.
Sampling of the bentonite was carried out as follows: slices of half cylinders of bentonite between the embedded coupons were collected in sterile bags. After all 5 layers were collected, the samples were sealed in a double layer of MYLAR to keep them in an anoxic environment. The cutting of the bentonite

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was carried out with spatulas and knifes of different sizes disinfected with 3% bleach solution and rinsed with sterile water. The surfaces of cleaned spatulas and knifes were sampled to assess potential contaminations. The bentonite samples were shipped and stored at 4°C until cultivation. All other samples (i.e., samples of swabs and cuts of black spots for DNA isolation), were kept at -20°C until DNA isolation.

Figure 3: Pictures of Module 13.

From left to right. Opening of transport canister in glovebox (left), removal of top lid (middle), removal of stainless steel case (right). The exposed filter exterior was sampled twice with swabs.

DNA extraction from swabs

Swabs were prepared for DNA extraction as described by Engel *et al.*[9] using a DNeasy PowerSoil Kit except for using a bead beater (Precellys24, Bertin Technologies, Montigny-le-Bretonneux, France) at 5.5 m/s for 45 seconds and for storing the recovered elution in 60 µL at -20°C rather than as 2x 30µL aliquots. In order to recover 60µL, 65µL were applied to the silica filter tubes to elute the genomic DNA.

DNA extraction from bentonite

Bentonite samples were prepared for DNA extraction together with the samples for cultivation, as described above. The modified extraction method was described by Engel *et al.*[9]. A mass of 2 ± 0.2 g of bentonite from the inner and outer layer were used for DNA extraction with the DNeasy PowerMax Soil Kit (Qiagen, Hilden, Germany). A modification to the manufacturer's protocol included use of glycogen (molecular biology grade, Merck Calbiochem, Darmstadt, Germany) and 0.1 x volume of 5M NaCl, (molecular biology grade) to precipitate DNA. In addition, a negative control (so called 'kitome') was prepared to track potential contamination introduced during the extraction.

DNA amplification with PCR for MiSeq DNA sequencing

515F-MiSeq
5'-TCG TCG GCA GCG TCA GAT GTG TAT AAG AGA CAG **GTG CCA GCM GCC GCG GTA A**-3'

Pro806bR-MiSeq
5'-GTC TCG TGG GCT CGG AGA TGT GTA TAA GAG ACA G**GG ACT ACN VGG GTW TCT AAT**-3'

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By the end of June 2018, this primer pair covered 76.8% of the SILVA Ref NR Database, of which 87.7% of Bacteria and 53.2% of Archaea are covered – considerably better than the V4-V5 region primers used for bentonite and swabs.
We used the PCR reaction mix KOD Hot Start (Merck KGaA, Darmstadt, Germany), which contains the following: a 1x concentration of a proprietary Buffer for KOD Hot Start DNA polymerase, 1.5 mM $MgSO_4$, 0.2 mM of each dNTP's, 0.3 µM of each 515F-MiSeq and Pro806bR-MiSeq primers and 0.02 U/µL KOD Hot Start polymerase and 10µL of a DNA template. In addition, the PCR conditions are listed in the tables below (**Table 1** and **Table 2)**.

Table 1: Composition of PCR solutions used to amplify the 16S rRNA gene V4 region.

	50µL=40µL MM + 10µL DNA µL per sample	final c
DEPC H2O	20	-
10x Buffer	5	1x
2 mM dNTP's	5	0.2 mM
25 mM MgSO4	3	1.5 mM
5 µM Primer +	3	0.3 µM
5 µM Primer -	3	0.3 µM
KOD Polymerase 1U/µL	1	0.02 U/µL
Total Vol [µL]	40	

Table 2: PCR temperature profile with 35 cycles in total to amplify the 16S rRNA gene V4 region.

temp	duration	
95	2 min	
95	25 sec	X34
50	15 sec	
70	15 sec	
70	3 min	

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1.3 Results

1.3.1 Bulk bentonite community

The results from the bulk bentonite correspond to the community that was identified within bentonite by extracting DNA from pieces of the compacted clay and differentiating samples obtained from the interior of the module (interior) or close to the edge of the module (exterior).

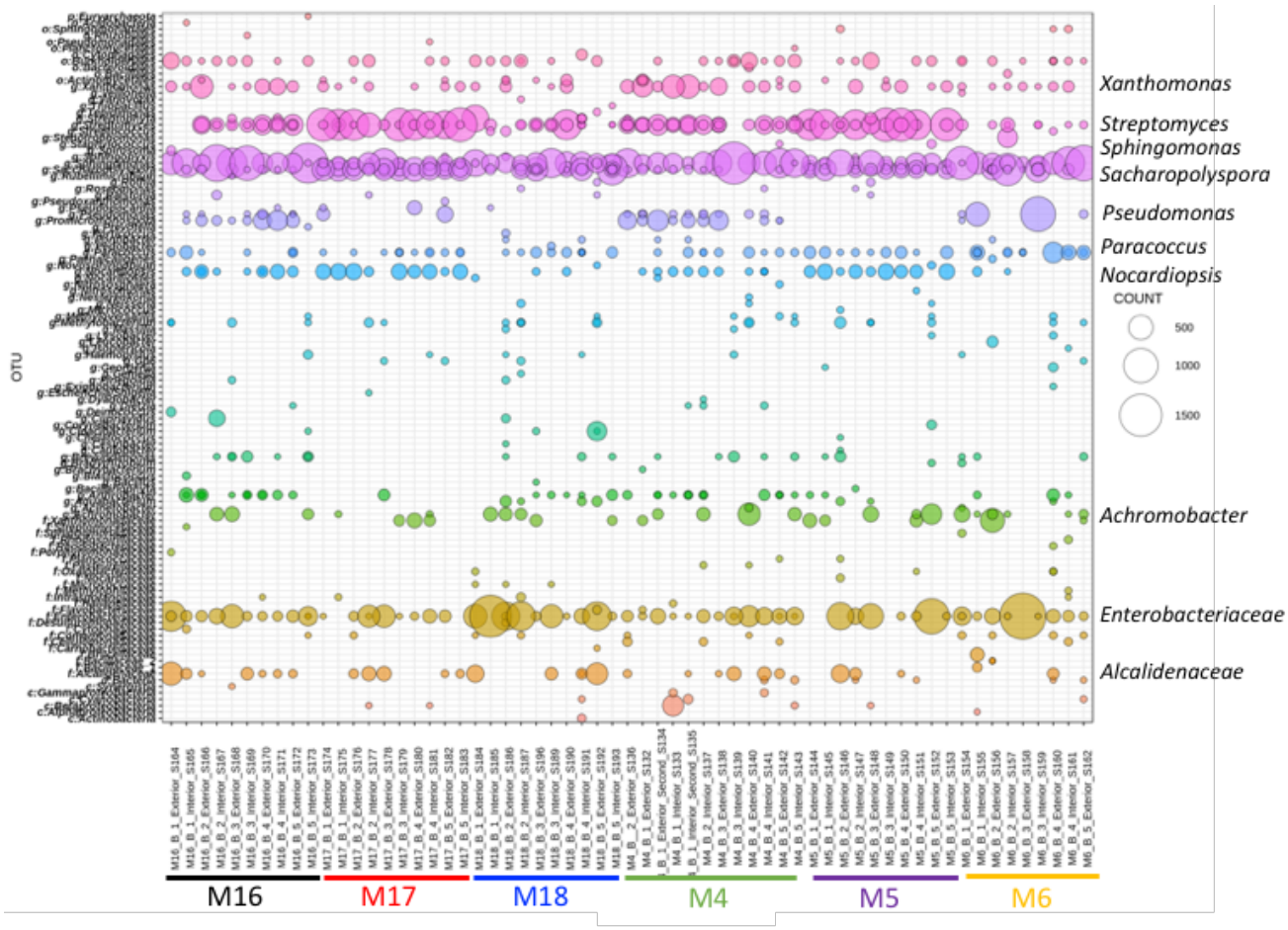

Figure 4: Abundance of OTUs for bulk bentonite across 6 modules and interior/exterior location.

The patterns are readily apparent when considering a large number of bentonite samples (Figure 4). More specifically, the dominant OTUs found in bulk bentonite of modules M4-6 and M16-18 belong to the genera *Streptomyces, Sphingomonas* and *Sacharopolyspora* with contributions from *Paracoccus*, *Nocardiopsis*, *Pseudomonas*, *Xanthomonas*, and *Achromobacter*. A previous sequencing effort using a separate primer set identified these OTUs at the species level as *Paracoccus marcusii*, *Pseudomonas stutzeri*, *Xanthomonas axonopodis*. Differences depending on the bentonite position (interior or exterior) were not readily observable. Interestingly, the major OTUs correspond to aerobic microorganisms (*Paracoccus, Xanthomonas, Streptomyces, Sphingomonas, Sacharopolyspora, Nocardiopsis*). This result suggests that either residual oxygen persists within the bentonite or there is substantial bias in DNA extraction. Furthermore, the contribution of sulfate-reducing bacteria was minimal.

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1.3.2 Steel or copper coupon regions

The microbial community associated with bentonite in the vicinity of the coupons was ascertained by using sterile swabs to sample the bentonite. Thus, the coupon was removed and the bentonite in contact with the coupon was typically discolored. DNA extraction yielded a greater amount of DNA from those regions as compared to bulk bentonite. The microbial community was dominated by the same OTUs identified in the bulk bentonite samples (Figure 5). There was no evidence for sulfate-reducing bacteria in this region.

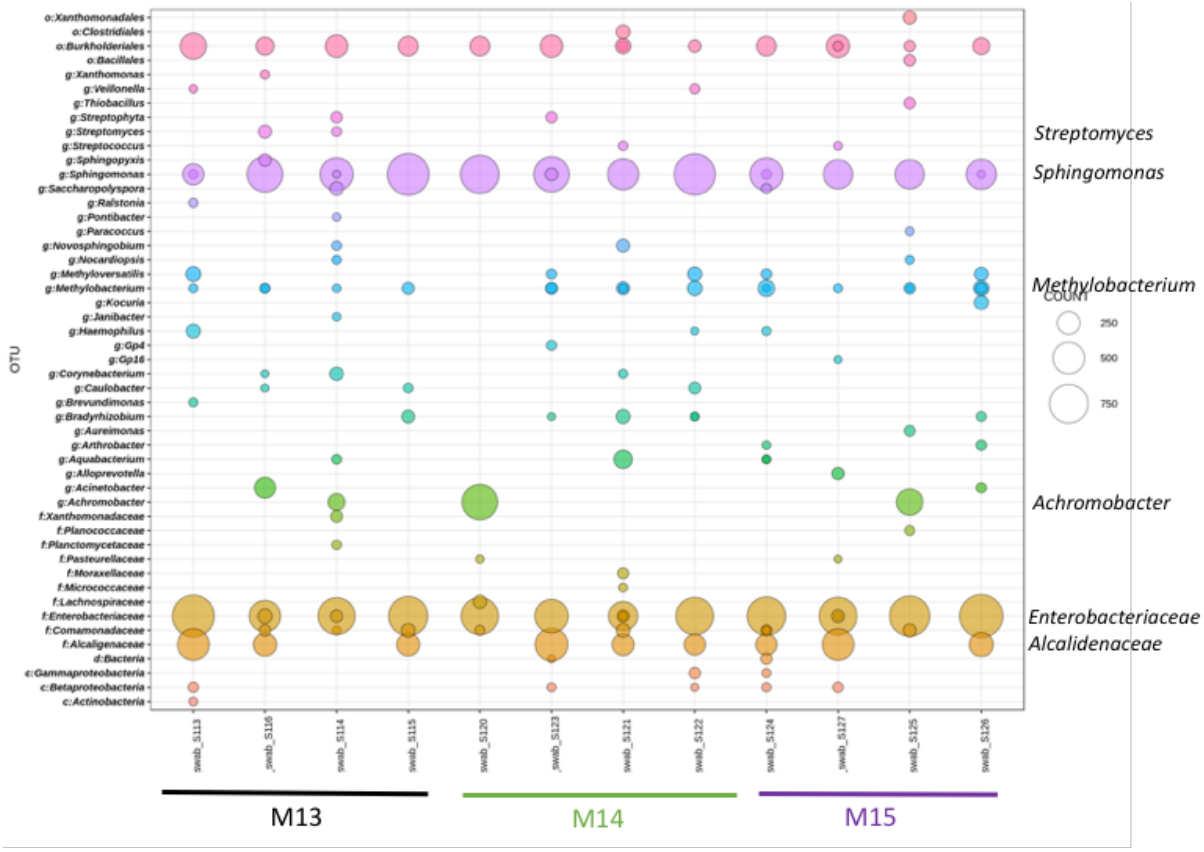

Figure 5: Abundance of OTUs associated with bentonite in the coupon imprint region.

1.3.3 Points of interest

A number of points of interest were sampled at the surface of the bentonite. They included black spots that were swabbed or cut out or other discolored regions (white areas). The OTUs detected bentonite points of interest are dominated by *Pseudomonas* and *Ralstonia* and, for the modules incubated the longest, a large contribution from *Desulfocapsa* (Figure 6). Additionally, in some samples, there is a small contribution from *Desulfosporosinus, Dethiobacter, Desulfuromonas,* and *Desulfitibacter*. Many of the latter are able to reduce intermediate valence sulfur compounds (sulfite, thiosulfate). The presence of *Pseudomonas* is not surprising, as it is found everywhere, including in previous timepoints. The large difference between the community in the bentonite bulk and the points of interest suggest that in the specific points, anoxic conditions prevail and allow for the development of sulfate-reducing bacteria and the associated sulfur-cycling community. Many of these genera are also found in the porewater microbial community, suggesting that the porewater is the source of these organisms.

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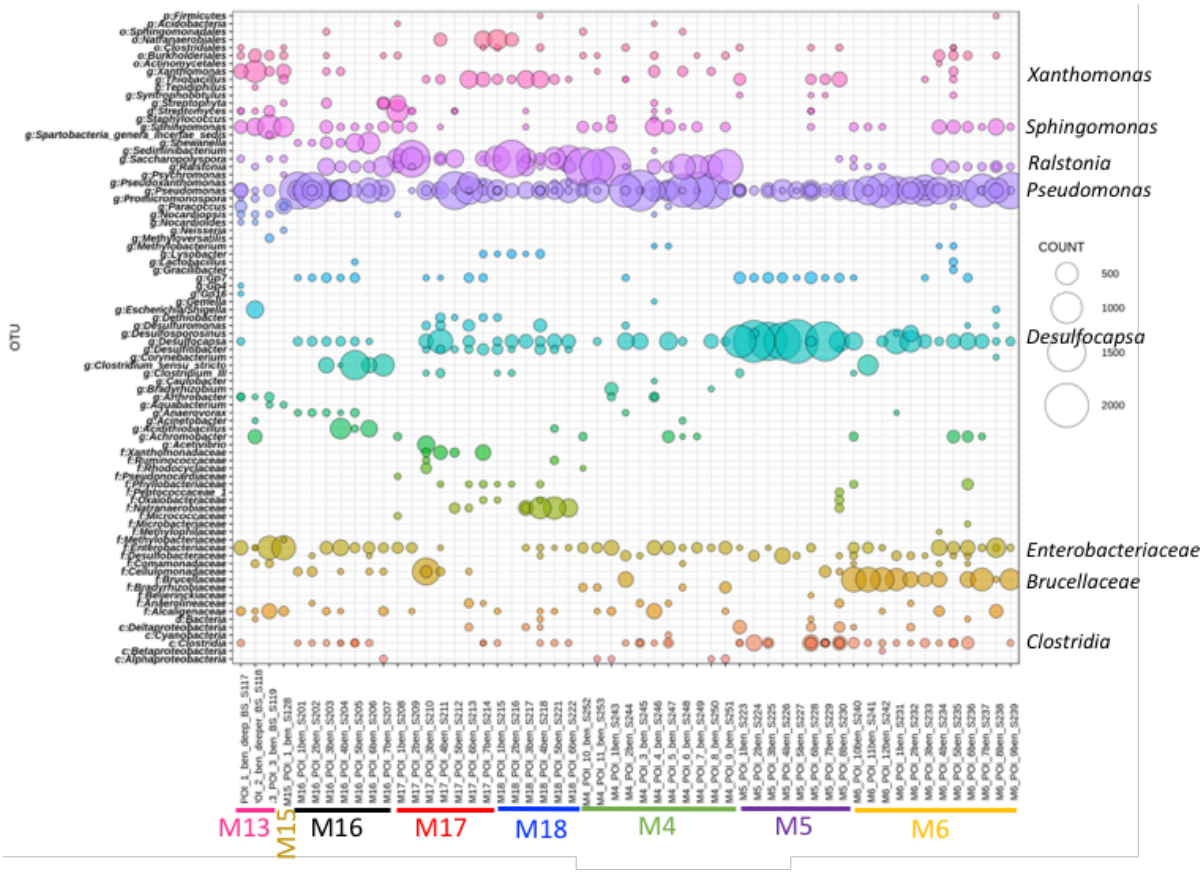

Figure 6: Abundance of OTUs associated with bentonite points of interest.

1.3.4 Module surface

Swabs taken from the modules exterior are dominated by OTUs known to thrive in the porewater of BIC-A1. This includes *Pseudomonas, Desulfotomaculum* and *Desulfusporosinus,* an SRB OTU related to the family of Desulfobulbaceae, but also Micrococcaceae, a family from the phylum Actinobacteria. Thus, the module surface is representative of the Opalinus Clay community, with a few exceptions (Micrococcaceae) that could be contaminants.

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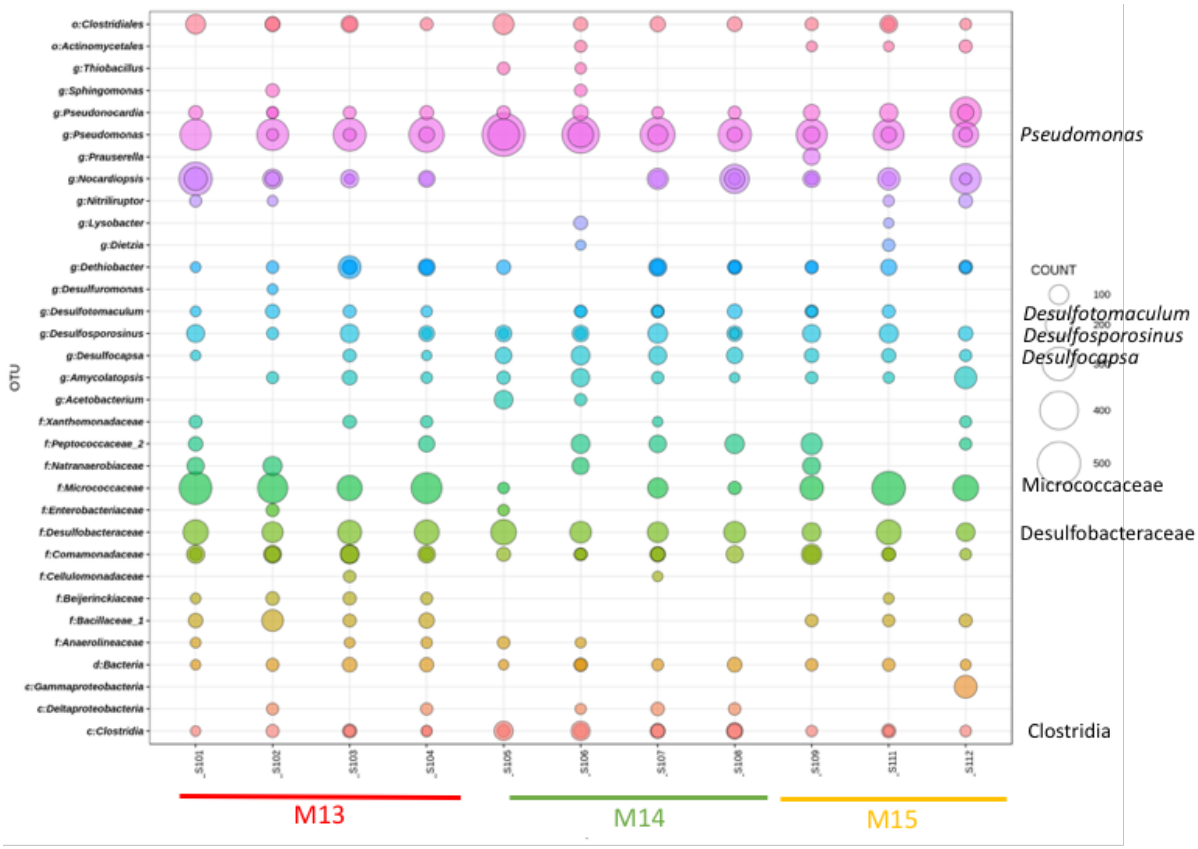

Figure 7: Abundance of OTUs associated with the surface of the module.

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2 Discussion

DNA extractions with the DNeasy kit were shown to yield a sufficient amount of DNA of sufficient purity for PCR-based amplification of the 16S rRNA gene. The PCR protocol was based on amplification of the V4 region. For which the primers (515F and 806bR) cover most of the bacteria but also 53.2% of Archaea.

The results of DNA sequencing from the module surface are consistent with previous results and the microbial community was similar to that observed previously in the porewater. *Pseudomonas*, the group of SRBs and the Peptococcaceae (c.f. Bagnoud *et al.*[13]) are all present and seem to be interconnected, cycling the carbon between each other, as proposed in that publication. The SRBs and Peptococcaceae act as primary biomass producers and Pseudomonas as the major biomass degrader.

The most salient result is the observed shift in the microbial community when comparing the bulk bentonite and the surface black spots. The bulk bentonite harbors primarily aerobic organisms such as *Streptomycetes* and *Sphingomonas*. In contrast, on the surface of bentonite, the black spots exhibit a population of strict and facultative anaerobes such as *Ralstonia* and *Pseudomonas* and SRB that contributed little to the community in the bulk bentonite. We interpret these data as the persistence of oxygen inside the bulk bentonite (most likely as adsorbed O_2), while anaerobic conditions are established on the surface of the bentonite, allowing sulfate-reduction to proceed (hence the presence of black areas/spots). At the longer deployment durations, some of the modules were entirely covered with a black layer, suggesting the colonization and growth of SRB. Near the coupons, we observed no evidence of change in the microbial community relative to bulk bentonite, despite a discoloration. We attribute the discoloration to secondary minerals derived from abiotic steel corrosion. Thus, it appears that SRB travel from the porewater, through the module case and through the module filter but may not grow within the bentonite very effectively.

As the results stand, there is no evidence for microbially-induced corrosion in bentonite in this experimental setup, despite the presence of viable SRB as demonstrated by cultivation in both deployed modules and the initial bentonite blocks (Deliverable D2.14). This could be due to the conditions being unfavorable for the growth and activity of SRB. Thus, SRB are undeniably present but in low abundance and are not active. The relevant question is: why are they inactive? One possible reason could be related to the abundance of aerobic organisms within the bentonite bulk. DNA sequencing revealed a higher contribution of aerobes to the microbial community in bentonite than expected, such as OTUs belonging to *Xanthomonas, Streptomyces, Sphingomonas,* and *Paracoccus.* A possible explanation could be the persistence of adsorbed molecular oxygen on the clay. This occurrence would favor aerobic organisms and would exclude the activity and growth of sulfate-reducing bacteria.

Other explanations also exist for the inactivity of SRB within the bentonite. The pore space and water activity could be too low to allow microbial growth. And other explanations exist for the presence of aerobic organisms in the bentonite. They could be dormant (inactive) but still viable, which would be supported by the cultivation results. Regarding SRB, the question remains as to whether porewater-derived microorganisms would be able to colonize an increasingly thick layer of bentonite as time progresses or whether their activity will remain limited to a thin layer at the interface of bentonite and the porewater.

Finally, It is important to note that the robustness of interpretation of the microbial community results is challenged by the relative high counts of PCR-related artefacts, many of which are prominent within the OTUs of the bentonite interior and exterior samples. Thus, these results are to be confirmed with future time points, particularly those pertaining to the abundance of SRB within bulk bentonite. It

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remains to be seen whether evidence for SRB growth will extend into the bulk bentonite over longer periods of time.

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3 Conclusions

The molecular biological methods offer deeper insights into the diversity of the microbial population than strictly cultivation. The SRB sphere of influence seems restricted to the porewater, the module case, module filter, and the surface of bentonite. Within bentonite, mostly aerobic organisms are identified by 16S rRNA amplification. At the moment, there is no clear explanation for this finding but we hypothesize i) the persistence of O_2 adsorbed to the bentonite or (ii) the persistence of inactive aerobic microbes or (iii) an experimental artefact in DNA extraction from bentonite. Cultivation of SRB from the bentonite only indicates that they are viable but does not provide proof of their activity.

4 Acknowledgement

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5 References

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