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Methane generation in the Mt Terri URL and the underlying microbial processes

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Publishable Summary

Methanogenesis is one of the potential microbial processes in deep geological repositories after closure. It could have several impacts on the repository environment due to the consumption and production of gases and induced changes in pH which can affect the surrounding geochemistry. It is potentially less harmful than sulfate reduction in a repository environment as it does not produce any corrosive substances and can consume hydrogen gas.

In-situ experiments at Mont Terri URL have shown that there is a diverse microbial community within borehole water which is capable of utilising hydrogen gas. Methanogens have been identified in this community at very low abundance (<0.1%), however the experiment showed no evidence for the onset of methanogenesis with no increase in methane concentrations over time. The composition of the microbial community varies with time, but there is no increase in the relative abundance of methanogens. Further *in-situ* experiments using a porous sand:bentonite medium showed dominance of methanogenic archaea and fermenting bacteria after nine months of incubation. Laboratory microcosm experiments where the initial sulfate concentrations were very low also show no evidence for methanogenesis even after sulfate depletion. There is no increase in methane concentration and at the end of the experiment the microbial community is dominated by one family of sulfate-reducing bacteria. Further analysis of the geochemical conditions in both experiments suggests that the bicarbonate concentration is too low in these systems which makes methanogenesis thermodynamically unfavourable. The effect of bicarbonate concentration on the onset of methanogenesis is being investigated using a series of laboratory microcosm experiments. Further to evidence that methanogenesis is extant in the presence of sand/bentonite, experiments are also ongoing to assess the impact of trace metal availability, which are essential for enzyme co-factors in methanogenesis.

Contents

1 Introduction

Methanogenesis is the formation of methane by microbes known as methanogens. Methanogens are from the Archaea domain which is distinct from eukaryotes and bacteria. Methane may have various impacts on a nuclear waste repository including changes to gas volume, methanogenesis would result in a net reduction of the gas volume within a repository reducing the pressure burden for the engineered barriers and host rock. Methane may also promote microbial growth by providing a carbon source upon cell death and necromass breakdown. Any increases in microbial activity can lead to changes in pH which can induce corrosion and geochemical changes including the precipitation and dissolution of minerals [1].

The objectives of WP1 include reducing the uncertainty surrounding the impact of microbial processes on radionuclide, chemical and gas release as well as quantifying the consumption and production of gases due to these processes. This section of work focuses on the ability of the Mont Terri and Opalinus clay environment to support and sustain methanogenesis with the aim of reducing uncertainty associated with methanogenic processes.

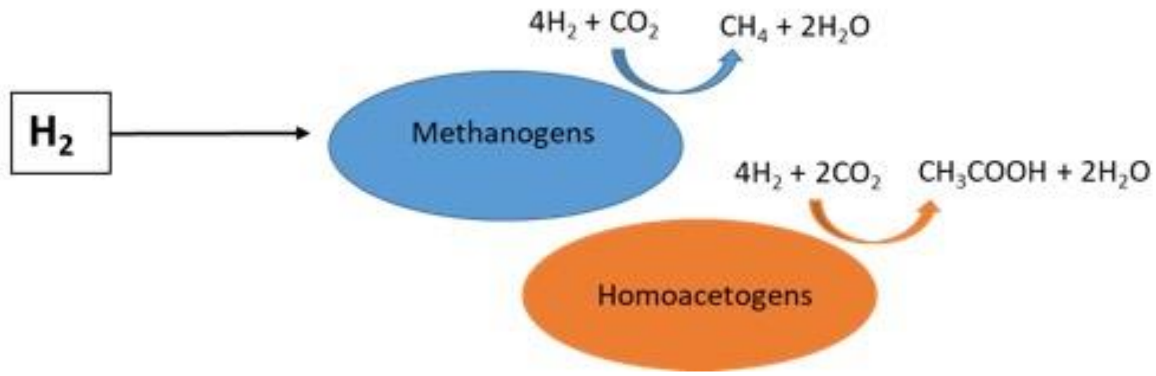

Figure 1 Microbial metabolism of hydrogenotrophic methanogens and homoacetogens

Previous *in-situ* borehole experiments in Opalinus clay have confirmed the existence of sulfate-reducing bacteria which can utilise H_2 [2]. The presence of sulfate is known to inhibit methanogenesis via substrate competition from sulfate-reducing bacteria [3,4], however it is possible that, during the lifetime of the repository as anoxic conditions are established, electron acceptors such as iron and sulfate will be depleted allowing methanogenesis to become the most dominant microbial process. The impact of this microbial process within the context of a deep geological repository is not fully understood. Thus, the goal of this work was to investigate the potential for *in situ* methanogenesis.

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2 In-situ borehole experiments

2.1 Materials and methods

In situ experiments took place in the BRC-3 bioreactor within the shaly facies of the Opalinus Clay formation at Mont Terri Underground Research Laboratory for 350 days. The bioreactor is designed to allow free flow of porewater into the borehole with a packer to isolate the interval from the URL gallery and so maintain anoxic conditions. Two polyamide lines (2mm diameter) have been placed in the interval to allow sampling of the borehole.

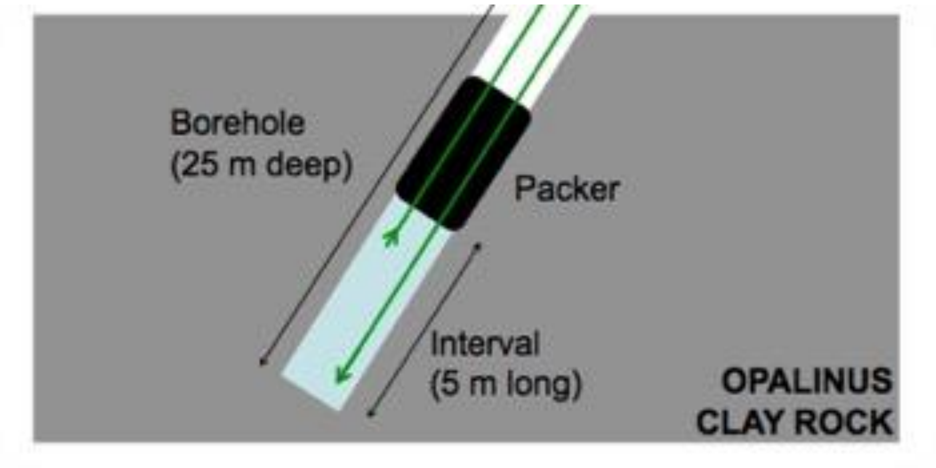

Figure 2 Diagram of the BRC-3 bioreactor in-situ

Every 7 to 15 days approximately 800 mL of borehole water was extracted, samples were collected directly into sterile, N_2-filled serum bottles to maintain anoxic conditions prior to analysis. The remaining volume was filtered immediately for DNA extraction.

The volume was replaced with sterile artificial porewater devoid of sulfate (see Table 1 for composition) to maintain pressure within the borehole (approximately 800 mL per sample). This was followed by H_2 injection (3 L).

Table 1: Artificial porewater composition, pH adjusted to 8.

Compound	Concentration (g/L)
KCl	0.16
$MgCl_2.6H_2O$	4.68
$CaCl_2.2H_2O$	2.78
NaCl	30
$NaHCO_3$	0.042

2.1.1 Geochemical analyses

Gas samples were collected in serum vials containing mercury (II) chloride or sodium azide for hydrogen and methane quantification respectively. Gas chromatography with flame ionization detection (GC-FID) was used to analyse the samples (Varian 450-GC, Agilent, Santa Clara, USA). The $\delta^{13}C\text{-}CH_4$ was analysed using Cavity ring-down spectroscopy (g2201-I Analyzer, Picarro, USA). Aliquots for aqueous analysis are filtered (0.22 µm) prior to addition of reagents. Sulfate concentration was measured using an ion chromatograph (IC) equipped with an IonPac AS111-HC (DX-3000, Dionex, USA) with a KOH gradient from 0.5 to 30 mM for elution. Sulfide and iron(II) were measured using

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spectrophotometry (UV-2501PC, Shimadzu CO., Japan). Sulfide measurement was made using the method of Cline (1969)[5], iron (II) analysis follows the method of Stookey (1970)[6]. Solid sulfate was recovered as a barium sulfate precipitate to measure $\delta^{34}S\text{-}SO_4$ using a Delta V™ Isotope Ratio Mass Spectrometer (Thermo Fisher, USA). The redox potential of unfiltered water was measured using an IntelliCAL™ MTC101 standard gel filled ORP electrode (HACH). Solution pH was measured periodically in experiments by an Orion 815600 ROSS Combination pH electrode (ThermoScientific), calibrated daily at pH 4, 7 and 10. Alkalinity was calculated using titration with 0.01 M HCl until pH 4.5 was achieved.

2.1.2 Biological analyses

Flow cytometry was used to determine the microbial cell density. Water samples are filtered (30 µm) and fixed with 1.5% paraformaldehyde. The fixed cells are stained with a 1:1000 final dilution of SYBR® Green to stain the live cells in the sample.

For 16S rRNA gene amplicon sequencing (V3-V4 region), water is filtered through a sterile 0.22 µm polycarbonate membrane. The DNA is extracted using a modified version of the phenol-chloroform classical extraction following the method of Bagnoud et al., 2016. Sequencing was performed at the Lausanne Genomic Technologies Facility (GTF, University of Lausanne, Switzerland) using Illumina MiSeq (Illumina, USA). The obtained sequences are processed and identified using the UPARSE OTU [7] and taxonomy was prescribed using the RDP database.

2.2 Results

2.2.1 Geochemical evolution of water

The concentration of methane remains low at < 20 µM with no sustained increase in concentration.
Over the course of 350 days, the sulfate concentration was successfully reduced from 14 mM to a minimum of 4 mM. Sulfide concentrations show an increase although still at a magnitude lower than the sulfate concentration. The pH varies between 7.3 and 8.5 which may reflect dissolution/precipitation of mineral phases in the surrounding Opalinus clay. Increases in H_2 concentration coincide with sampling days where H_2 injections occurred with variation in concentration likely to be reflecting the uncertainty in volume addition and in sample extraction. Concentrations of Fe(II) remain low from the initial time point to ~200 days. The concentration of Fe(II) then increases reaching a maximum at 250 days before returning to ~1 µM until the end of the sampling period. The results of the alkalinity analysis suggest a decrease in alkalinity over time.
The high value for the initial time point for the redox values reflects the addition of oxic artificial porewater (to unclog sampling lines) however redox values then decrease and remain negative for the duration of the sampling period. Cell density fluctuates throughout the sample period, possibly reflective of the ability of the microbial populations to recover and grow between sample points as well as any changes in redox potential, for example we see a marked increase in cell density following the input of oxic water.

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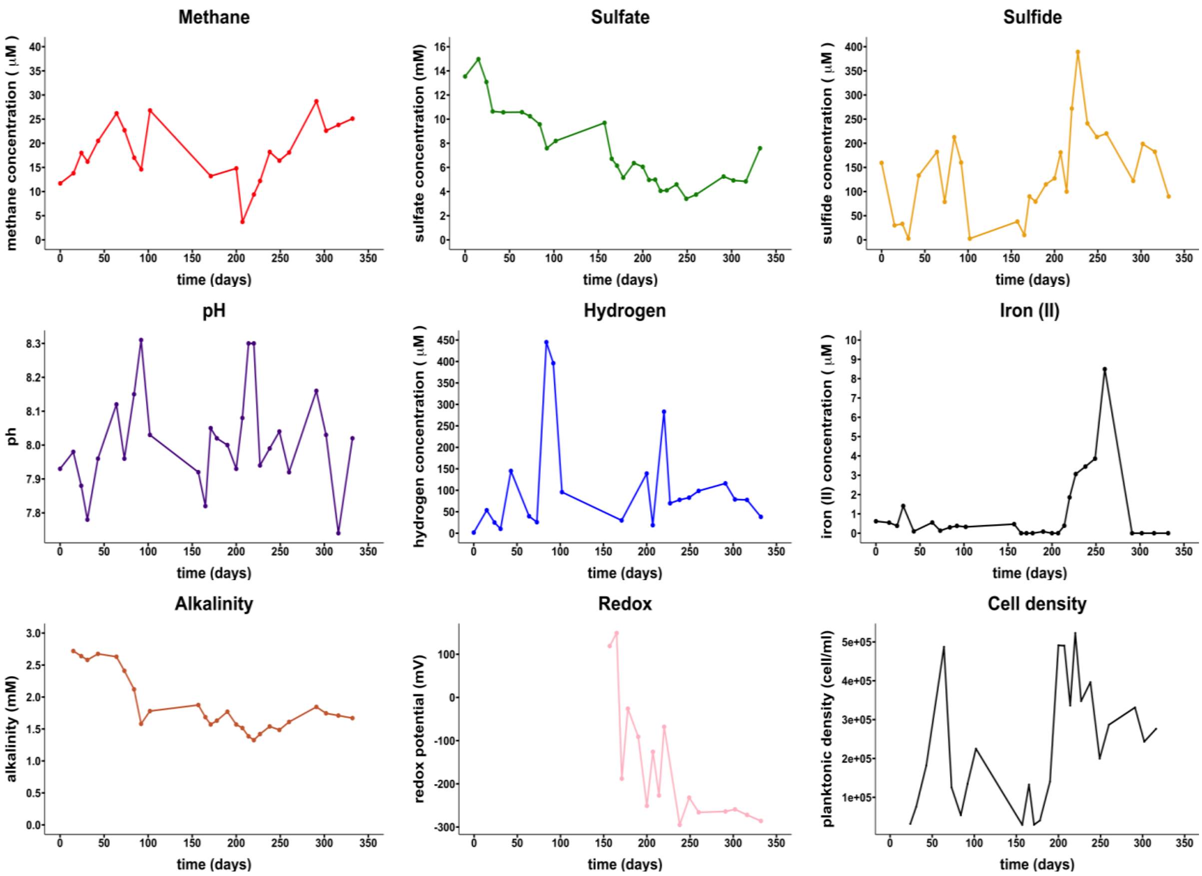

Figure 3 In-situ geochemical evolution of water

2.2.2 Composition of the microbial community

The structure of the microbial community varies over time, but is consistently dominated by sulfate-reducing bacteria (see Figure 4). Methanogens and acetogens were identified within some samples however they made up less than 0.1% of the total reads throughout the sampling period with no evidence for an increase in dominance of these microorganisms.

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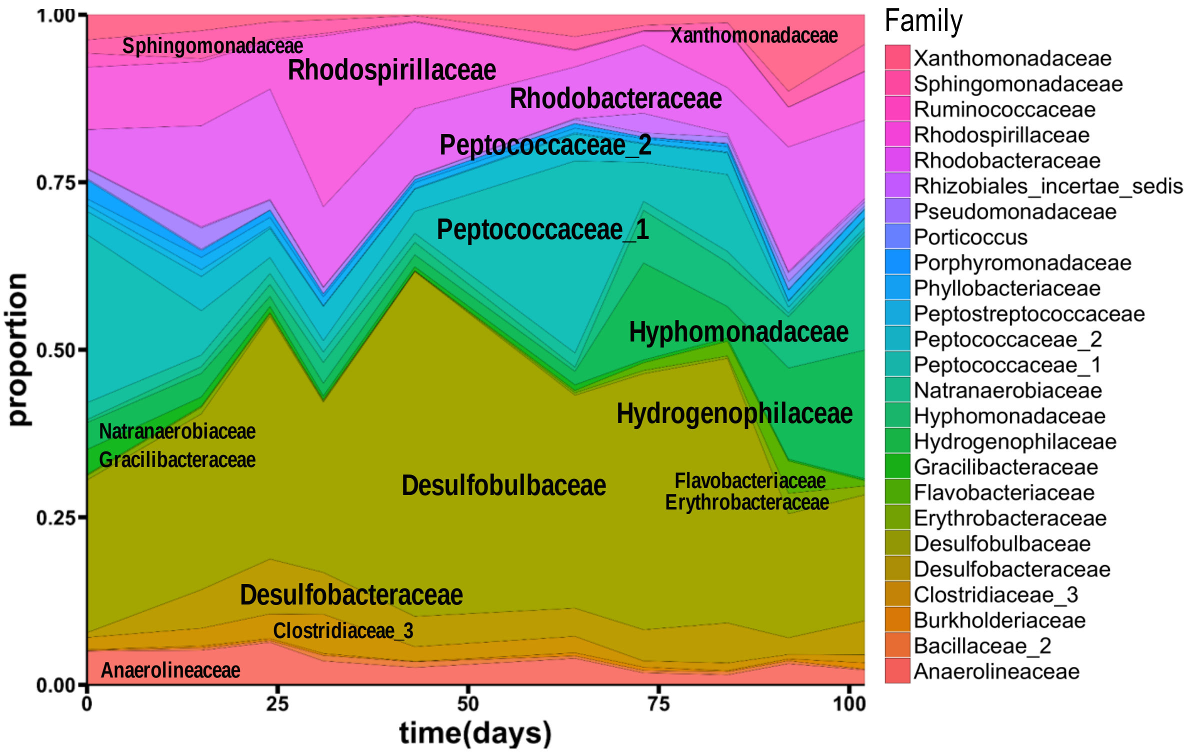

Figure 4 Microbial composition of MA borehole in-situ community over time

2.3 *In-situ* experiments using sand:bentonite porous medium

In-situ experiments comprised of a porewater saturated porous medium (80% sand: 20% bentonite, w/w) and H_2 addition were incubated for nine months. DNA was then extracted from 19 sample points within the bioreactor (Figure 5) and 16S rRNA sequencing undertaken. The results show that the most abundant sequences are associated with methanogens, in particular *Methanosarcina,* and fermenters, such as *Pseudomonadaceae*.

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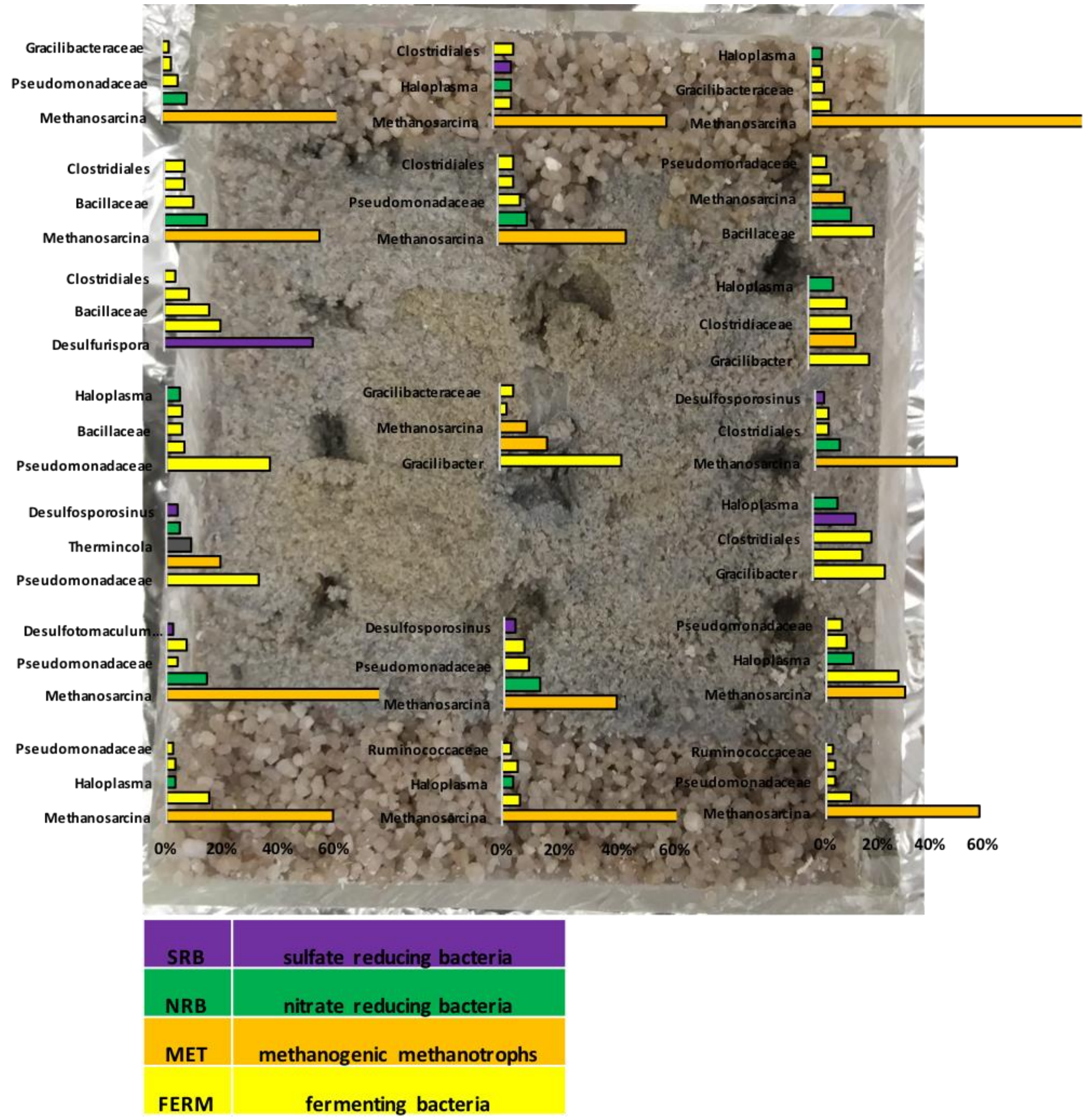

Figure 5 Results of 16S rRNA sequencing of the In-situ bioreactor with porewater saturated sand:bentonite mixture after nine months of incubation

2.4 Summary of findings

Methanogens and acetogens have been identified in the microbial community of the MA borehole, however their relative abundance remained below 0.1% throughout the duration of the experiment. There is no sustained increase in methane or acetate concentration which suggests that any activity by these microorganisms is very minor in comparison to the activity of sulfate-reducing microorganisms. The *in-situ* bioreactor of the MA-A experiment suggests that, after the depletion of sulphate, methanogenesis becomes prevalent within the microbial community from the Opalinus Clay porewater within the sand:bentonite bioreactor. Work is ongoing to determine the geochemical conditions required for this to occur.

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3 Laboratory experiments

As no evidence was found for significant methanogenesis or an increase in the proportion of methanogens in the *in-situ* experiments with Opalinus Clay, microcosm experiments were set up in the laboratory with the aim of creating a sulfate-free environment in which methanogenesis might be able to proceed.

3.1 Materials and methods

3.1.1 Opalinus Clay

A core from the sandy facies of the Opalinus Clay geological formation at Mt Terri Rock Laboratory (St-Ursanne, Switzerland) was recovered during the drilling of BMA-A2 borehole, with pressurized air used as the drilling fluid. The core was conditioned in mylar bags and stored at -20 °C. Clay from the interior of the core was recovered under sterile conditions using a scalpel for use in experiments.

3.1.2 Experimental set up

Duplicate microcosms were set up in 200 mL serum bottles containing either $40gL^{-1}$ or $20gL^{-1}$ of Opalinus clay from the BMA-A2 borehole. To these, 90 mL of artificial porewater (APW) and 1 mL of 0.1% resazurin dye was added. The pH was adjusted to pH 8 and the solution was autoclaved prior to addition to the serum bottles (see Table 1 for APW composition). An additional set of duplicate bottles contained only the APW and resazurin dye was prepared. All bottles were flushed with N_2 for 30 minutes and then flushed with an H_2:N_2 mix (20:80%) for 10 minutes. 10 mL of borehole water was added as inoculum to the experiments (taken from borehole BPC-C2, Mont Terri, Saint-Ursanne, Switzerland). Single control experiments were set-up using 100 mL of APW. All set-up and handling was done under a flame to maintain sterile conditions. Experiments were incubated in the dark at 19°C for the duration of the experiment.

3.1.3 Microbial community in borehole water from BPC-2 (inoculum)

Porewater was extracted from the BPC-C2 borehole at Mont Terri (Saint-Ursanne, Switzerland) and anaerobic conditions were maintained. Water was stored overnight at 4°C prior to use. It was checked for living cells using SYBR Green nucleic acid stain (Sigma-Aldrich, USA) on an epifluorescence microscope. For the preparation, 1 mL of sample was filtered on a black polycarbonate membrane (0.22 μm), rinsed three times with PBS, and finally stained with a solution of SYBR Green I (1:200 dilution) and polyvinylalcohol (moviol) [8]. 900 mL of BPC-2 borehole water was filtered through a 0.22 um pore size filter and the filter was retained for DNA analysis to determine the composition of the original microbial community. Aliquots of unfiltered borehole water were used for measurement of pH, Fe^{2+}, SO_4^{2-} and H_2S to establish geochemical conditions prior to incubation.

3.1.4 Geochemical analyses

Aqueous Fe^{2+} was measured following the photometric method of Stookey, 1970 [6] whereby 900 μL of ferrozine is added to 100 μL of sample. Samples were then left to develop for 20 minutes prior to measurement on the UV-vis spectrophotometer (UV-2501PC, Shimadzu CO., Japan) at 562 nm. Sulfide was also measured using UV-vis spectrometry according to the method of Cline, 1969 [5]. Sulfate was measured using an ion chromatograph equipped with an IonPac AS11-HC column (DX-3000, Dionex, USA) and with a KOH gradient from 0.5 to 30 mM for the elution. Solution pH was measured periodically in experiments by an Orion 815600 ROSS Combination pH electrode (ThermoScientific), calibrated daily at pH 4, 7 and 10.

At the end of the experiment, samples were combined into four groups prior to filtering to ensure sufficient DNA recovery for sequencing: 1) clay – inoculated; 2) clay – non-inoculated; 3) no clay –

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inoculated, and 4) no clay – non-inoculated. The liquid phase was filtered using 0.1 μm polycarbonate filters for the end time point to retain sufficient biomass for sequencing which was then placed in 1.5 mL Eppendorf tubes containing 500 μL of Lifeguard preservation solution (MoBio Laboratories) and stored at - 20°C prior to DNA extraction. The solid phase clay samples were dried under vacuum in anaerobic conditions, the clay was then ground in to a fine powder and transferred to 10 mL serum bottles to maintain anaerobic conditions prior to spectroscopic analyses.

3.1.5 X-ray Absorption Sulfur XANES

X-ray absorption near edge structure (XANES) spectra were collected from the inoculated and non-inoculated clay samples at Stanford Synchrotron Radiation Lightsource, beam line 4-3. Multiple XANES spectra were averaged to improve signal-to-noise ratio, normalized and plotted using Athena, Version 0.9.26 [9]. Linear combination fitting (LCF) was used to analyse the spectra and assign mineral phases.

3.1.6 Microbial community analysis

DNA was extracted from five samples, these consisted of the unaltered source rock sample from the BPC-2 borehole, the inoculated clay sample, the non-inoculated clay sample, the sample inoculated but without clay and the non-inoculated sample without clay. Extractions were undertaken using a previously described phenol-chloroform-isoamylalcohol extraction followed by a glycogen-assisted ethanol precipitation (see [2] for detailed methodology).

Of these, two samples yielded sufficient DNA for sequencing (unaltered source rock sample and the inoculated clay sample). Samples were sent for sequencing at the RTL Laboratory (Lubbock, Texas, USA) where Illumina Truseq adapters and indices were attached to the DNA fragments in a two-step PCR amplification targeting the 16S rRNA gene (V4 region) [10]. Pooled amplicons were single end sequenced on the Illumina MiSeq platform (2 x 250 bp) generating around 40,000 clusters of data. Illumina adapter sequences were removed and the trimmed reads were processed using the USEARCH programme (version 10.1) [7]. Dereplication, clustering, chimera filtering and singletons removal was performed simultaneously on the dataset. Operational taxonomic units (OTUs) were defined by minimum of 97% sequence identity between the putative OTU members. OTUs were allocated to a taxon using UTAX command and the RDP database using a confidence value of more than 0.6 for classification to balance sensitivity and error rate in the prediction (OTUs not classified to the level of phylum or classified as archaea were excluded from subsequent analysis). The entire dataset was then allocated to the OTUs and reported in the OTU table with the taxonomy and abundance of the OTUs.

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3.2 Results

3.2.1 Geochemical evolution of water

The results of the aqueous analysis show that sulfate is present in both the clay and inoculum from the borehole. Where clay and inoculum are both present, sulfate concentrations decrease due to sulfate-reducing bacteria. There is no evidence for sulfide in the aqueous phase, however there is an increase in Fe(II) concentration in solution over time which may be due to interaction between iron and sulfur species. Notably, no sulfate-reducing activity is detectable in the non-inoculated samples despite the presence of non-sterile clay.

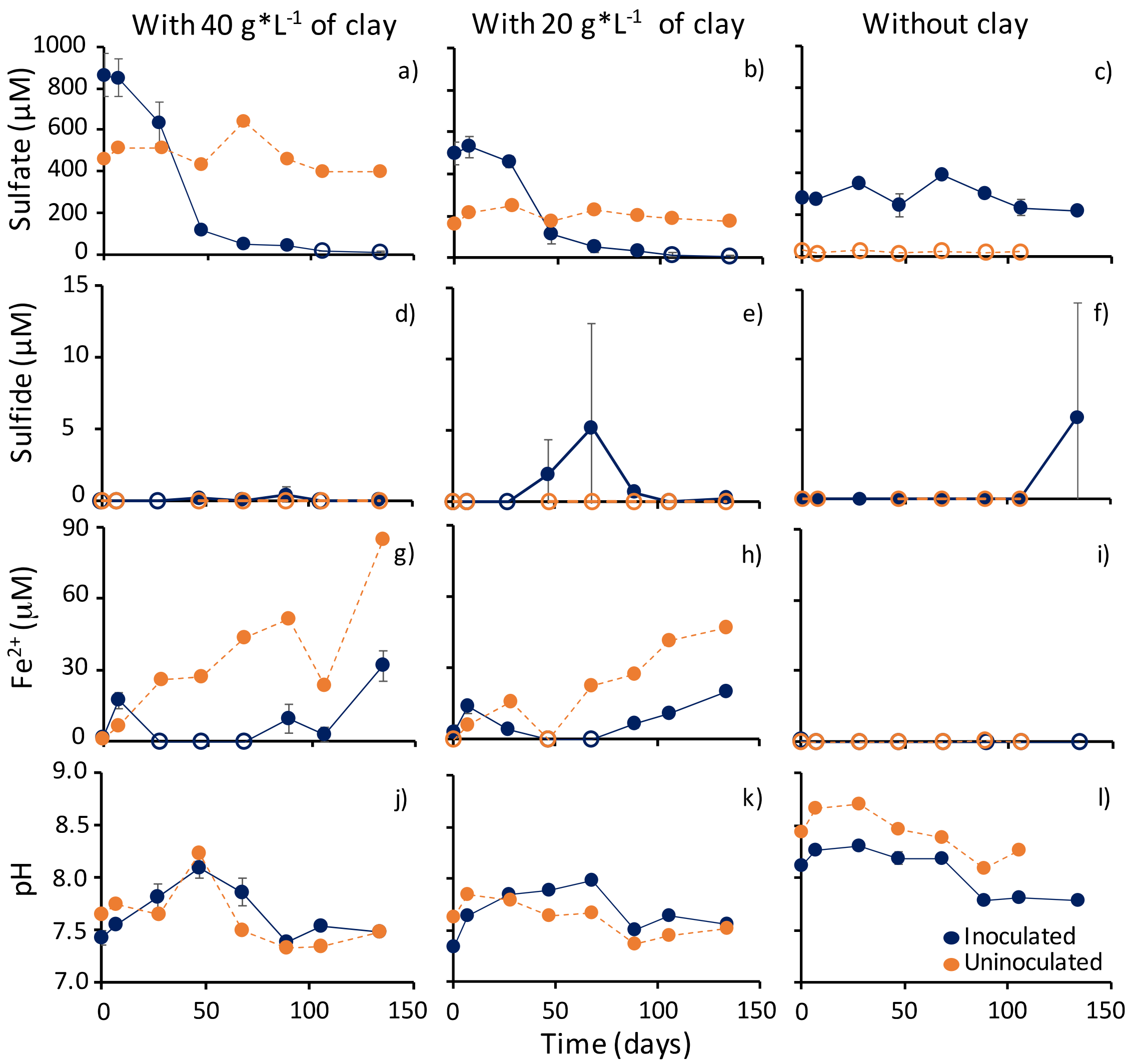

Figure 5 Geochemical analyses of inoculated and non-inoculated systems under three experimental conditions: with 40g L^{-1} of clay; with 20g L^{-1}) of clay; and without clay: a,b,c) sulfate concentration; d,e,f) sulfide concentration; g,h,i) Fe^{2+} concentration; and j,k,l) pH. Inoculated samples shown in blue with continuous line, non-inoculated in orange with dashed line. Unfilled data points represent values below the limit of detection for the method used. Error bars on the inoculated system (dark blue) denote the standard deviation of duplicate samples, single samples were used for non-inoculated.

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3.2.2 Microbial community composition

The microbial community of the borehole at the time of sample collection shows a low-diversity community with several dominant OTUs. After the incubation period, over 98% of the total reads are prescribed to a single OTU, the *Desulfobulbacea* family. This family is comprised of known sulfate reducers and contains microorganisms which are able to utilise H_2 gas as their electron donor [2].

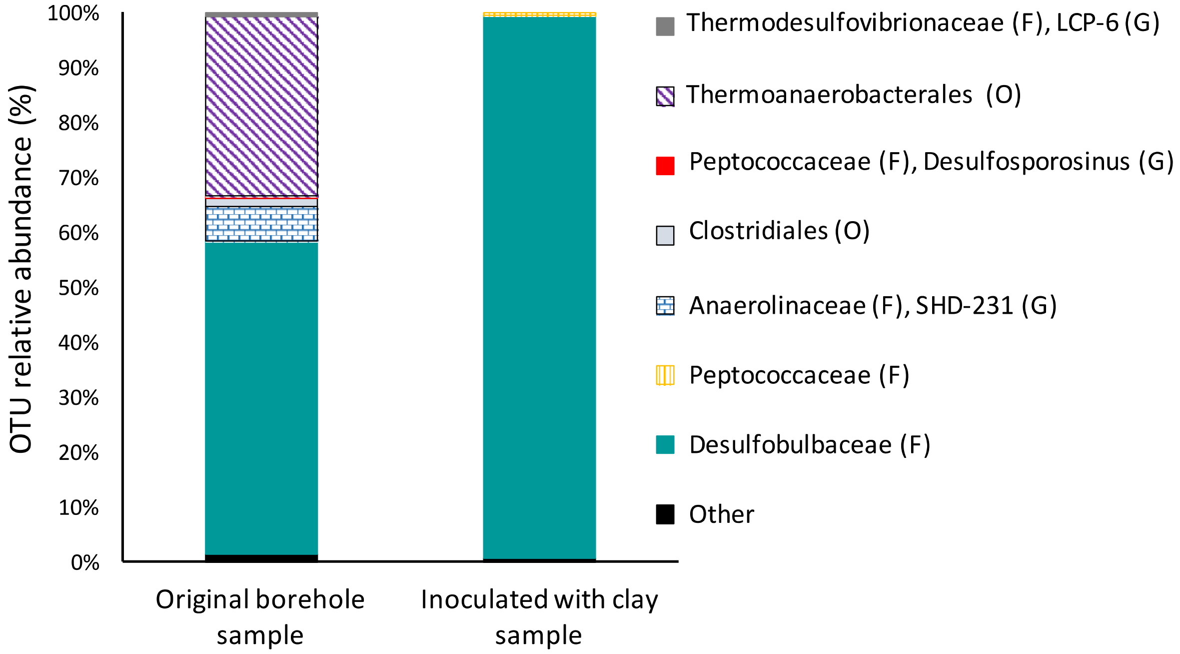

Figure 6 Microbial community composition showing individual OTUs as a percentage of the total reads. OTUs which accounted for less than 0.005% of the total reads were grouped together as 'Other'. (O) – order; (F) – family; (G) – genus.

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3.2.3 Changes to Opalinus Clay

Sulfur in Opalinus clay

Results of the sulfur speciation suggest that most sulfur is associated with pyrite in the source rock, the inoculated and non-inoculated samples. However, the proportion of FeS increased significantly post-incubation in the inoculated sample compared to the other samples.

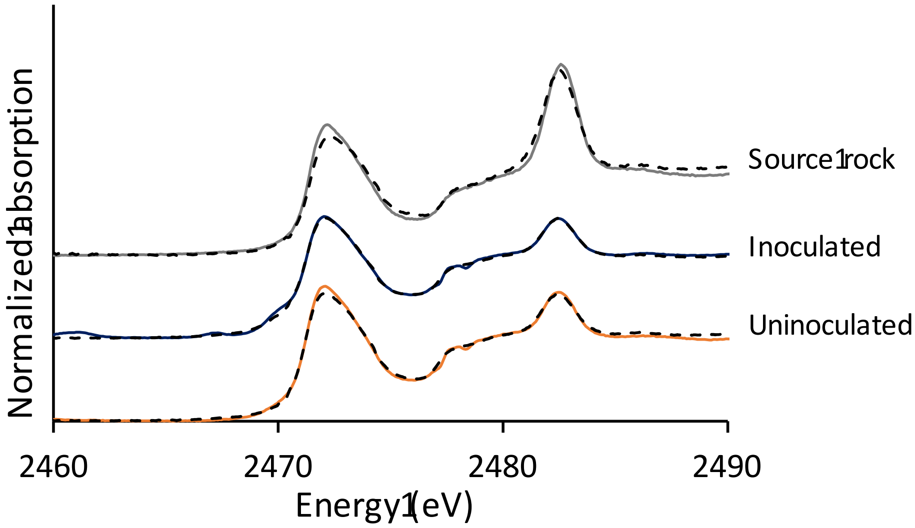

Figure 7 The speciation of sulfur within Opalinus clay

Table 2 Sulfur containing phases identified by XANES shown as a percentage of total for inoculated and uninoculated samples. R-factor of <0.05 suggests good fit with data. Number in brackets shows uncertainty in measurement, (-) denotes model compounds not used for LCF.

Sample	Pyrite (%)	Elemental sulfur (%)	Sulfate (%)	FeS (%)	R-factor
Source rock	82(1)	0(<1)	18 (<1)	-	0.0077
Inoculated	86(1)	0(<1)	7 (<1)	7 (<1)	0.0012
Uninoculated	90(2)	2(1)	7 (<1)	-	0.0024

3.3 Summary of findings

The results of the microcosm experiments suggest that even under sulfate-depleted conditions there is no increase in methane concentration, the microbial community composition at the end of the experiment shows dominance of one family of sulfate-reducing bacteria and no methanogens.

Furthermore, the study has provided insight in to some of the areas of uncertainty around the impact of sulfate-reducing bacteria. The formation of iron sulfide (FeS) suggests that if sufficient Fe^{2+} is available in the porewater then sulfide may be removed from solution, decreasing the corrosion potential and improving the safety case. This study has been published in *Applied Clay Science* [11].

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4 Conclusions

Results of the *in-situ* borehole experiments show that methanogens and acetogens are found at very low abundance (<0.1%) as part of the microbial community, however no change to the relative proportion of these microorganisms was detected throughout the duration of the experiment. Although the sulfate concentration was successfully reduced to less than 4 mM during the *in situ* experiment no evidence for methanogenesis and acetogenesis was recorded.

Further *In-situ* experiments using a porewater saturated porous medium (80% sand: 20% bentonite, w/w) and H_2 addition show evidence for the dominance of methanogens. Within nine months, the biofilm was dominated by methanogenic archaea including *Methanosarcina* and *Methanoculleus*. Further *in-situ* tests are being complemented by laboratory experiments with sulphate-depleted porewater from Mont Terri URL. The laboratory experiments will focus on the importance of trace metals and nutrient availability to the onset and dominance of methanogenic archaea.

The results of the laboratory microcosm experiments display no evidence for the onset of methanogenesis. Methane concentrations were measured for the duration of the experiment however no increase in concentration was recorded. There is also no significant increase in the acetate concentration over time suggesting that acetogens are not active in the community. The microbial composition after the incubation period supports these conclusions as it is dominated by one family (*Desulfobulbaceae*), which accounts for over 98% of the total reads. No methanogenic or acetogenic microorganisms were identified.

One area of uncertainty within WP1 is the ability of sulfate-reducing bacteria to create a feedback loop where the reduction of sulfate with hydrogen gas by sulfate-reducing bacteria produces hydrogen sulfide which then corrodes the waste-containing canisters which would in turn increase the hydrogen gas available for consumption by sulfate-reducing bacteria. The results of the microcosm study suggest that if sufficient Fe^{2+} is available, sulfide will be retained in the solid phase as FeS. This would lead to a reduction in hydrogen sulfide in solution and so a reduction in corrosion rates.

Further investigation suggests that the onset of methanogenesis is also controlled by the bicarbonate concentration [12], the bicarbonate concentration in the *in-situ* and microcosm experiments was too low so methanogenesis remained thermodynamically unfavourable. Current laboratory-based experiments are ongoing to determine the concentration of bicarbonate required, and the impact of trace metals and vitamin availability on methanogenesis. If methanogenesis is recorded in these experiments it will be possible to use the composition of artificial porewater in the *in-situ* bioreactor at Mont Terri with the aim of encouraging methanogenesis.

4.1 Factors affecting methanogenesis in Opalinus clay

1. Availability of suitable electron donors

Under repository conditions, there is expected to be sufficient H_2 gas production which can be used as an electron donor. Known methanogens can utilise H_2 and have been detected at very low abundance in the *in-situ* borehole experiments.

2. Bicarbonate concentration

The bicarbonate concentration is important within a methanogenic system, because it serves as a pH buffer and as an electron acceptor during hydrogenotrophic methanogenesis [12]. The artificial porewater used in both experiments in this report used a low bicarbonate concentration. Thermodynamic calculations suggest that a slightly higher bicarbonate concentration is more favourable.

3. The presence of more energetically favourable terminal electron accepting processes

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Opalinus clay and porewater contains high concentrations of sulfate and so sulfate-reducing bacteria are the dominant microorganisms within the community. Although methanogenesis can co-occur with other electron accepting processes, methanogens will not dominate the microbial community whilst more energetically favourable electron acceptors remain. Even after the depletion of sulfate in the microcosm experiments there was no evidence for an increase in the proportion of methanogens suggesting that there are other factors which can influence the onset of methanogenesis.

4. Availability of water and space

Unaltered Opalinus Clay is incredibly compacted resulting in a very low pore size (0.01 -0.02 μm) and low water activity [13], however the existence of fractures may provide sufficient space to allow microbial growth.

5. Availability of trace metals and vitamins

Nickel is a key metal co-factor in key enzymes involved in methanogenesis. Where the concentration of Ni is too low methanogenic activity will be inhibited. Conversely where the concentration is too high there is evidence of a toxic effect.

5 Acknowledgement

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