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MIND

Microbiology In Nuclear waste Disposal

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DELIVERABLE 2.11

<Microbial cement deterioration boundaries>

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Publishable Summary

Cementitious materials have been used for centuries in many construction and engineering applications because of their long-term durability. Also for the geological disposal of radioactive waste, cementitious materials are used in many parts of the engineered barrier. Consequently, the interactions and the evolution of these materials with other repository materials, the host rock and its ground water, need to be assessed. Organic acids (e.g. acetate), carbon dioxide and sulphur compounds, originating from the waste and host rock or produced by microbes in the repository, can be corrosive towards cementitious materials resulting in Ca^{2+} leaching and a decrease in the original high alkaline pH. The latter will give rise to lower pH niches on the concrete were microbial activity will be enhanced and which in turn can have a possible impact on the mineralogy and chemistry of the cementitious materials. Interestingly, microbial processes can either have a detrimental effect on or be beneficial for the functional performance of the cementitious materials used within a geological disposal.

In this study, anoxic batch experiments were performed to study the microbial community present in Boom Clay borehole water in a cementitious environment. The objective of this study was to investigate whether this microbial community could affect in a positive or negative way the long-term evolution of the cementitious materials present in the engineered barrier of a geological repository for radioactive waste. This preliminary study demonstrated that the high pH conditions imposed by the OPC CEM I inhibit microbial sulphate and nitrate reduction. However, SEM analysis indicated the presence of intact cells in the suspension on top of cement and putative biofilm structures on the cement. This suggest that the high pH environment does not completely eliminate the microbial population. Interestingly, in sulphate reducing conditions, a pH decrease from > 12 to pH 10 was observed in one replicate harbouring clearly a larger microbial community in the suspension on top of the cementitious material compared to the other samples. However, the precise mechanism remains unclear and more detailed chemical and microbial analysis and of the structure of the OPC CEM I is planned.

Contents

1 Introduction

The Belgian Agency for Radioactive Waste and Fissile Materials (Organisme National des Déchets Radioactifs et des Matières Fissiles / Nationale Instelling voor Radioactief Afval en Splijtstoffen or ONDRAF/NIRAS) proposes deep geological disposal for low- and intermediate-level long-lived radioactive waste (LILW-LL) (such as Eurobitum), vitrified high-level waste and spent nuclear fuel in a poorly indurated clay formation (Boom Clay or Ypresian clay) considering a depth of 200m, 400m or 600m. The disposal system would consist of a combination of a natural and engineered barrier system [1]. Various parts of the engineered barrier consist of cementitious materials. Cement is one of the matrices used for the immobilization of low- and intermediate level long-lived (LL-LILW) radioactive waste. In addition, the primary waste packages will be placed in a prefabricated concrete monolith after which the voids are filled with mortar and the waste monolith will be closed with a concrete lid [2]. For the disposal of vitrified high-level radioactive waste (HLW) and spent fuel, the 'supercontainer' is selected as reference design in Belgium. This concept foresees the emplacement of primary waste packages in a carbon steel overpack surrounded by a massive Ordinary Portland Cement (OPC) buffer and, if needed, a stainless steel envelope [1]. The waste will be placed in a disposal gallery lined with concrete wedge blocks and after waste emplacement, all voids will be filled probably with mortar [1]. To avoid direct contact between the disposal waste package and the Boom Clay, a concrete end plug is foreseen at the end of the disposal galleries. Disposal galleries will be sealed, but it is not yet defined whether a bentonite or a concrete seal or a combination of both is preferable [1].

The waste monolith will be designed such that it serves as permanent shielding for the workers during the operational phase, and the concrete will slow down radionuclide release. As such the concrete waste monolith provides a first safety barrier [1, 3]. Similarly, the supercontainer will form a first safety barrier by providing a permanent shielding for the workers during the operational phase. Additionally, in this case, the integrity of the engineered barrier system has to be ensured at least for the duration of the thermal phase of the waste after emplacement in the repository (several hundreds of years for vitrified HLW and a few thousands of years for spent nuclear fuel) [1]. In addition, in both cases, the high pH originating from the cementitious materials results in the passivation of the steel, hence reduces the uniform anaerobic corrosion rate (i.e. the main corrosion mechanism) [1]. Consequently, the interactions and the evolution of these materials with other repository materials, the host rock and its ground water, need to be assessed [1,2].

Several geochemical processes can affect the chemical evolution of concrete buffer such as chloride ingress, sulphate attack, alkali-silica reactions, carbonation and Ca^{2+} leaching, which are mostly followed by alteration of the microstructure what might change the physical properties (e.g. transport and mechanical properties) of the concrete [4]. Moreover, these processes can result in a local decrease in pH [4], leading to niches where growth of microorganisms can be possible. In addition, when the alkaline plume reaches the interface of Boom Clay with the disposal gallery, chemical reactions are likely to occur. For instance, some primary minerals Boom Clay minerals are unstable at high pH and tend to dissolve, forming new secondary minerals. Possibly this will lead to the consumption of OH^- ions. Moreover, the CO_2 will diffuse, driven by the relatively high partial pressure within the Boom Clay, towards the cementitious repository and react with the high concentration of calcium from concrete to form calcite at the interface of the repository and the Boom Clay. The combination of chemical reactions results in lowering of pH to maximally 10.5 [5]. This pH of 10.5 is not expected to be limited for microbial activity [6, 7], thus, microbial activity might be present at the Boom Clay - disposal gallery interface of the repository. Furthermore, concrete has a low capacity for deformation under tensile stress, leading to the formation of (micro)cracks through which microorganisms are able to migrate and at the surfaces of cracks, they are able to form a biofilm [8, 9]. Microbial degradation of cementitious materials in a wide variety of conditions is commonly known [10], and might result in a loss of alkalinity, erosion, spalling of the concrete skin,

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corrosion of reinforcing bars, loss of water- or airtightness, and collapse [11]. In addition, microorganisms can produce organic and inorganic acids, which can be corrosive towards cementitious materials and can lead to a decrease in pH. On the other hand, microbial metabolic activity can lead to the formation of calcium carbonate [8], which can result in partially clogging of the fractures, as the molar volume of calcite is larger than that of portlandite [4].

Previous studies already indicated that the microbial community present in Boom Clay borehole water is metabolically diverse and able to thrive in several environments [12, 13] (see also MIND deliverable 1.3). In this study, anoxic batch experiments were performed to study the microbial community present in Boom Clay borehole water in a cementitious environment. The objective of this study was to investigate whether this microbial community could survive and be active in these conditions and could affect in a positive or negative way the long-term evolution of the cementitious materials present in the engineered barrier of a geological repository for radioactive waste.

2 Materials & Methods

2.1 Characteristics of the Boom Clay Borehole water

The HADES (High-Activity Disposal Experimental Site) underground research facility is located at a depth of 225 meters in the core of the Boom Clay, in Mol, Belgium (Figure 1). Boom Clay is a marine sediment from the Rupelian period (approximately 35 million years ago), dominated by illite and smectite clay minerals. It is a silty clay or argillaceous silt and presents a layered structure with (limited) variations in grain size (alternating clayey silt and silty clay layers), organic matter content and carbonate content. These variations are the consequence of depositional processes (sedimentary, from marine origin) and subsequent limited burial history. The band thickness ranges between 10 centimetres and 2 meters. A detailed overview of the geology is given in [14].

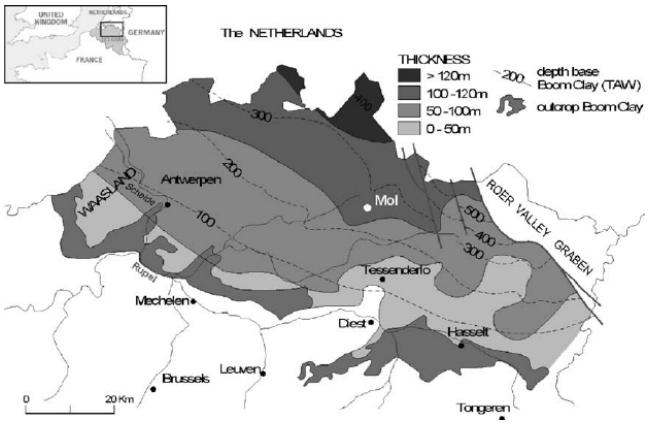

Figure 1: Occurrence and thickness of the Boom Clay formation in Belgium. The HADES underground research facility is indicated in white [15]

The bulk mineralogy of the Boom Clay is shown in Table 1. Qualitatively, the mineralogy is the same over the entire Boom Formation but the quantities of the minerals present at each clay layer vary according to the precise sedimentological build-up of the deposit. Organic matter from marine origin is present is small quantities (ca. 0.5 %) while organic particles from terrestrial origin can reach a few percentage (3-5 %) in some horizons [16]. The clay contains about 20 wt % of water that mainly consists of about 15 mM $NaHCO_3$ and 115 mg/l dissolved organic carbon. At a temperature of 16 °C, it has a slightly alkaline pH around 8.5 and a pCO_2 of $10^{-2.62}$ bar (Table 2)[17].

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Table 1: Mineralogical composition of the Boom Clay [16].

Mineral	Amount
Quartz	20-60 %
Clay minerals	22-56 %
Illite	5-18 % of bulk
Smectite	7-24 % of bulk
Mixed ilite-smectite	7-23 % of bulk
Plagioclase	10-15 %
Potassium-feldspar	10-15 %
Kaolinite	10-15 %
Chlorite	1-4 %
Pyrite	0-3 %
Carbonates	
Siderite	0-1 %
Dolomite	0-1.5 %
Calcite	0-4 %
Anatase	> 1 %

Table 2: Average Boom Clay pore water composition at a temperature of 16°C [17], the composition of the Boom Clay pore water of filter TD-116E that was used as background medium, and the composition of the pore water of filter TD-11D-23 used as inoculum. Concentrations are presented in mg/l unless specifically stated otherwise.

Component	Average Boom Clay pore water	TD-116E	TD-11D-23
HCO_3^-	879	859	843
Na^+	359	357 ± 36	389 ± 39
Cl^-	26	21.5 ± 1.4	28.2 ± 1.8
K^+	7.2	11.4 ± 1.5	8.6 ± 1.3
Si	3.4	4.4 ± 0.5	5.8 ± 0.6
F^-	3.0	2.86 ± 0.29	2.98 ± 0.30
SO_4^{2-}	2.2	2.78 ± 0.32	80.9 ± 3.6
Ca^{2+}	2.0	3.65 ± 0.38	2.86 ± 0.30
Mg^{2+}	1.6	3.07 ± 0.31	2.54 ± 0.25
Br^-	0.6	0.94 ± 0.23	1.11 ± 0.23
Fe	0.2	0.282 ± 0.035	0.53 ± 0.06
Al	0.0006	0.051 ± 0.006	0.063 ± 0.007
pH	8.5	8.56	7.88
TOC (mg C/l)	115	72	142
Alkalinity (meq/l)	15.1	15.5	15.0

The water used to carry out the experiments was borehole water from the piezometer called TD-116E (previously reported as SPRING). The piezometer is placed horizontally in the Boom Clay, and is located in the Test Drift part of the HADES URF, at ring 116, pointing towards the east. It is entirely made of stainless steel and contains in total four large surface filter screens made from high porosity seamless filter tube made by "Krebsöge", quality: SIKA R5, material: 1.4404 (AISI 316 L/B), pore size distribution: 7 to 16 µm. This piezometer was designed in 1999 to provide sufficient quantities of representative Boom Clay pore water as feed and reference material for laboratory experiments [17]. The chemical composition of the pore water used in this study is presented in Table 2.

The inoculum for the experiments was collected from another piezometer. In a recent study, the microbial population present in borehole water collected via a vertical piezometer TD-11D (previously reported as MORPHEUS) was characterised in detail [12]. The piezometer, installed in 2001, was designed to study the variability of the Boom Clay pore water composition underneath the HADES research facility. It allows pore water sampling at 12 different stratigraphic levels of the Boom

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Clay (clayey/silty, organic rich/poor, carbonate rich/poor). All the porous filter screens of this piezometer are made out of "Schumatherm" filters, used for its chemically inert characteristics [17]. The chemical composition of the pore water used during this study is presented in Table 2. The microbial population present in borehole water from filter 23 was determined as the most representative for the microbial community present in that piezometer [12], hence it was selected as inoculum for the experiments carried out in this study (Figure 2).

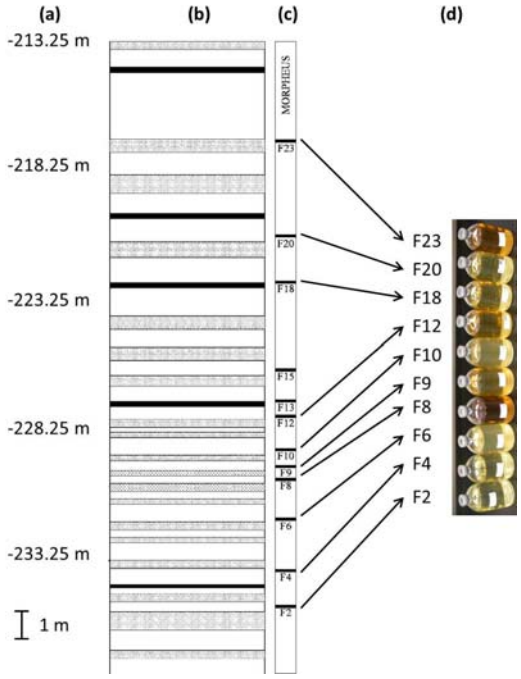

Figure 2: Schematic view of the vertical piezometer TD-11D, used for harvesting the microbial inoculum from Boom Clay borehole water, indicating depth below sea level (a), Boom Clay stratigraphic layers (b) (white = clayey, grey = silty, black = including septaria, very silty), piezometer filters, as indicated by Fxx (c) and corresponding samples in septum bottles (d), as indicated by arrows.

2.2 Culture conditions

All batch experiments were performed in triplicate in anoxic conditions. To this end, all preparations were performed in an anoxic glove box with a manually controlled atmosphere of 99% argon and 1% hydrogen. In addition, as glass quickly dissolves at high pH, all batch experiments were performed in 250 ml polystyrene culture flasks as these showed little to no effect after continuous exposure to pH 11 for 30 days (Thermofisher Scientific). Furthermore, preparatory manipulations were performed to completely sterilize the natural Boom Clay pore water collected from piezometer TD-116E was used as background medium for all experiments. The pore water was 0.22 µm filter sterilized to exclude a false positive signal from the present microbial community during flow cytometry analysis. In addition to filter sterilization, the pore water was autoclaved. Afterwards, it was transferred to the anoxic glove box and to deoxygenate by overnight mixing on a magnetic stirrer. However, exposure of Boom Clay pore water to air may lead to a loss of dissolved carbon dioxide due to the re-equilibration with the atmospheric CO_2 partial pressure. This will result in a pH increase from a pH of ~ 8.4 up to ~ 9.3 when it is in equilibrium with air [18]. In this study, preparatory manipulations to completely sterilize the culture medium made that the final medium was equilibrated with air. However, it was not supplemented with additional bicarbonate, therefore an initial pH of ~9.5 was obtained during these experiments. In addition, this resulted in a decreased buffering capacity of the Boom Clay pore water [18]. In addition, the preparatory manipulations were carried out in air, hence this could have resulted in a partial oxidation of the dissolved organic matter, resulting in lower molecular weight constituents [19, 20].

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First, 10 g of Ordinary Portlandite cement (OPC) CEM I was added in the culture flasks and hardened by adding 5 ml Boom Clay pore water of piezometer TD-116-E. After 1 month, 45 ml natural Boom Clay pore water collected from piezometer TD-116E (as prepared mentioned above) was added to the hardened OPC CEM I. The pore water was supplemented either with 10 mM sodium sulphate and 10 mM calcium lactate or with 100 mM sodium nitrate and 15 mM sodium formate. In the experiments with sodium nitrate, after 49 days, samples were supplemented with 10 mM sodium acetate. Samples were inoculated with 5 ml (10 % v/v) Boom Clay borehole water from piezometer TD-11D-23 providing the microbial community or with an additional 5 ml pore water collected from piezometer TD-116E in the negative control samples. The culture flasks were closed with caps containing a membrane that allows gas exchange with the atmosphere, and incubated at room temperature (ca. 22°C) without shaking in the anaerobic glove box (99% Ar/1% H_2) to maintain anoxic conditions throughout the experiment. In addition, similar experiments without the presence of OPC CEM I were initiated.

2.3 Chemical analysis

Nitrate, nitrite, sulphate, acetate, formate and lactate were measured with a Dionex ion chromatography system equipped with a Dionex IonPac AS11-HC anion exchange column, a Dionex IonPac AS11-GC guard column and conductivity detector. Before measuring, samples were diluted 40 times in 1 ml and filtered using 0.45 µm Acrodisc® PSF syringe filters. The eluent gradient program was 1 mM sodium hydroxide for 8 min, increasing to 15 mM sodium hydroxide during the following 10 min, increasing to 30 mM sodium hydroxide in the following 10 min, in the next 10 min increasing to 60 mM sodium hydroxide and finally hold for 2 min at 60 mM sodium hydroxide. The chromatograms were collected and processed with the Chromeleon version 6.5 software. With those settings, the IC data obtained for sulphate reducing conditions were more difficult to interpret compared to those of the nitrate reducing conditions. It is expected that lactate is oxidized to acetate by sulphate reducing microorganisms, therefore concentrations of both compounds should be monitored. However, the retention time for lactate and acetate only differs by 0.7 minutes what makes it more difficult to accurately resolve both peaks and determine truthful concentrations (Figure 3). A further optimization of the protocol and selection of suitable dilutions is necessary.

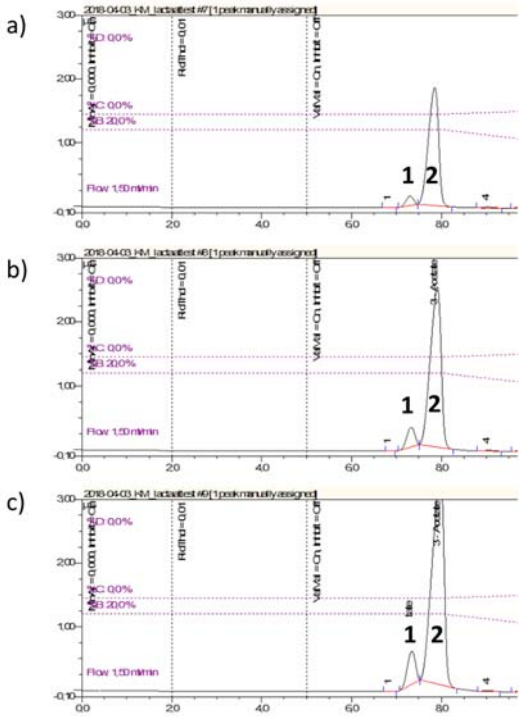

Figure 3: IC chromatogram showing the standard solution with lactate (1) and acetate (2) at a) 6.25 mg/l; b) 12.5 mg/l and c) 25 ppm. A red line represents the baseline.

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2.4 Microbiological analysis

Flow cytometry was used to count the total amount of microbial cells present in the borehole water on top of the hardened OPC CEM I. Samples were diluted in 0.22 µm filter sterilized Evian potable water. Double stranded DNA was stained with SYBR® Green I (10 000 x concentrate in 0.22 µm filtered dimethyl sulfoxide) (ThermoFisher Scientific, Belgium) (final concentration of 1x concentrate) and incubated in the dark for 20 min at 37°C. Flow cytometry was performed using a C6 Accuri™ flow cytometer (BD Biosciences, Belgium), which was equipped with four fluorescence detectors (530/30 nm, 585/40 nm, >670 nm and 675/25 nm), two scatter detectors and a 20-mW 488-nm laser. The flow cytometer was operated with Milli-Q filtered water (Merck Millipore, Belgium) as sheath fluid. Samples were analysed in a fixed volume mode of 50 µl and the threshold was fixed on the green fluorescence (FL1-H at 1000). Dilutions which had between 200 and 2000 events per µl were considered representative and used for the calculation of cell counts. After flow cytometry measurement, data were analysed with the BD Accuri C6 Software.

2.5 Scanning electron microscopy (SEM)

SEM of the solid cement surface and of the cells in the pore water on top of the OPC CEM I was performed after 89 days with nitrate as electron acceptor and after 41 day with sulphate as electron acceptor. One replicate of each condition was sacrificed to analyse biofilm formation on the OPC CEM I and to visualize intact cells in the pore water. The latter was done by concentration of 1 ml of the pore water on a Whatman® Nuclepore™ track-etched polycarbonate membrane with 0.1 µm pore diameter (Merck, Belgium). To analyse biofilm formation on the OPC CEM I, the culture flask was sliced with a Dremel. Afterwards, a small piece of wet cement was excised with a sterile razor blade and a pair of tweezers. The OPC CEM I piece was washed with 10 mM MgSO4 for 10 min to remove residual microbial cells that are not attached to the cement.

Samples were fixated overnight with solution containing 0.3 M gluteraldehyde and 0.132 M sodium cacodylate. Afterwards, excess gluteraldehyde was removed by washing the filter with sodium cacodylate solution (0.150 M) twice. Subsequently, cells were dehydrated using an ascending graded series of ethanol solutions (30%, 50%, 70%, 90%, 95% v/v), followed by a final solution of 100% ethanol which was replaced two times (minimum 10 min between each solution). Drying of the samples was performed twice with hexamethyldisilazane for 2 min, followed by air drying for at least one hour. Finally, the cement piece was mounted on a copper stub using carbon conducting tape and nail polish. Samples were sputter-coated with gold (20 nm) in one cycli of 200 s (4 mbar Argon, 50 mA, 1 kV) (Scancoat Six, BOC Edwards B.V., Dongen, The Netherlands). SEM analysis was performed on a Phenom ProX (Phenom-World, The Netherlands), equipped with a backscatter electron detector at a working distance of 20 mm and a 10 or 15 kV acceleration.

3 Results & Discussion

In this study, anoxic batch experiments were performed to study the microbial community present in Boom Clay borehole water in a cementitious environment.

In a first set of batch experiments, sulphate was added as electron acceptor and the sulphate reduction activity of the microbial community in the presence of cement was studied. Oxidation of Boom Clay – although limited until 1 meter of the concrete-clay interface – is unavoidable during the construction and operation of a geological repository of radioactive waste [19]. The oxidative dissolution of pyrite will result in the presence of sulphate and thiosulphate in Boom Clay pore water. Thiosulphate however, is metastable and will further react to form additional sulphate. Concentrations of ca. 10 mM SO_4^{2-} were observed in different parts of the HADES URF [19], hence this concentration was added in the batch experiment performed in this study. Sulfate-reducing microorganisms are key players in microbially induced anaerobic corrosion of steel. This group of

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microorganisms is able to take away electrons from the steel (Fe^0) and/or the H_2 and reduce sulphates to sulfides (S^{2-}) or HS^-, which can create corrosive agents (such as H_2S) [21].

In another set of batch experiments, the nitrate reduction capacity of the microbial community in the presence of cementitious material was tested. In the Belgium disposal concept, nitrate will be present at some parts of the Boom Clay – disposal gallery interface as it will leach from bituminized waste, an important fraction of the intermediate level long-lived waste in Belgium (discussed in MIND deliverable 1.3). Conservative scoping calculations indicated that the maximum NO_3^- concentrations reach between 0.5 and 1 M at the Eurobitum disposal gallery interface, and decrease fast within the host formation (e.g. 0.1 M at a distance of 5 m in the Boom Clay) [34]. The purpose of the present batch experiments was to pinpoint the effect of a cementitious environment on the microbial processes. Therefore a concentration of 0.1 M $NaNO_3$ was selected as this concentration likely enhances microbial activity, while concentrations of 1 M can hamper the microbial community [22]. Nevertheless, in future experiments, the combined effect of high salt concentrations and the presence of cementitious materials should be investigated. Microbial nitrate reduction can result in an accumulation of nitrite, shown to be able to chemically oxidise pyrite and possibly dissolved organic matter from the Boom Clay host rock [23, 24]. Oxidation of these Boom Clay components, makes that its reducing capacity will be decreased and mobility of certain radionuclides can be increased. In addition, if hydrogen – which will be produced by the anaerobic corrosion of steel and radiolysis of water – is used as electron donor to fuel microbial denitrification, it could result in a net gas consumption.

To obtain optimal conditions for microbial growth, an electron donor was added to the medium. The batch experiments with nitrate as electron acceptor were supplemented first with 15 mM formate and after 49 days 15 mM acetate was added, while 10 mM lactate was added in the samples with sulphate as eletron acceptor. Important to note is that IC measurements of day 0 of the batch experiments, indicated that acetate was naturally present in the Boom Clay pore water. However, the precise concentration could not be determined as the sample was too diluted. Nevertheless, the present acetate could be used as additional electron donor.

3.1 Evolution of the pH

In situ Boom clay water has a slightly alkaline pH of ~ 8.4 [18]. It is expected that the cementitious materials used during geological disposal of radioactive waste will induce an alkaline plume resulting in a pH > 12.5 for thousands of years [5]. Afterwards the pH will gradually drop to pH 10. The alkaline plume will penetrate the first 1-3 meters of the Boom Clay, increasing the pH at the interface of the Boom Clay with the concrete disposal gallery, to max pH 10.5, while further down in the Boom Clay, the pH will not change and will remain ~ 8.4 [5]. In this study, a pH of ~ 10 was targeted to mimic conditions representative for the Clay-concrete interface of the disposal gallery. To this end, an excess of Boom Clay pore water compared to OPC CEM I (ratio 5:1) was used to enable (partial) buffering of the expected pH increase. However, as aforementioned, the preparatory manipulations of the Boom Clay pore water, resulted in the equilibration with air and an initial pH of ~ 9.5 was obtained. In addition, this resulted in a decreased buffering capacity of the Boom Clay pore water [18].

In the samples with microbes but without OPC CEM I, no change in pH 9.5 was observed in the presence of nitrate, while in the presence of sulphate, the pH decreased from ~ 9.5 to ca. pH 7 after 94 days. In the conditions with OPC CEM I, a rapid increase of pH was observed: within 14 days, the pH of the supernatans raised up to pH > 12 in all samples with OPC CEM I. In conditions with OPC CEM I and with nitrate as electron acceptor, the pH remained > 12 in all samples up to 89 days. In the presence of sulphate and OPC CEM I, the pH remained > 12 up to 94 days in all sterile control samples and in one sample inoculated with the Boom Clay microbial community. On the other hand, in one replicate containing the Boom Clay borehole water microbial community, the pH dropped to pH 10 after 41 days and remained 10 up to 94 days and in another replicate containing the microbial

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community and cement, the pH dropped to pH 10.44 after 94 days. Putative further decrease in pH will be monitored during future samplings.

3.2 Total microbial cell count

Flow cytometry enables a fast fingerprinting of microbial communities in aquatic samples and allows the detection of shifts within these microbial communities [25]. In this study, flow cytometry was used to monitor the evolution of the total cell counts in the liquid during the batch experiments. Total cell count is based on SYBR® Green I which has a strong affinity for dsDNA but also a low affinity for ssDNA and RNA [26] and enters and stains all cells with nucleic acids independent of membrane integrity [25].

When sulphate was added as electron acceptor, in the absence of OPC CEM I, the total cell count remained constant for 94 days (Figure 4, Figure 5). The presence of OPC CEM I and the consequent high pH (> 12) seem to have a lethal effect on the sulphate reducing microbial community present in Boom Clay pore water as the total cell count in the supernatans strongly decreased in 2 replicates (Figure 4, Figure 5). On the other hand, in one of the three replicates a clear microbial community was observed in the presence of OPC CEM I. In this sample, the total cell count based on flow cytometry indicated a rapid decrease of ± 2 log during the first 15 days but afterwards, the total cell number remained quite constant (Figure 4, Figure 5). The reason for the variation in replicates remains unclear and more detailed analysis of the present microbial community is necessary.

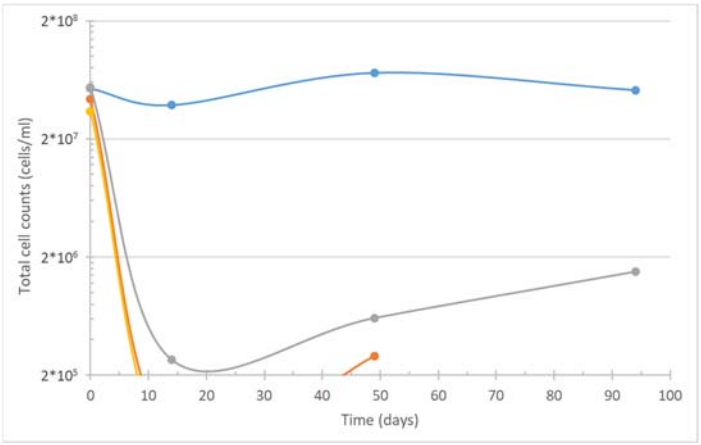

Figure 4: Evolution of the total cell count based on flow cytometry analysis of the suspension of samples supplemented with sulphate as electron acceptor in the absence of OPC CEM I (blue line) or of the replicates in the presence of OPC CEM I (orange, grey and yellow line). Results for condition without OPC CEM I (blue line) are averages and standard deviation of three replicates (first three points) and two replicates (last sampling point). For conditions with OPC CEM I (orange, grey and yellow line) results are shown for each replicate separately. The Y-axes crosses at the minimum relevant cell concentration needed to perform statistical analysis.

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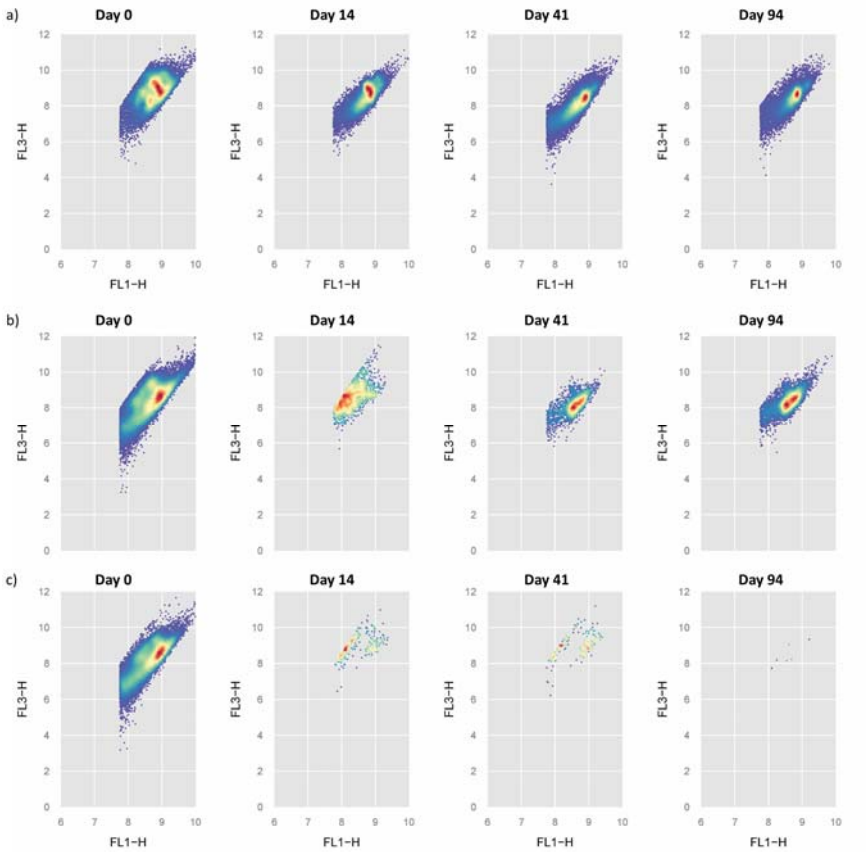

Figure 5: Flow cytometry dot plot of the supernatans after staining with SYBR® Green I at different time point with sulphate as electron acceptor a) in the absence of OPC (one replicate) or b,c) in the presence of OPC (two replicates). Plots are visualisation of the logaritmically transformed dataset for the fluorescence bivariates FL1-H (green fluorescence, 530/30 nm) and FL3-H (red fluorescence, > 630 nm). Blue and green correspond to areas of lower cell density, red and orange are areas of high cell density, and yellow is mid-range.

When nitrate was added as electron acceptor, in the absence of OPC CEM I, a clear microbial community was visible (Figure 7). The cell concentration decreased ca 0.5 log during the first 49 days, while it remains constant afterwards (Figure 6). The presence of OPC CEM I and the consequent high pH > 12 seem to be lethal for the nitrate reducing microbial community present in the Boom Clay borehole water as the total cell count in the supernatans was strongly decreased and only few cells remained in suspension after 89 days (Figure 6, Figure 7). In the samples supplemented with sodium nitrate, no clear difference between the replicates was observed and all three showed a similar inhibitory effect of the cementitious environment.

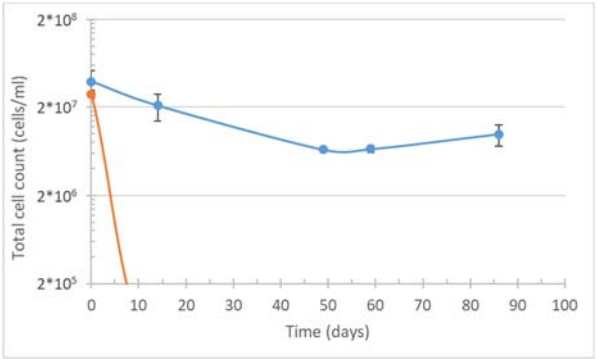

Figure 6: Evolution of the total cell count based on flow cytometry analysis of the suspension of samples supplemented with nitrate as electron acceptor in the absence of OPC CEM I (blue line) or in the presence of OPC CEM I (orange line). Results are mean and standard deviation of three replicates The Y-axes crosses at the minimum relevant cell concentration needed to perform statistical analysis.

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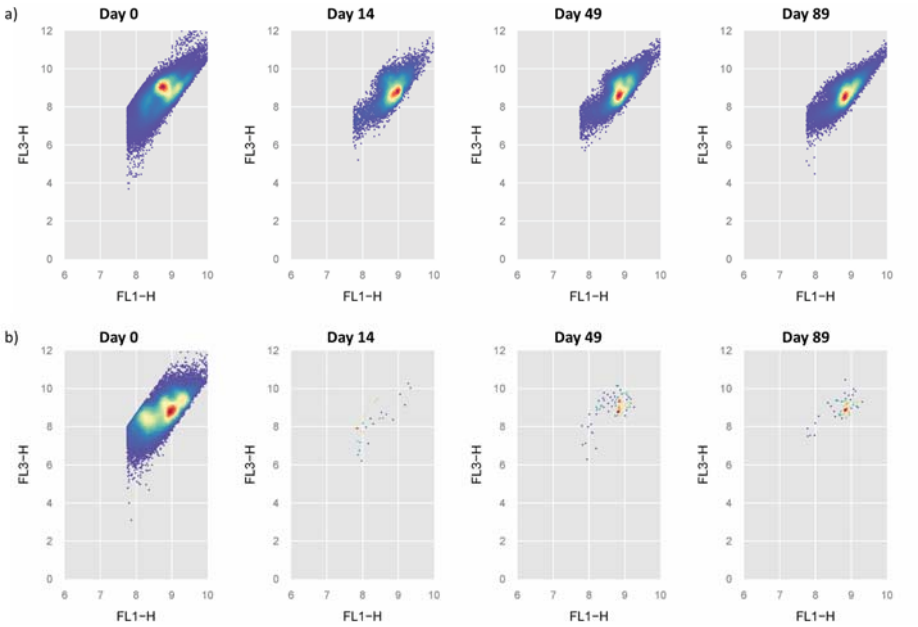

Figure 7: Flow cytometry dot plot of the supernatans of one replicate after staining with SYBR® Green I at different time point with nitrate as electron acceptor a) in the absence of OPC or b) in the presence of OPC. Plots are visualisations of the logaritmically transformed dataset for the fluorescence bivariates FL1-H (green fluorescence, 530/30 nm) and FL3-H (red fluorescence, > 630 nm). Blue and green correspond to areas of lower cell density, red and orange are areas of high cell density, and yellow is mid-range.

The presence of intact cells in the suspension was confirmed with SEM analysis of the suspension of one replicate from the nitrate reducing conditions after 89 and one replicate from sulphate reducing conditions (not the one where a clear microbial community was present with flow cytometry) after 41 days. Figure 8 demonstrates that there are intact cells present in both samples with pH> 12. Although there are much less cells in the supernatans compared to the conditions without OPC CEM I, this indicates that high pH alone does not eliminate the presence of all microorganisms.

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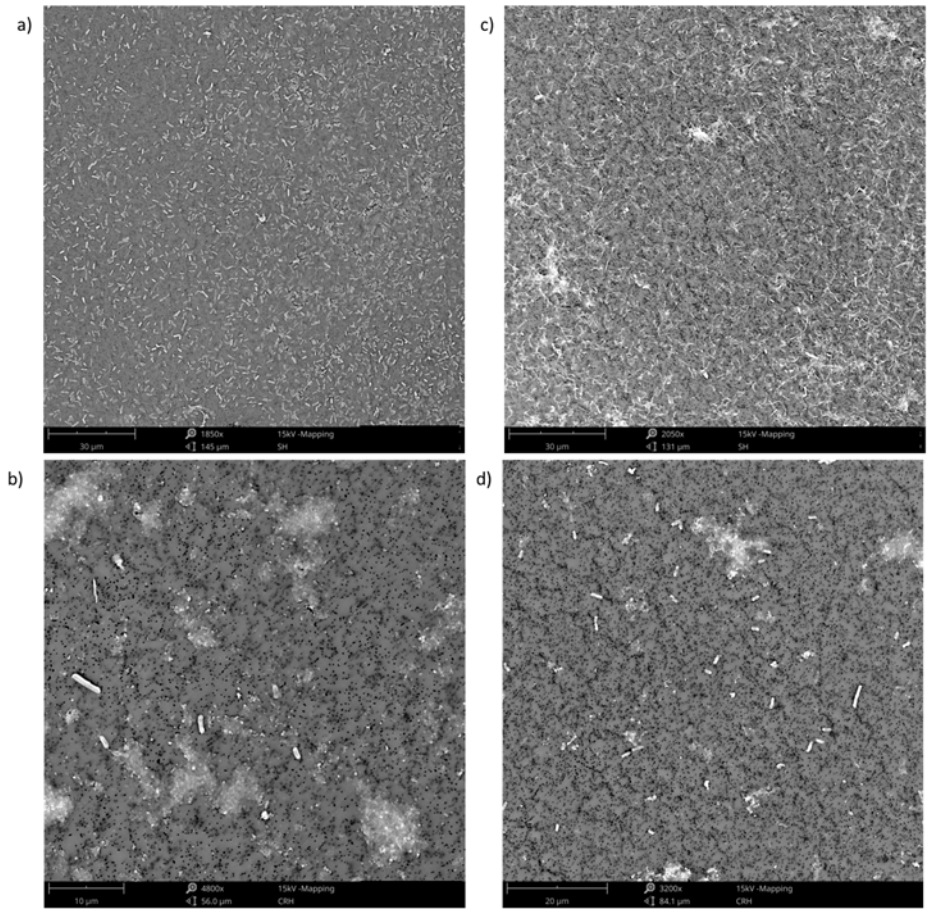

Figure 8: SEM picture showing the presence of intact cells in the suspension in sulphate reducing conditions after 41 days a) in the absence of OPC CEM I and b) in the presence of OPC CEM I and in the suspension in nitrate reducing conditions after 89 days c) in the absence of OPC CEM I and d) in the presence of OPC CEM I.

3.3 Microbial metabolism

Possible ongoing microbial sulphate reduction processes were analysed by monitoring sulphate, lactate and acetate concentrations at the beginning of the batch experiments, after 14 and 41 days with IC. As indicated in the materials & methods section, the lactate and acetate peaks were more difficult to resolve accurately, thus no truthful concentrations could be determined to calculate for example the C-balance.

Without OPC CEM I a clear sulphate reduction was observed as after 14 days, the sulphate concentration halved (from 10mM to ± 5 mM) in samples with microorganisms compared to the sterile control (Figure 9 a) and lactate was completely consumed and resulted in the production of acetate. Thus, incomplete lactate oxidation to acetate occurred according to the following reaction:

$2\ CH_3CH(OH)COO^- + SO_4^{2-} \rightarrow 2\ CH_3COO^- + HS^- + HCO_3^- + H^+$ ($\Delta G0'$ (pH 7) = -160.1 kJ/mol SO_4^{2-}) [27]

This reaction could also explain the observed decrease in pH. Indeed, in the samples with microbes but without OPC CEM I, in the presence of sulphate, the pH decreased from 9.5 to ca. pH 7 after 94 days. It is not expected that the 1 % hydrogen present in the atmosphere of the anaerobic glove box served as electron donor to reduce the ± 5 mM sulphate that was still present after 14 days. To carry out hydrogenothropic sulphate reduction, ($4H_2 + SO_4^{2-} \rightarrow HS^- + 4\ H_2O$), protons are consumed, hence a pH increase is expected instead of a pH decrease as observed in our experiments. Another possible electron donor could be the Boom Clay dissolved organic matter, although it is shown to be highly recalcitrant for sulphate reduction, even after oxidation of the clay [28]. Therefore, more research is necessary to determine the precise electron donor used in our experiments.

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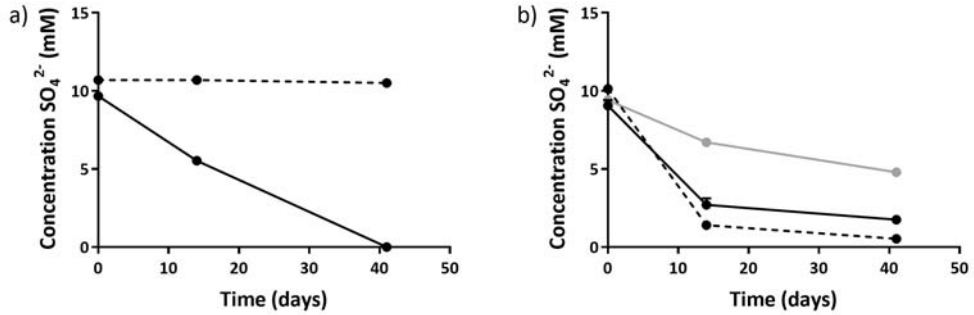

Figure 9: a) Evolution of sulphate concentration during the batch experiments without OPC CEM I. The black dotted line represent the values of one sterile control, while the mean and standard deviation of the three replicates inoculated with the microbial community of Boom Clay borehole water are shown full black lines. b) Evolution of sulphate concentration during the batch experiments in the presence of OPC CEM I. The black dotted line represent the mean and standard deviation of three sterile control samples, the full black line represent the mean and standard deviation of two replicates inoculated with the microbial community of Boom Clay borehole water but without a clear microbial community in suspension and the grey full line presents the data of one replicate inoculated with the microbial community of Boom Clay borehole water showing a clear microbial community in suspension.

For the samples with OPC CEM I, results are not conclusive yet. Sulphate concentrations decreased in all samples, also in the sterile controls (Figure 9 b). Lactate was supplemented as calcium lactate and in the presence of high Ca^{2+} concentrations sulphate can built in the OPC CEM I [29], hence this might explain the observed decrease in sulphate concentration. However, the precise cause of this rapid decrease needs to be investigated more in detail. Interestingly, this decrease seemed much slower in the sample harbouring clearly a larger intact microbial community in the supernatans. After 41 days, sulphate concentrations only halved instead of completely removed as in the negative controls (Figure 9). Also in the other 2 biological replicates, sulphate concentrations seemed slightly higher compared to the negative control (Figure 9). The extracellular polymeric substances (EPS) produced by the microbial community has been shown to interact with Ca^{2+} and could play a critical role in the precipitation of $CaCO_3$ [30], thereby indirectly resulting in more sulphate in solution. In addition, carbonation might explain the observed decrease in pH from pH > 12 to pH 10 after 41 days [4]. The data regarding lactate and acetate were not conclusive, therefore, it remains unclear if the observed decrease in sulphate in the replicate harbouring clearly a larger intact microbial community in suspension can be attributed to a chemical reaction or the microbial sulphate reduction. Therefore, more detailed analysis is planned to elucidate the precise ongoing mechanism.

When the microbial community of the Boom Clay borehole is provided with 100 mM nitrate without exposure to OPC CEM I, the nitrate concentration reduced from 100 mM to ca. 45 mM within 14 days and was completely removed after 49 days (Figure 10 a). Incomplete denitrification of nitrate to nitrite seems to be the dominant nitrate reduction mechanism as the N balance calculated from NO_3^- and NO_2^- concentrations remained constant (Figure 10 a, Figure 11). Possibly this incomplete denitrification can be attributed to the excess of nitrate that is present compared to the amount of electron donor. Indeed, it was observed that decreasing the C/N ratio below a certain threshold (depending on the microbial population and the type of organic compound) resulted in an accumulation of nitrite [13, 31, 32].

Initially, 15 mM formate was added as electron donor to fuel microbial metabolism and was completely oxidized within 14 days (Figure 10 b). Formate could serve as electron donor for nitrate reduction according to the following reaction kinetics:

$HCOO^- + NO_3^- \rightarrow NO_2^- + HCO_3^-$ ($\Delta G_0'$ (pH 7) = -161.9 kJ/mol) (1) [33]

This reaction kinetics suggests that with 15 mM too little formate was present to reduce 55 mM nitrate, therefore other electron donors could be used in the nitrate reduction process. This could be the 1 % H_2 present in the atmosphere of the anaerobic glove box. Hydrogen could diffuse in the

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culture bottles via the cap, which contains a membrane to allow gas exchange. A possible reaction with hydrogen as electron donor is:

$H_2 + NO_3^- \rightarrow NO_2^- + H_2O$ ($\Delta G_{0'}$ (pH 8) = -81 kJ/mol (2) [34]

Another possible electron donor could be the Boom Clay dissolved organic matter, although it is shown to be highly recalcitrant for denitrification [13]. However, all preparatory manipulations carried out in this study could have resulted in a partial oxidation of the dissolved organic matter, resulting in lower molecular weight constituents [19, 20], which are more prone to biodegradation. In addition, as mentioned above, acetate was observed in the Boom Clay borehole water nd the presence of acetate has been shown to facilitate the degradation of larger DOM into smaller organic molecules [13].

However, further research is necessary to determine the precise electron donor that was used by the microbial community.

It has been shown previously that acetate is a preferential electron donor for nitrite reduction to nitrous oxide [35]. However, when the samples were supplemented with additionally 10 mM acetate after 49 days no acetate consumption was observed during the following 10 days (Figure 10 d). The lack of microbial nitrite reduction can be attributed to the high nitrite concentration (ca 100 mM) accumulated during nitrate reduction, which can be inhibiting microbial activity [13]. This is also in accordance with the observed decrease in total cell counts in the period where nitrite concentrations accumulate in the suspension (Figure 6).

In the presence of OPC CEM I, conditions with pH > 12, no microbial nitrate reduction occurred (Figure 10 c &d), indicating that the high pH inhibited the nitrate reducing microbial community present in the Boom Clay borehole water. In addition, formate nor acetate was oxidized in those conditions (Figure 10 c & d).

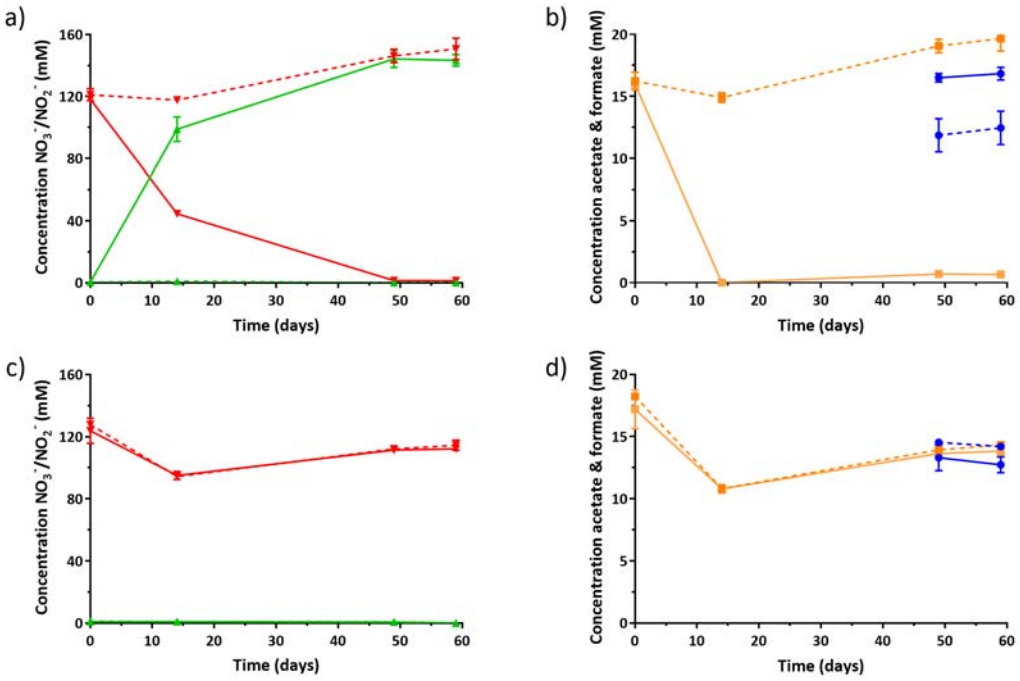

Figure 10: Concentrations of nitrate, nitrite, formate and acetate at different time points during the experiments with nitrate as electron acceptor a-b) in the absence of OPC CEM I and c-d) in the presence of OPC CEM I. Nitrate concentrations are presented as red lines, nitrite concentrations are shown as green lines, orange lines represent formate concentrations and blue lines correspond to acetate concentrations. Negative control samples are presented as dotted lines and samples inoculated with the microbial community of Boom Clay borehole water are shown as full lines. Values represent mean and standard deviation of three replicates.

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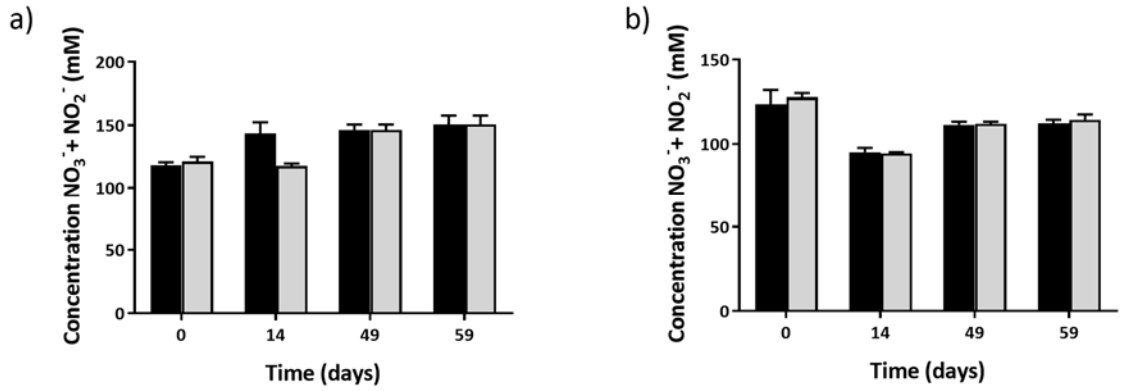

Figure 11: N balance originating from NO_3^- and NO_2^- concentrations of samples a) in the absence of OPC CEM I inoculated with the Boom Clay microbial community (black bars) and in the sterile control (grey bars) and b) in the presence of OPC CEM I inoculated with the Boom Clay microbial community (black bars) and in the sterile control (grey bars). Results are the mean and standard deviation of three biological replicates.

3.4 Biofilm formation on OPC CEM I

SEM analysis was performed on a small piece of OPC CEM I from 1 replicate after 89 days and 41 days with respectively nitrate and sulphate as electron acceptor. Figure 12 shows detailed pictures of biofilm formation on OPC CEM I in both nitrate and sulphate reducing conditions. In addition, overview pictures indicate biofilm formation at several places of the OPC CEM I. It should be noted the samples where incubated without shaking (only when subsamples were taken, the bottles were thoroughly shaken) and the hardened cement layer comprised the entire bottom of the culture flask. Therefore, cells could have settled by sedimentation on the OPC CEM I and subsequently embedded by EPS, thus this biomass is not solely formed as the result of a biofilm forming process [36]. Biofilm formation is known to enhance survival against a range of environmental stresses [37], hence possibly enables the microbial community to withstand the high pH environment originating from the OPC CEM I. However, further experiments are necessary to elucidate if viable cells are present within the biofilms.

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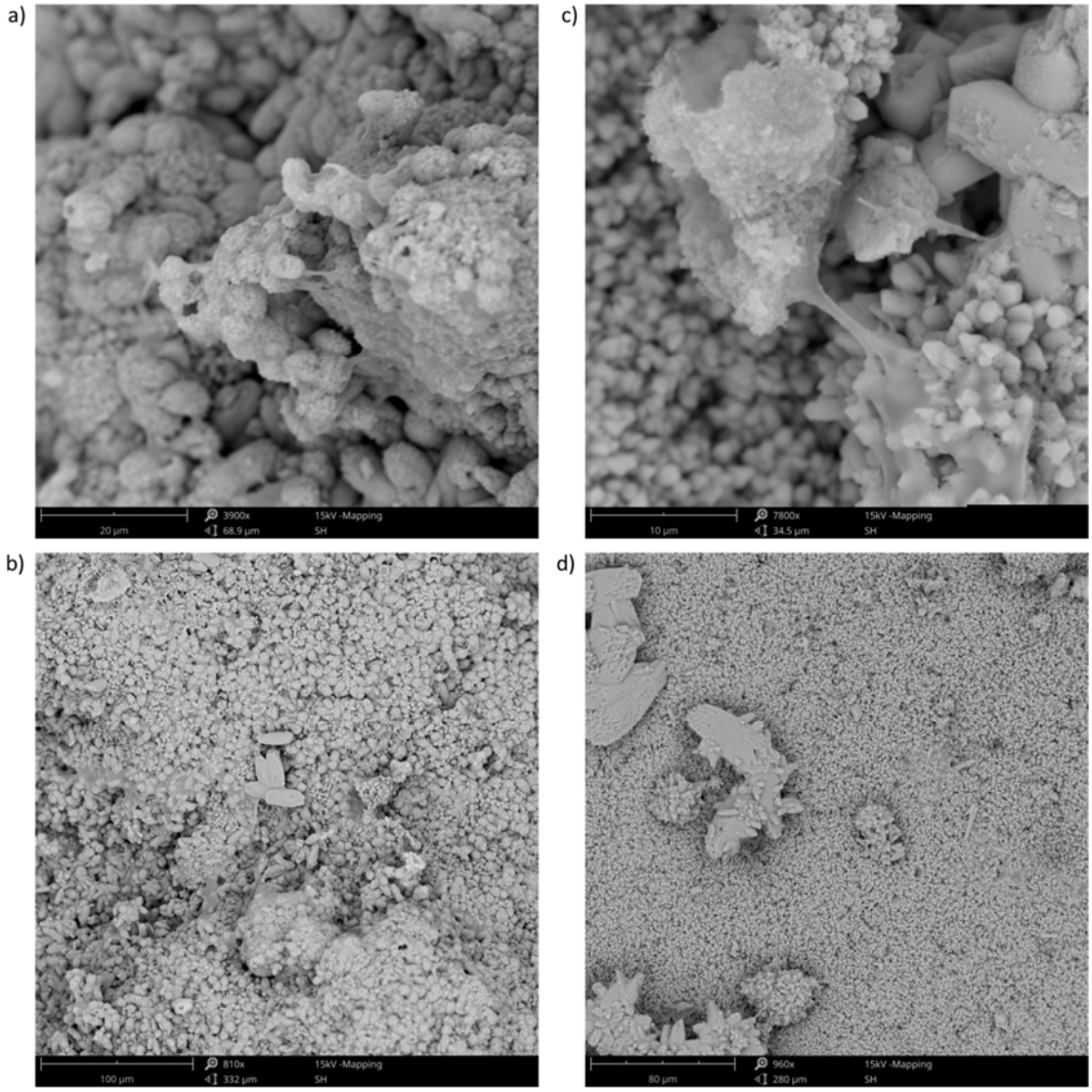

Figure 12: SEM pictures of one replicate exposed to OPC CEM I in a,b) sulphate reducing conditions for 41 days and c,d) nitrate reducing conditions for 89 days.

4 Conclusions

This preliminary study demonstrated that the high pH conditions imposed by the OPC CEM I inhibit microbial sulphate and nitrate reduction. However, SEM analysis indicated the presence of intact cells in the suspension on top of cement and putative biofilm structures on the cement. This suggest that the high pH environment does not completely eliminate the microbial population. Therefore, if niches arise with lower pH values, nitrate and sulphate reduction could be induced. In addition, at the interface between the Boom Clay – gallery liner, the pH is not expected to be higher than 10.5, hence microbial activity could be present. Interestingly, in sulphate reducing conditions, a pH decrease from > 12 to pH 10 was observed in one replicate harbouring clearly a larger microbial community in the suspension on top of the cementitious material compared to the other samples. This pH decrease can possibly result in more optimal conditions for other species within the microbial community. However, the precise mechanism remains unclear and more detailed chemical and microbial analysis and of the structure of the OPC CEM I is planned.

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