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Bacterial sulphide-producing activity in water saturated iron-rich Rokle and iron-poor Gaomiaozi bentonite at wet densities from 1750 to 1950 kg m^{-3}

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Bacterial sulphide-producing activity in water saturated iron-rich Rokle and iron-poor Gaomiaozi bentonite at wet densities from 1750 to 1950 kg m^{-3}

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Abstract

The bentonite buffer that will contain copper canisters with spent nuclear fuel in Sweden's future repository must maintain a high density to be able to sufficiently protect the canisters. Naturally occurring sulphide-producing bacteria (SPB) in the bentonite and in surrounding groundwater may potentially threaten the integrity of the canisters by dissimilatory production of sulphide, a substance corrosive to metals such as copper. Bacterial sulphide-producing activity has been observed to decrease with increasing wet density but it is not yet clear whether there is a specific wet density above which bacterial activity is not impacting the integrity. Such a value may vary between different types of bentonite Previous work with Wyoming MX-80, Asha and Calcigel bentonite have resulted in good understating of the density threshold for microbial activity in saturated MX-80, Asha and Calcigel bentonite clay. However, for other types of bentonite very little is known about microbial activity as a function of clay density. It has been observed that sulphide production, analysed as formation of copper sulphide on copper discs, ceased at a lower wet density in the iron-rich Asha bentonite compared to iron poor Calcigel and MX-80 bentonites. This work investigated the effect from one iron-rich Rokle bentonite and one iron-poor Gaomiaozi (GMZ) bentonite on sulphide producing activity over a range of three wet densities, 1 750, 1 850 and 1 950 kg m^{-3}. This work also analysed the cultivability of SPB over the tested ranges of wet densities in the two studied bentonites.

Test cells of titanium were used in the experiment which consisted of a cylinder with a piston inside. The cells were filled with bentonite clay powder with addition of a bacterial cocktail consisting of three different species of SPB, except controls without added SPB. The bentonite clay powder was compacted to a specific volume and then water saturated with a salt solution through inlets at the bottom and top of the test cells. When the clay had reached the planned wet densities and was fully water saturated, the piston and bottom lids were removed. A 40 µm pore size titanium filter was attached to the bottom lid and kept the clay from swelling into the inlet hole. This filter was at start of the experiments replaced with a copper disc that simulated a copper canister. On the opposite clay core side to the copper disc, $^{35}SO_4^{2-}$ together with lactate were added. The test cells were then closed again and the force transducer, piston and top lid were refitted. The test cells were harvested in series after up to 78 days from the addition of $^{35}SO_4^{2-}$ and lactate. The radioactivity of the $Cu_2^{35}S$ that had formed on the copper discs was located and quantified using electronic autoradiography. Samples were taken from different layers of the bentonite core and analysed for distribution of ^{35}S, sulphate, acetate, lactate and most probable number of SPB.

Sulphide-producing bacteria could be cultivated from all tested wet densities and there were significant numbers of inherent sulphide-producing bacteria in both tested clays. The radiotracer methodology indicated a very low sulphide-producing activity in Rokle at all tested densities except for the sample with the lowest wet density that had a locally high sulphide production. For the GMZ, there was significant sulphide-producing activity at all three densities tested. A cut-off in sulphide-producing activity by SPB was observed over increasing wet density that correlated with a loss of sulphide production.

Contents

1   Introduction

In the Finnish and Swedish repository concepts for geodisposal of spent nuclear fuel (SNF) the bentonite barrier has an important function in maintaining the integrity of the copper canisters isolating the SNF (SKB 2010). In the repository, a highly compacted bentonite with a bulk wet density between $1\,950$ and $2\,050$ kg m^{-3} is projected. The bentonite is intended to hinder outward transport of radionuclides and inward transport of corrosive groundwater components, and is required to act as a buffer against rock movements. The presence and activity of sulphide-producing bacteria (SPB) have been detected in groundwater at repository depth (Hallbeck and Pedersen 2012, Pedersen et al. 2014) as well as in various types of commercially available bentonites including Asha, Calcigel and Wyoming MX-80 (Svensson et al. 2011). Sulphide-producing bacteria have been found in a full scale demonstration repository (Arlinger et al. 2013), in various pilot and full scale tests of bentonite performance (Karnland et al. 2009, Lydmark and Pedersen 2011) and in the Boom Clay formation (Bengtsson and Pedersen 2016). In a future SNF repository, the dominant long-term copper corrosive agent may, therefore, be sulphide. The anaerobic bacterial processes of concern are consequently 1) the dissimilatory reduction of sulphate or sulphur to hydrogen sulphide, 2) disproportionation of thiosulphate to sulphide and sulphate and 3) desulphurylation of organic-S by various sulphide-producing bacteria (SPB).

If given good growth conditions, i.e. carbon and energy sources and sulphate, the SPB can rapidly multiply several orders of magnitude and then the produced sulphide over time may become large. Therefore, it is required to test the present hypothesis that sulphide-producing activity of SPB will be very slow or nil in the planned bentonite density range. Bacterial sulphide-producing activity has been demonstrated to decrease with increasing density of bentonite (Motamedi et al. 1996, Motamedi 1999, Stroes-Gascoyne et al. 1997). Previous work with Wyoming MX-80 bentonite suggested that bacterial sulphide-producing activity and cultivability cease somewhere in the range of $1\,900$–$2\,100$ kg m^{-3} wet density, but the exact cut-off density remained to determine (Masurat et al. 2010, Pedersen et al. 2000a, b). Variables of importance for such activity, in addition to bentonite density can be pore space and pore water composition, transport conditions to and from the bentonite boundaries, usability of the naturally occurring organic matter present in the bentonite and H_2 from corroding metals, H_2 and CH_4 from geological sources and temperature. Mineral compositions of bentonites may also have a large influence on survival and activity of SPB.

Until now, our laboratory research on survival and activity of SPB in bentonite as functions of wet density has been performed with Wyoming MX-80, Calcigel and Asha bentonites at wet densities of $1\,500$ to $2\,000$ kg m^{-3} (Bengtsson and Pedersen 2017). The worked performed has provided a good understanding of viability and activity of bacteria in the tested range of densities. It was observed that sulphide production, analysed as formation of copper sulphide on copper discs, ceased at a lower wet density in the iron-rich Asha compared to the more iron poor Calcigel and MX-80 bentonites.

This work investigated the effect from one iron-rich Rokle bentonite and one iron-poor GMZ bentonite on sulphide producing activity over a range of three wet densities from $1\,750$ to $2\,000$ kg m^{-3}. This work also seeks to evaluate the cultivability of SPB over the tested ranges of wet densities in the two studied bentonites. Favourable growth conditions were obtained by the addition of lactate which is a preferred carbon and energy source for most SPB. Pure cultures of SPB were added to ensure that all experiments except controls included approximately an equal number of SPB plus possibly already present SPB in the commercial clays.

Cylindrical test cells made of titanium were used in the experiments. The cells were filled with the respective bentonite clay powder with addition of a bacterial cocktail consisting of three different species of SPB, except control cells that were filled with clay without added SPB. The bentonite clay powders were compacted to a specific volume and then water saturated. The test cells were opened in an anaerobic atmosphere and a copper disc that simulated a copper canister was installed in the clay core bottom when the clay cores had reached the planned wet densities and were fully water saturated. On the other side of the clay core, opposite to the copper disc, $^{35}SO_4^{2-}$ together with lactate, which is a preferred carbon and energy source for SPB, were added. This radioactive substance was used as a tracer for bacterial reduction of sulphur in sulphate to sulphide. The test cells were sampled in series

after up to 78 days from the addition of $^{35}SO_4^{2-}$ and lactate. The radioactivity of $Cu_2{}^{35}S$ that had formed on the copper discs was located and quantified using electronic autoradiography. Samples were taken from different layers of the bentonite core and analysed for distribution of ^{35}S, sulphate and most probable number (MPN) of cultivable SPB.

2 Material and method

2.1 Experiments

This report describes two different, consecutive experimental series with two different bentonite types and three different species of added SPB exposed to varying levels of wet density. The experimental series was a continuation of the work described by Bengtsson et al. (2017) were experiments 1–5 are treated. To more easily refer to each experiment, a numbered list with information about bentonite types, planned densities and the time frame of the respective experiment 6 to 7 is given in Table 2-1.

Table 2-1. List of performed experiments with Rokle and GMZ bentonites with the planned wet and dry densities. Dry densities were calculated according to Karnland et al. (2006), see Section 2.5.

Experiment number	Year	Bentonite tested	Planned wet densities (kg m^{-3})	Planned dry densities (kg m^{-3})
6	2016	Rokle	1750, 1850, 1950	1112, 1260, 1408
7	2016–2017	GMZ	1750, 1850, 1950	1160, 1315, 1469

2.2 Chemical and mineralogical characters of the bentonites

The main mineralogical and chemical compositions of the clays are given in Table 2-2. Both clays have a high content of montmorillonite and minor amounts of various clay minerals. The literature about GMZ is less detailed than for Rokle which results in some points were no data could be found. The GMZ contains more silica and quartz than Rokle. Rokle also distinguishes from GMZ by a five times larger amount of iron. Sulphur was not found in Rokle (Karnland et al. 2006). Data could not be found regarding the amount of sulphate in GMZ. Both clays were selected and delivered by SKB. In another investigation, sulphur was reported to be present in Rokle in low amounts (Svensson et al. 2011).

Table 2-2. Average results from the XRD analyses of mineral compositions and the content of elements in the Rokle (Karnland et al. 2006) and GMZ (Ye 2016) bentonite materials expressed as weight percent of major element oxides, total carbon and sulphur of dry mass after ignition. LOI denotes the percent mass loss due to ignition. – = no data.

Component	Rokle	GMZ
Minerals		
Montmorillonite	69.4	75.4
Muscovite	2.8	–
Plagioclase	0.2	–
Pyrite	1.1	–
Quartz	2.5	11.7
Other	24	12.9
Elements		
S-total	0	–
S-Sulphate	0	–
S-Sulphide	0	–
SiO_2	46.6	67.43
Al_2O_3	12.9	14.20
Fe_2O_3	13.0	2.40
MgO	2.6	0.10
CaO	5.8	1.13
Na_2O	0.2	1.75
K_2O	1.0	0.73
TiO_2	4.1	0.12
P_2O_5	0.8	0.02
Total carbon	0.94	–
Organic carbon	0.02	–
LOI	12.7	–

Sulphate analysis was also performed on raw bentonite before the start of each experiment to determine the amounts of lechable sulphate in each bentonite type. This was done by dispersing 1 g of bentonite in 10, 20, 30, 40 and 50 mL of a 10 % $MgCl_2$ solution. The concentration of sulphate was then measured with the SulfaVer4 method on diluted or undiluted supernatant from the five different tubes. To ensure that the entire sulphate was leached from the bentonite the remaining supernatant after analysis was poured off and the experiment was repeated with new $MgCl_2$ solution. Sulphate could not be leached from these clays.

2.3   Test cells

Identical test cells were used to create saturated bentonite cores in series with different densities. A test cell consisted of a titanium cylinder of inner diameter 35 mm with top and bottom lid attached by six Allen screws for each lid. A piston operated inside the cylinder (Figure 2-1). When the piston was at its most extended position, a confined cavity of height 20 mm was produced inside the cylinder (Figure 2-2). This cavity was filled with Rokle or GMZ bentonite powder (see Section 2.5). The test cells are described in detail in Bengtsson et al. (2017). Each test cell was given a unique code with information of the planned bentonite wet density, addition of SPB and incubation time: TC = test cell, 1 750–1 950 = 1 750 to 1 950 kg m^{-3} bentonite wet density, (+/−) = with or without adding of SPB, d = days of incubation. Two different incubation times were used for each experiment to be able to analyse microbial sulphide producing activity as a function of time.

Figure 2-1. *View of all parts included in a test cell. All parts in contact with the bentonites were made of titanium. See text for details. WS = water saturation.*
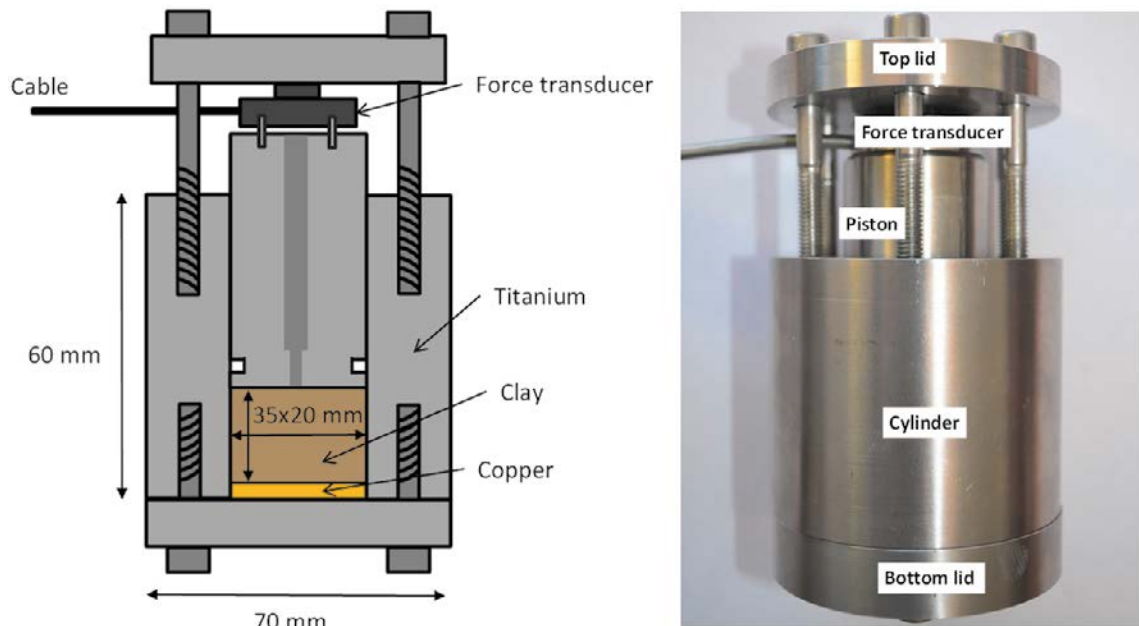
Figure 2-2. Left: A schematic cross section of a test cell. Right: An assembled test cell, spacers are not mounted.

2.4 Bentonite suspensions

Bentonite suspensions were prepared as described in Bengtsson et al. (2017) with the following modifications.

The bacterial species *Desulfotomaculum nigrificans* (DSM 574) was exchanged by *Desulfotomaculum guttoideum* (DSM 4024) due to that it was difficult to produce viable cultures of *D. nigrificans*. *D. guttoideum* is a spore-forming sulphate-reducing bacterium that has many similarities with *D. nigrificans* that also form spores, but is easier to cultivate.

The Rokle bentonite was delivered in a 15-kg bag containing grinded clay with granulates in the range from 0–4 mm. Consequently, the clay had to be further grinded before it could be added to the test cells. This was carried out manually with a mortar until a fine powder was created.

2.5 Compaction and water saturation of bentonite

The day before compaction of the bentonite the water content was determined on both non-doped and the SPB-doped bentonite batches by heating 3×1 g of each batch in aluminium bowls in 105 °C for 20 h. The average of the weight difference before and after heating for the three replicates was thus equal to the initial water content of each bentonite batch. The amount of bentonite (m_{solids}) needed to obtain the planned wet density for each test cell was calculated using the following equation (from Karnland 2010).

$$m_{solids} = V_{total} \times \rho_m - m_{max\ water}$$

Where ρ_m is the saturated density, m_{solids} is the mass of the solids, m_{max} water is the maximum possible mass of water, and V_{total} is the total volume of all components (solids and water). Grain densities were obtained from Karnland (2006) for Rokle and from Sun et al. (2014) for GMZ. The analysed dry and wet densities agreed with calculated values ($\pm$ 1 %).

Each test cell was assembled and thereafter water saturated as described in Bengtsson et al. (2017).

2.6 Addition of $^{35}SO_4^{2-}$, SO_4^{2-} and lactate

All work performed with additions of $^{35}SO_4^{2-}$ and lactate as well as the cleaning and insertion of the copper discs were carried out in the anaerobic box. Batches of $Na_2^{35}SO_4$ (PerkinElmer, cat. no. NEX041H010MC, 10 mCi (370MBq), specific activity: 1 050–1 600 Ci (38.8–59.2 TBq mmol^{-1} sodium sulphate in 1 mL water) was distributed over the test cells by pipette to a pore water concentration of 0.05 µM. In addition, a 5.7 M lactate solution was added to a final calculated pore water lactate concentration of 28 mM in all test cells and the water saturation medium was added with 3 mM Na_2SO_4. This concentration corresponds to from 1 to 1.5 µmole gdw^{-1} with the highest value in the highest density with the smallest pore water volume. For more details see Bengtsson et al. (2017).

2.7 Sampling and analysis

At the sampling date, the pressure logging in the force transducer software was stopped, the force transducer was removed together with the top lid and screws. Recorded pressures during the water saturation phase and incubation phase can be seen in Appendix 1 and 2. The top lid was then attached again, however with shorter screws to be able to push the piston all the way to the bottom. The test cells were moved to a fume hood and the bottom plates were carefully removed. The piston was then pressed up by turning the screws so that the edge of the copper disc became visible (Figure 2-3). An overview of analyses carried out on the copper discs and the bentonite cores is shown in Table 2-3. All analyses were performed according to the methods previously described in Bengtsson et al. (2017) with the addition of analysis of lactate and acetate of clay samples taken in profiles through the clay core (also described in Bengtsson et al. (2017), described below.

Weight and volume parameters that was applied at start of the experiment for each test cell can be found in Table 2-4. After the experiment, the wet densities were calculated as total weight of the clay/volume of the test cell.
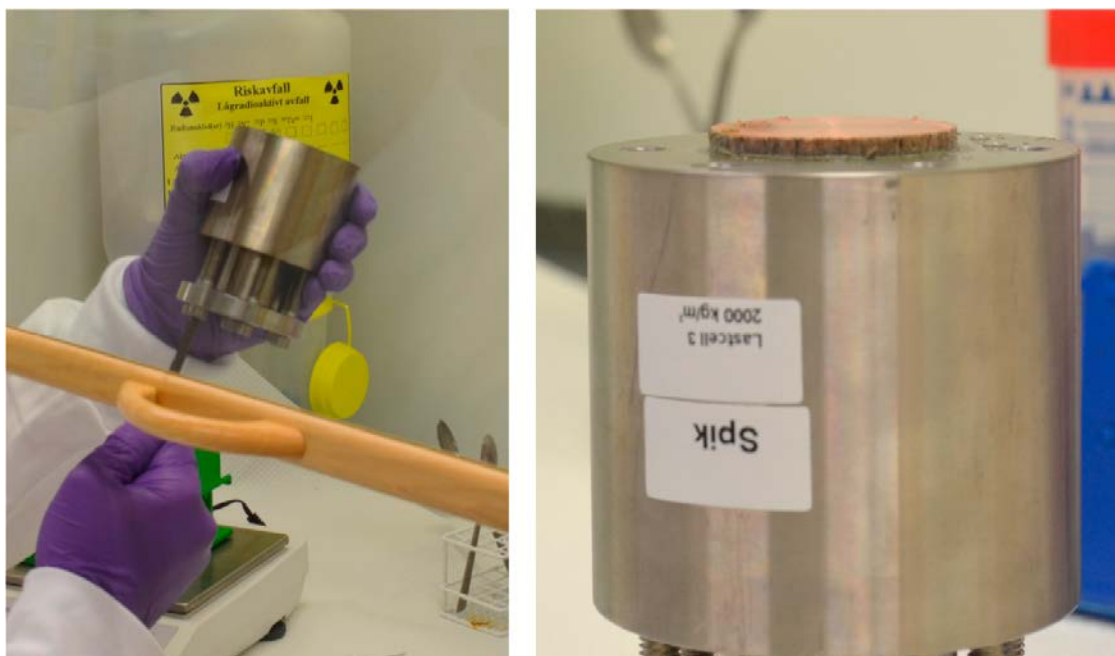

Figure 2-3. *Sampling of a test cell in the previous experiment 2 (T8 2000 (+) 47d) as described in Bengtsson et al. (2017). Left: pushing the piston up by tightening screws. Right: copper disc completely pushed out of the test cell.*

Table 2-3. Overview of performed analyses for all test cells.

Analysis	Method
$Cu_2{}^{35}S$ on copper discs	2D surface autoradiography
Most probable number	Cultivation
Distribution of ^{35}S in bentonite cores	Liquid scintillation counting
Distribution of sulphate in bentonite cores	Turbidimetric method by precipitation of $BaSO_4$
Distribution of acetate and lactate in bentonite cores	Enzymatic spectrophotometric methods

Table 2-4. Weight and volume parameters applied at start of the experiment for the water saturated Rokle and GMZ bentonite in each test cell. (gdw = gram dry weight, %ww = percent wet weight).

Bentonite type	Exp. no.	Test cell code	Test cell volume (cm³)	Amount of bentonite (gdw)	Pore water volume (mL)	Water content (%ww)
Rokle	5	TC41 1750 (–) 78d.	19.54	21.57	12.63	36.93
Rokle	5	TC42 1950 (–) 78d.	19.54	27.32	10.79	28.31
Rokle	5	TC43 1750 (+) 33d.	19.54	21.73	12.47	36.46
Rokle	5	TC44 1750 (+) 78d.	19.54	21.73	12.47	36.46
Rokle	5	TC45 1850 (+) 33d.	19.54	24.62	11.53	31.88
Rokle	5	TC46 1850 (+) 78d.	19.54	24.62	11.53	31.88
Rokle	5	TC47 1950 (+) 33d.	19.54	27.52	10.58	27.77
Rokle	5	TC48 1950 (+) 78d.	19.54	27.52	10.58	27.77
GMZ	6	TC51 1750 (–) 77d.	19.54	23.17	11.02	32.24
GMZ	6	TC52 1950 (–) 77d.	19.54	29.35	8.75	22.97
GMZ	6	TC53 1750 (+) 33d.	19.54	22.67	11.53	33.71
GMZ	6	TC54 1750 (+) 77d.	19.54	22.67	11.53	33.71
GMZ	6	TC55 1850 (+) 33d.	19.54	25.69	10.46	28.93
GMZ	6	TC56 1850 (+) 77d.	19.54	25.69	10.46	28.93
GMZ	6	TC57 1950 (+) 33d.	19.54	28.71	9.39	24.64
GMZ	6	TC58 1950 (+) 77d.	19.54	28.71	9.39	24.64

2.7.1 Acetate and lactate analyses

Acetate and lactate concentrations were determined with the enzymatic UV method (kit no. 10148261035 for acetate and kit no. 10139084035, for lactate; Boehringer Mannheim/R-Biopharm AG, Darmstadt, Germany) using a Genesys 10UV spectrophotometer (Thermo Fisher Scientific) for detection. The analyses were tested on two clays with increasing amounts of added lactate or acetate. There was a very good agreement between added and analysed amounts of these compounds (Figure 2-4 and Figure 2-5). The background values for lactate and acetate in Asha and Rokle clays without addition of lactate or acetate were ~0.1 and ~0.5 μmol gdw^{-1}, respectively. The background for acetate in GMZ was determined to 0.7 μmol gdw^{-1}.

Figure 2-4. *Added amounts of lactate to Rokle and Asha bentonite clays and the corresponding analysed amounts. The respective linear regression lines are shown.*
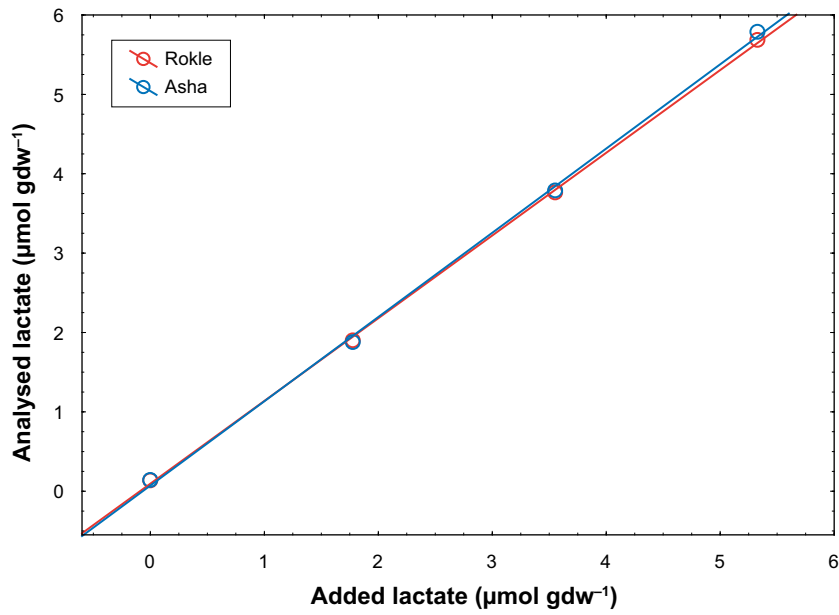

Figure 2-5. *Added amounts of acetate to Rokle and Asha bentonite clays and the corresponding analysed amounts. The respective linear regression lines are shown.*
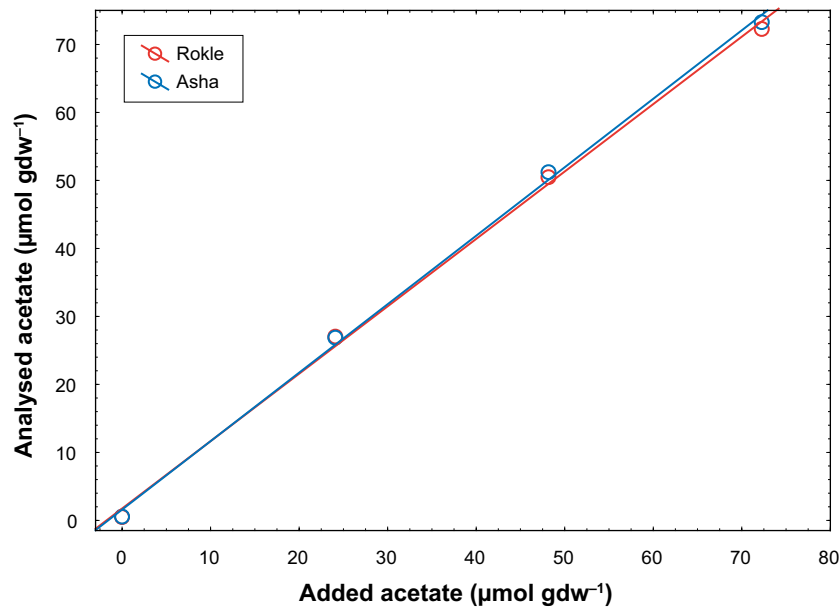
2.8   Isotope calculations

Measured radioactivity counts were recalculated to mol ^{35}S in the form of $Cu_2{}^{35}S$ via Equation (2-1), based on a maximum Instant Imager detector efficiency of 2 % (manufacturer's specification), as follows:

$$n = \left(\frac{DPS}{\lambda} \middle/ N_A \right) \times 50 \tag{2-1}$$

where n is the number of mols, λ is the decay constant for ^{35}S, NA is Avogadro's constant and DPS is decays per second. The decay constant, λ, was calculated with Equation (2-2), as follows:

$$\lambda = \frac{\ln(2)}{t_{1/2}} \tag{2-2}$$

where $t_{1/2}$ is the half-life of ^{35}S (i.e. 7.55×10^6 s). Adjustment for decay of ^{35}S during the experimental time was made with Equation (2-3), as follows:

$$A = A_0 \times e^{-\lambda t} \tag{2-3}$$

where A is activity at time t, A_0 is activity at time zero and t is elapsed time. The amounts of $Cu_2{}^{35}S$ were then calculated using the total elapsed time between start of experiment and the measurement dates. The isotope dilution caused by the added, nonradioactive sulphate to the pore water was calculated by dividing the added concentration of sulphate (3 mM) by the concentration of radioactively labelled sulphate at the start of the experiments. The amount of $Cu_2{}^{35}S$ was multiplied by the isotope dilution factor to obtain the total amount of Cu_2S produced per copper disc. The amount of Cu_2S in mol on copper discs was calculated as follows: (Gross counts)/(count time in min)/60 s/min/λ/$N_A \times 50 \times$ (isotope dilution factor). The factor of 50 is attributed to the Instant Imager's count efficiency of 2 %, as specified by the manufacturer.

2.9   Data processing, graphics and statistics

Data processing, statistical analyses and data visualizations were performed using Microsoft Office Excel 2016 (Microsoft Corporation, Redmond, USA) and Statsoft Statistica v 13 (Statsoft, Tulsa, USA) software.

3 Results

3.1 Copper discs

For the Rokle bentonite, only TC44, which had a low density (1 750 kg m^{-3}) and the longest incubation time (78 days), had a substantial accumulation of surface radioactivity on the copper disc (Table 3-1).

In the GMZ case, the accumulation of $Cu_2{}^{35}S$ was almost as high for the low density as the high density copper surfaces (1 750 and 2 000 kg m^{-3}) (Table 3-1). The intermediate density with 1 850 kg m^{-3} clay had more than ten times less surface radioactivity than some of the test cells with lower or higher density (Table 3-1). Similar results has previous been reported for other bentonite clays in the experiments series, as in Bengtsson et al. (2017), with no clear understanding on why high densities occasionally have more accumulation of surface radioactivity than lower densities. New information indicates that bentonite clays can immobilise sulphide which may have contributed to the variability in the amount of sulphide that reaches the clay (Pedersen et al. 2017). In the experiments 6 (Rokle) and 7 (GMZ) that are reported here, however, the lactate and acetate analysis offers a new dimension to the interpretation of the results with a high accuracy (see discussion and Section 3.3).

Table 3-1. Rokle and GMZ analysed wet densities, radioactivity detected on the copper discs recalculated for half-life of the isotope and the total amount of copper sulphide on the discs calculated from the surface activity and the isotope dilutions ($[SO_4^{2-}]/[^{35}SO_4^{2-}]$).

Test cell code	Analysed wet density (kg m^{-3})	Surface activity (kBq)	Total amount of Cu$_2$S (nmole)
Rokle			
TC41 1750 (–) 78d.	1 676	0.41	0.14
TC43 1750 (+) 33d.	1 692	0.05	0.02
TC44 1750 (+) 78d.	1 670	577	180
TC45 1850 (+) 33d.	1 838	0.06	0.02
TC46 1850 (+) 78d.	1 830	0.09	0.03
TC42 1950 (–) 78d.	1 924	0.15	0.05
TC47 1950 (+) 33d.	1 932	0.02	0.01
TC48 1950 (+) 78d.	1 940	0.02	0.01
GMZ			
TC51 1750 (–) 77d.	1 692	1 570	1 690
TC53 1750 (+) 33d.	1 715	243	247
TC54 1750 (+) 77d.	1 716	425	436
TC55 1850 (+) 33d.	1 832	34.2	38.2
TC56 1850 (+) 77d.	1 824	20.5	21.7
TC52 1950 (–) 77d.	1 910	54.2	64.6
TC57 1950 (+) 33d.	1 945	151	167
TC58 1950 (+) 77d.	1 929	1 110	1 170

3.2 MPN-samples

The MPN (Most Probable Number) results demonstrate how well the SPB community survived in the two different bentonites during incubation. In contrast to the accumulated ^{35}S measurements on copper discs viable SPB could be cultivated from all tested densities (Figure 3-1). There were approximately 100–1 000 more SPB in GMZ compared to Rokle in 1 750 and 1 850 kg m^{-3} clay cores. The MPN of SPB did not change over density for the Rokle clay cores There were SPB in all test cells without SPB inoculation. The MPN numbers in these test cells were somewhat lower than the inoculated test cells which should be expected because addition of laboratory SPB will add to the MPN of inherent SPB.
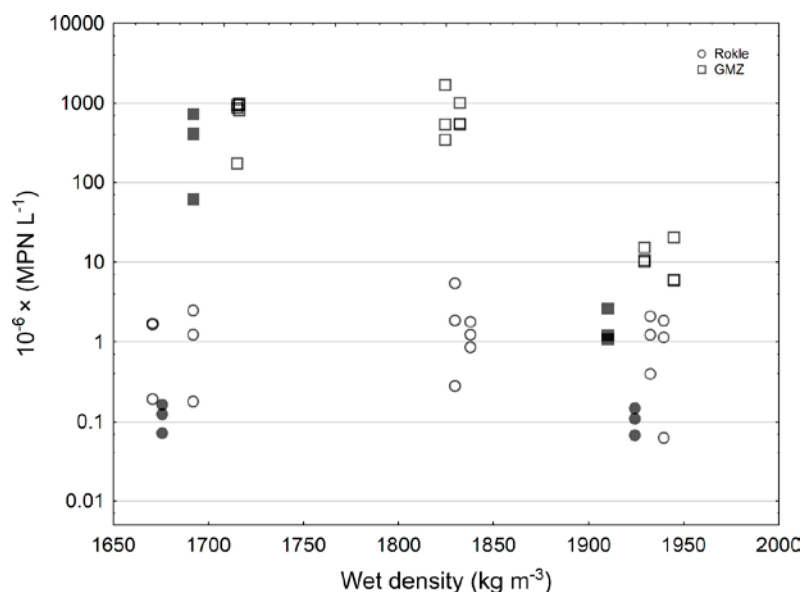
Figure 3-1. *Most probable numbers of sulphide producing bacteria in pore water of bentonite cores for two clays over wet density. Filled markers indicate control bentonite samples without bacterial addition.*

3.2.1 Rokle

The MPN analysis shows that SPB did survive in all test cells with Rokle bentonite and the numbers were fairly equally distributed in the clay cores (Table 3-2). There was no clear decrease in MPN numbers with an increase in wet density for this clay, similar to what was observed for Asha previously (Bengtsson et al. 2017).

3.2.2 GMZ

The MPN analysis shows that SPB did survive in all test cells with GMZ bentonite and the numbers were fairly equally distributed in the 1 750 and 1 850 kg m^{-3} clay cores (Table 3-3). There was a 100 times decrease in MPN numbers in the 1 950 kg m^{-3} clay cores compared to the lower densities, similar to what was observed for Asha (Bengtsson and Pedersen 2017).

Table 3-2. Most probable numbers of SPB in pore water of Rokle the bentonite cores.

	Position					
	Bottom **(1 mm from copper discs)**		**Middle** **(5 mm from copper discs)**		**Top** **(10 mm from copper discs)**	
Test cell code	**$10^{-6} \times$ (MPN L^{-1})**	**Lower–upper 95 % confidence interval**	**$10^{-6} \times$ (MPN L^{-1})**	**Lower–upper 95 % confidence interval**	**$10^{-6} \times$ (MPN L^{-1})**	**Lower–upper 95 % confidence interval**
TC41 1750 (–) 78d.	0.12	0.05–0.46	0.16	0.05–0.60	0.07	0.03–0.18
TC43 1750 (+) 33d.	0.18	0.08–0.42	2.48	0.99–9.94	1.24	0.52–4.84
TC44 1750 (+) 78d.	1.71	0.57–6.85	0.19	0.09–0.45	1.65	0.55–6.59
TC45 1850 (+) 33d.	0.86	0.37–2.21	1.79	0.60–7.74	1.24	0.56–3.26
TC46 1850 (+) 78d.	0.28	0.11–0.95	5.44	1.81–17.5	1.87	0.62–8.10
TC42 1950 (–) 78d.	0.07	0.02–0.18	0.15	0.06–0.37	0.11	0.05–0.45
TC47 1950 (+) 33d.	0.40	0.20–1.19	1.23	0.51–3.47	2.11	0.70–8.42
TC48 1950 (+) 78d.	0.06	0.02–0.18	1.85	0.82–4.60	1.15	0.47–3.10

Table 3-3. Most probable numbers of SPB in pore water of GMZ the bentonite cores.

| Test cell code | Position | | | | | |
| | Bottom (1 mm from copper discs) | | Middle (5 mm from copper discs) | | Top (10 mm from copper discs) | |
	$10^{-6} \times$ (MPN L^{-1})	Lower–upper 95 % confidence interval	$10^{-6} \times$ (MPN L^{-1})	Lower–upper 95 % confidence interval	$10^{-6} \times$ (MPN L^{-1})	Lower–upper 95 % confidence interval
TC51 1750 (–) 77d.	414	155–1 290	62.0	22.5–169	732	282–2 200
TC53 1750 (+) 33d.	174	57.8–752	> 953	–	874	328–2 900
TC54 1750 (+) 77d.	958	359–3 170	> 988	–	> 816	–
TC55 1850 (+) 33d.	542	181–1 750	558	186–1 800	1010	380–3 350
TC56 1850 (+) 77d.	349	139–1 190	546	205–1 710	1700	729–4 190
TC52 1950 (–) 77d.	2.64	1.20–6.18	1.22	0.57–2.94	1.09	0.51–2.63
TC57 1950 (+) 33d.	6.04	2.26–18.9	20.5	8.79–50.6	5.92	2.22–18.5
TC58 1950 (+) 77d.	15.4	7.00–40.6	10.2	4.35–26.1	10.6	4.56–27.4

3.2.3 Distribution of ^{35}S in the bentonite cores

In Figure 3-2 to Figure 3-3 the distributions of added ^{35}S in the bentonite cores are shown as nanomoles per litre pore water. Results were adjusted for the half-life of the ^{35}S isotope and the scintillation instrument efficiency (36 %). The analysis method does not separate $^{35}SO_4^{2-}$ from $H^{35}S^-$ in the bentonite pore water. Further, the analysis does not detect immobilised ^{35}S isotope, e.g. as Fe^{35}S. The results show that the isotope generally was evenly distributed through the bentonite cores with some exceptions as explained for each experiment.

3.2.4 Rokle

The concentrations of the ^{35}S isotope was in the range of 100–140 nM for all test cells except TC47. The sampling of test cell 47 did not go well. Too much clay was acciently sampled in the first layer which introduced an error in all values for this test cell. The data for TC47 should, therefore, be ignored.
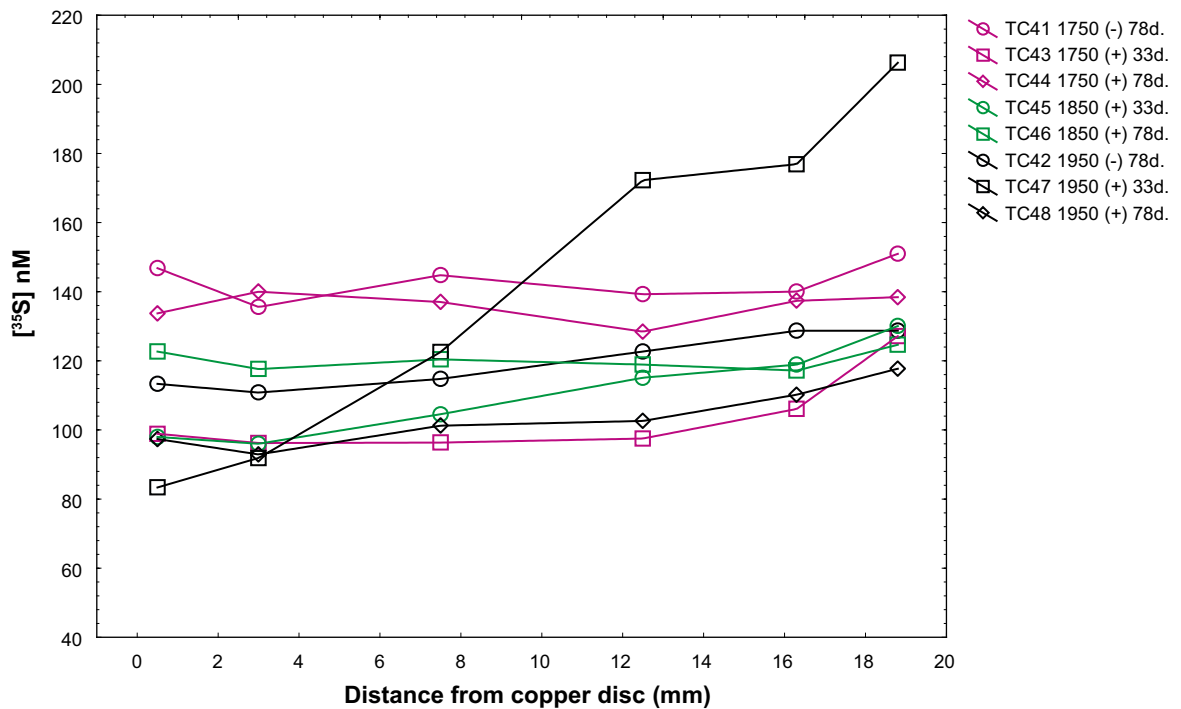

Figure 3-2. *Concentrations of ^{35}S in profiles in Rokle bentonite cores for each test cell. Test cell number, bentonite density, addition of bacteria (+/−) and incubation time according to symbol description.*

3.2.5 GMZ

The concentrations of the ^{35}S isotope was below the range found for Rokle for all test cells except TC57 (Figure 3-3). It appeared as if some of the ^{35}S isotope was immobilised and could not be leached for analysis. There was a large sulphide producing activity in all test cells as judged from the amount of $Cu_2{}^{35}S$ on the copper discs, also on the TC57 disc (Table 3-1). However, in difference to all other test cells, lactate was not consumed in TC57 as shown in Table 3-4.

During sampling of the GMZ test cells, black spots were found in the profiles of the clay cores (Figure 3-4). The colour likely was due to FeS locally produced by SPB. Bacteria typically grow in colonies and the results with black spots confirm that growth of SPB in this clay was heterogeneously distributed.
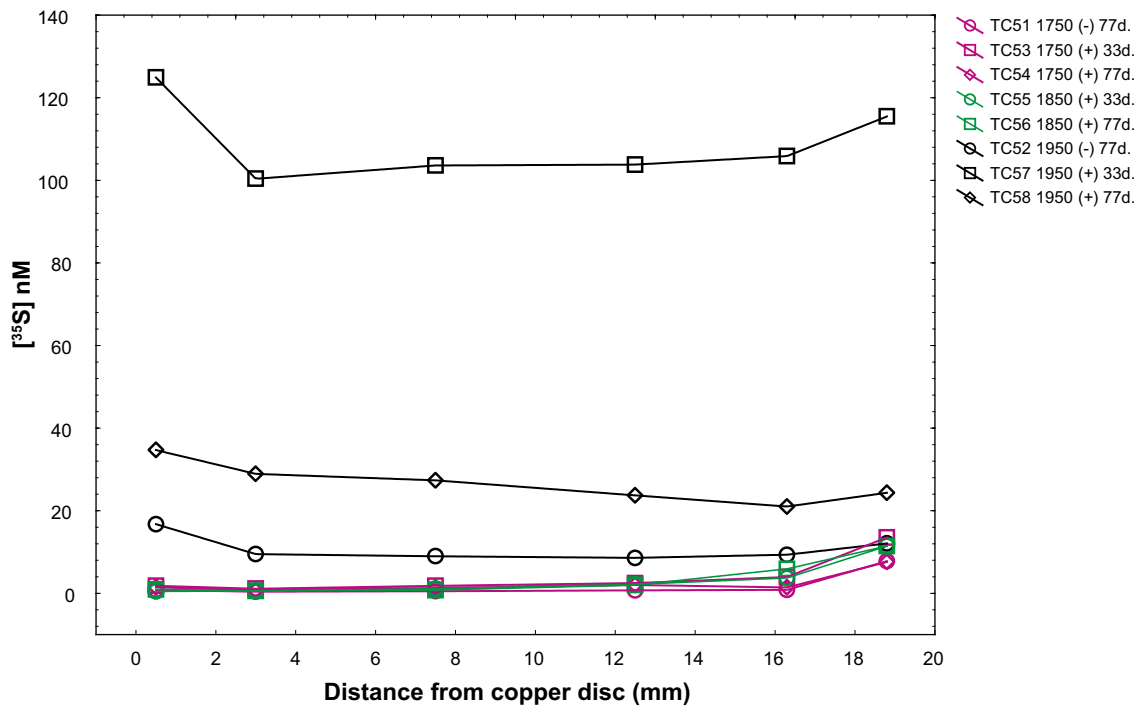

Figure 3-3. *Concentrations of ^{35}S in profiles in GMZ bentonite cores for each test cell. Test cell number, bentonite density, addition of bacteria (+/−) and incubation time according to symbol description.*

Figure 3-4. *Black spots observed during sampling of test cell with GMZ bentonite.*

3.3 Amounts and distribution of lactate, acetate and sulphate in the bentonite cores

3.3.1 Rokle

The concentrations of leachable sulphate show that approximately 0.5 to 1 µmol gdw^{-1} of the added sulphate (1 to 1.5 µmol gdw^{-1}) likely were reduced to sulphide (Table 3-4). The profiles of lactate amounts were horizontal in all test cells over the core length after 33 days demonstrating that lactate diffused to all parts of the clay core within a month (Figure 3-5). Similarly, acetate amounts were evenly distributed which indicates that bacterial oxidation of lactate, and natural organic matter in the clay, to acetate occurred throughout the clay core.

The test cell TC41 without bacterial addition had not consumed any lactate but had still produced intermediate levels of acetate. This formed acetate must then have been produced by bacterial degradation of organic matter in the clay. TC43 and 44 had similar levels of lactate consumption but TC44 had approximately twice as much produced acetate as had TC43. This again argues that acetate formation had taken place by utilizing organic matter present in the Rokle clay.

Comparing the lactate consumption and acetate production results with the accumulated $Cu_2{}^{35}S$ on the copper discs shows that only TC44 had accumulation levels that were above background. However, there must have been an ongoing bacterial activity in both TC41 and 43 as judged by the lactate consumption and acetate production values.

TC44 had one very high value of acetate next to the copper surface (Figure 3-5) that was matched by the radioactivity on the disc (Figure 3-6). In addition, the MPB of SPB was 10 times higher in the position close to the copper disc compared to the MPN in the other 1 750 kg m^{-3} test cells (Table 3-2).

Table 3-4. Average amounts of analysed sulphate in pore water of the clay cores, the calculated amount of added lactate at start of the experiment and the analysed amounts of lactate and acetate at end of the experiments. SD = standard deviation.

| Clay	Test cell code	Average sulphate (µmol gdw^{-1})	SD	Added lactate (µmol gdw^{-1})	Average lactate (µmol gdw^{-1})	SD	Average acetate (µmol gdw^{-1})	SD
Rokle	TC41 1750 (−) 78d.	0.06	0.10	16.7	16.0	0.70	7.20	2.77
Rokle	TC43 1750 (+) 33d.	0.73	0.69	16.4	9.66	0.47	4.38	2.70
Rokle	TC44 1750 (+) 78d.	0.43	0.60	16.4	9.21	0.92	11.7	10.6
Rokle	TC45 1850 (+) 33d.	1.11	1.12	13.3	11.9	1.00	3.31	1.28
Rokle	TC46 1850 (+) 78d.	0.65	0.68	13.3	14.4	0.88	4.79	1.45
Rokle	TC42 1950 (−) 78d.	0.10	0.06	11.3	8.35	0.34	4.04	1.32
Rokle	TC47 1950 (+) 33d.	0.84	1.05	11.0	12.8	2.01	2.73	0.94
Rokle	TC48 1950 (+) 78d.	0.74	0.93	11.0	9.31	0.65	3.96	1.97
GMZ	TC51 1750 (−) 77d.	0.03	0.06	13.6	0.97	0.33	21.6	2.77
GMZ	TC53 1750 (+) 33d.	0.02	0.05	14.5	1.05	0.61	35.8	7.04
GMZ	TC54 1750 (+) 77d.	0.04	0.06	14.5	0.88	0.57	38.0	5.11
GMZ	TC55 1850 (+) 33d.	0.05	0.12	11.6	0.84	0.44	26.3	4.13
GMZ	TC56 1850 (+) 77d.	0.07	0.14	11.6	0.76	0.25	27.5	3.11
GMZ	TC52 1950 (−) 77d.	0.13	0.14	8.50	5.62	0.91	7.85	2.64
GMZ	TC57 1950 (+) 33d.	0.24	0.30	9.32	11.7	0.91	6.73	3.00
GMZ	TC58 1950 (+) 77d.	0.09	0.09	9.32	4.88	0.70	13.7	3.13
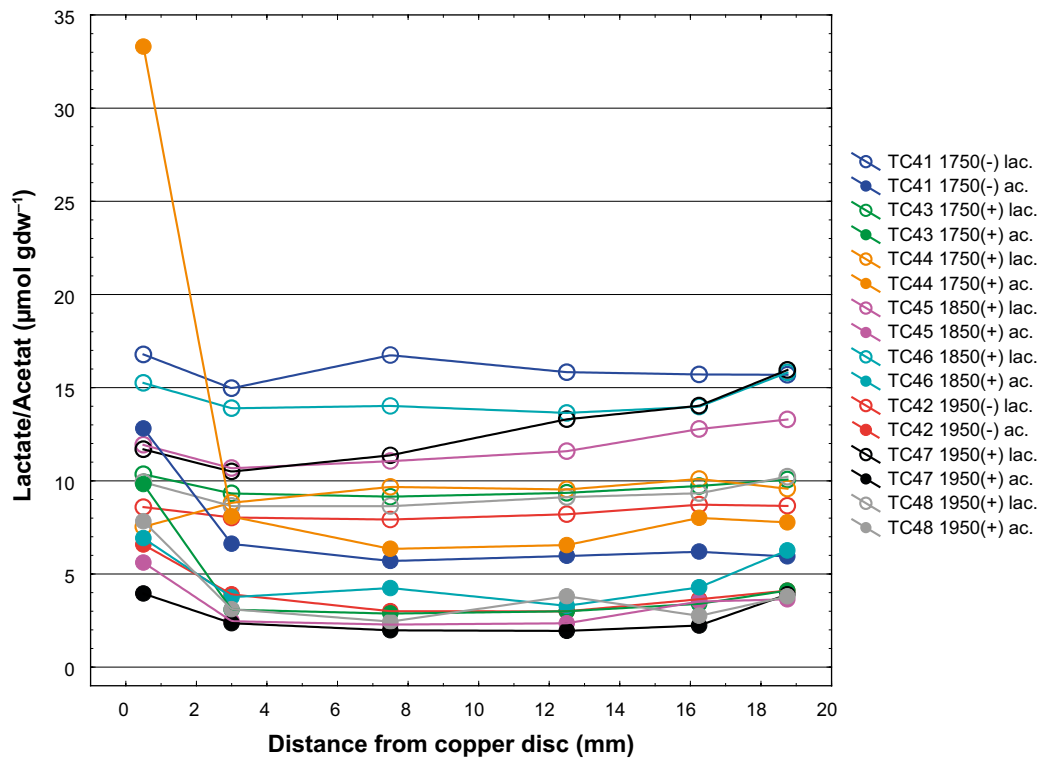
Figure 3-5. *Amounts of lactate and acetate in profiles of Rokle bentonite cores for each test cell. Test cell number, bentonite density, addition of bacteria (+/−) and incubation time according to symbol description.*
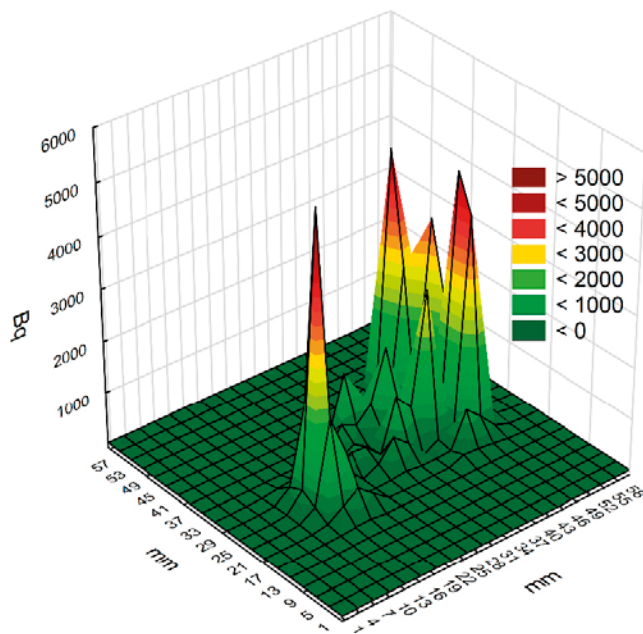

Figure 3-6. *Surface radioactivity on the copper disc of Rokle TC44 1750 (+) 78d.*

3.3.2 GMZ

The concentrations of leachable sulphate were very low, at the limit of detection for the Sulfaver method. All the added 1 to 1.5 µmol sulphate gdw^{-1} were likely reduced to sulphide. Analysed lactate values for the 1750 and 1850 kg m^{-3} test cells were similar; almost all added lactate had been consumed. The consumed amount of lactate links to high levels of produced acetate, >20 µmol gdw^{-1} for all 1750 and 1850 kg m^{-3} test cells. For the 1950 kg m^{-3} test cells, 50 % or more of the added lactate was still present after the experiment with exception for TC58, where approximately half of the added lactate had been consumed. Comparing these results to the accumulation of surface radio-activity, where the 1850 kg m^{-3} test cells had by far lower values than 1750 and 1950 kg m^{-3}, it can be concluded that bacterial activity indeed was on-going at 1850 kg m^{-3} wet density for the GMZ bentonite. The produced acetate amounts were more than two times higher than the amounts of added lactate for most test cells. Consequently, a large part of the analysed acetate must have originated from organic matter in the GMZ bentonite.
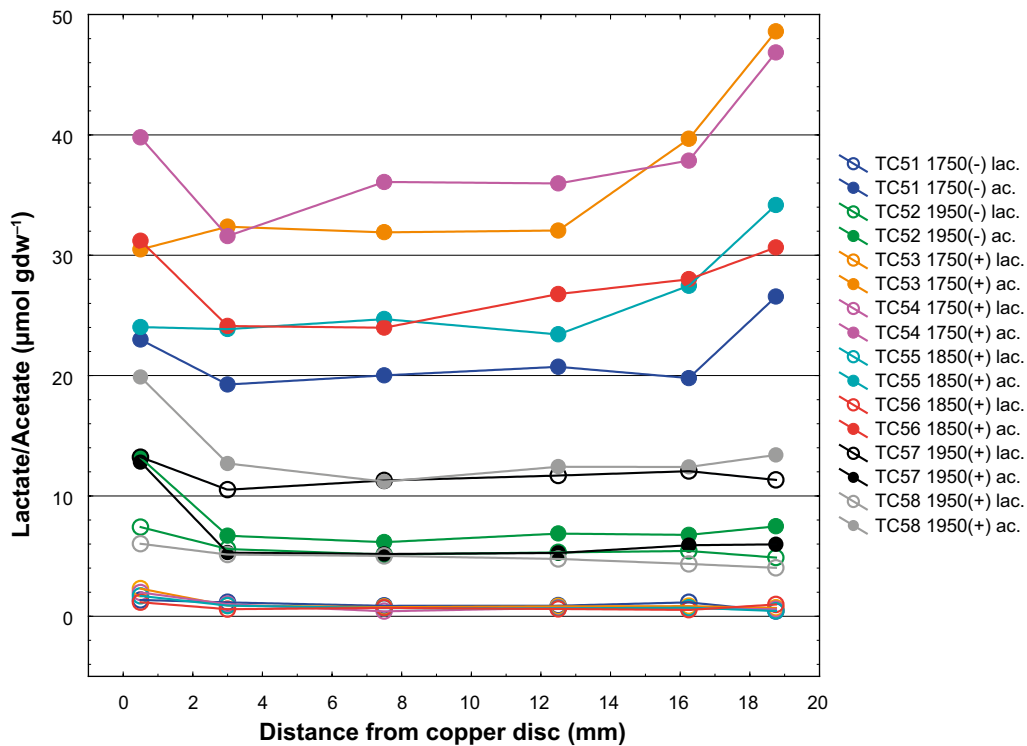

Figure 3-7. *Amounts of lactate and acetate in profiles of GMZ bentonite cores for each test cell. Test cell number, bentonite density, addition of bacteria (+/−) and incubation time according to symbol description.*

4 Discussion

Previously, three different clays have been investigated for bacterial sulphide-production as a function of wet density. The method for detection of bacterial activity in the investigated clay cores comprises analysis of production of copper sulphide on introduced copper discs. In this work, analysis of acetate production and lactate consumption was added. Rokle and GMZ did not contain leachable sulphate. Therefore, sulphate was added to know concentrations and the consumption of sulphate was analyses as well.

4.1 Experimental set-up

The experimental set-up was developed based on experiences from previous experiments using test cells made of stainless steel (Bengtsson and Pedersen 2017, Masurat et al. 2010, Pedersen 2010, Pedersen et al. 2000b). The test cells used in these experiments were made of titanium because this metal does not easily react with sulphide (Persson et al. 2011), which may be the case with stainless steel. Further, in earlier experiments, it was found that bacterial sulphide-producing activity in free water adjacent to the bentonite cores became very intensive and the produced sulphide interfered with the analysis of the sulphide production rates inside the bentonite cores (Pedersen 2010). Therefore, the present experimental equipment was constructed to exclude any free water in contact with the bentonite cores after the addition of carbon sources and $^{35}SO_4^{2-}$. Any production of $Cu_2^{35}S$ in the present experiments must have originated from bacterial sulphide-producing activity inside the bentonite cores.

4.2 Accumulation of $Cu_2^{35}S$

The copper surface radioactivity was low, close to detection in all Rokle test cells, except for test cell 44. This test cell is discussed below, in relation to the observed acetate production. In opposite, there was a large surface radioactivity on the copper discs in GMZ test cells. Sulphide-production was clearly ongoing at a relatively high rate in the GMZ test cells, compared to Rokle test cells.

Non-reactive transport is generally assumed in modelling of sulphide transport through the buffer and backfill (e.g. King et al. 2012). In the most recent experiments (Bengtsson and Pedersen 2017), it was observed that sulphide may not migrate as a non-reactive monovalent anion in the clay cores inside the test cells. A discrepancy was observed as the decrease in pore water sulphate concentration was much larger than the modelled decrease based on the amount of $Cu_2^{35}S$ on the copper discs. It was proposed that the investigated clays influenced transport of, or immobilised, HS^-. A similar effect was recently observed elsewhere for Wyoming MX-80 clay (Stone et al. 2016). This effect was recently confirmed in new experiments where bentonite clays were exposed to sulphide (Pedersen et al. 2017). Sulphide was found to reduce ferric iron in the montmorillonite type bentonites Asha, MX-80 and Calcigel under the formation of elemental sulphur, ferrous iron and iron sulphide. These reactions rendered an immobilisation capacity of the clays that was 40 μmole sulphide (g clay)$^{-1}$ or more, depending on the load of sulphide and type of clay. Therefore, there may have been some sulphide producing activity in all test cells where sulphate was consumed that escaped detection by the copper discs. Sulphate was indeed consumed in all test cells for all densities and clay types. (Table 3-4). Consequently, there was a contradiction between observed copper surface radioactivity and the amount of consumed sulphate.

The observed differences of accumulated of $Cu_2^{35}S$ (Figure 4-1) may depend on several different variables. Reactions of sulphide with metals, mostly iron, in the clays may have lessened the amount of sulphide that reached the copper discs compared to what was produced in the test cells. Finally, increasing wet densities generally had a mitigating effect on the sulphide production. There was, consequently, an increased stress on SPB by increasing wet density that resulted in a loss of high sulphide production at discrete wet density intervals that differed from clay to clay (Figure 4-1).
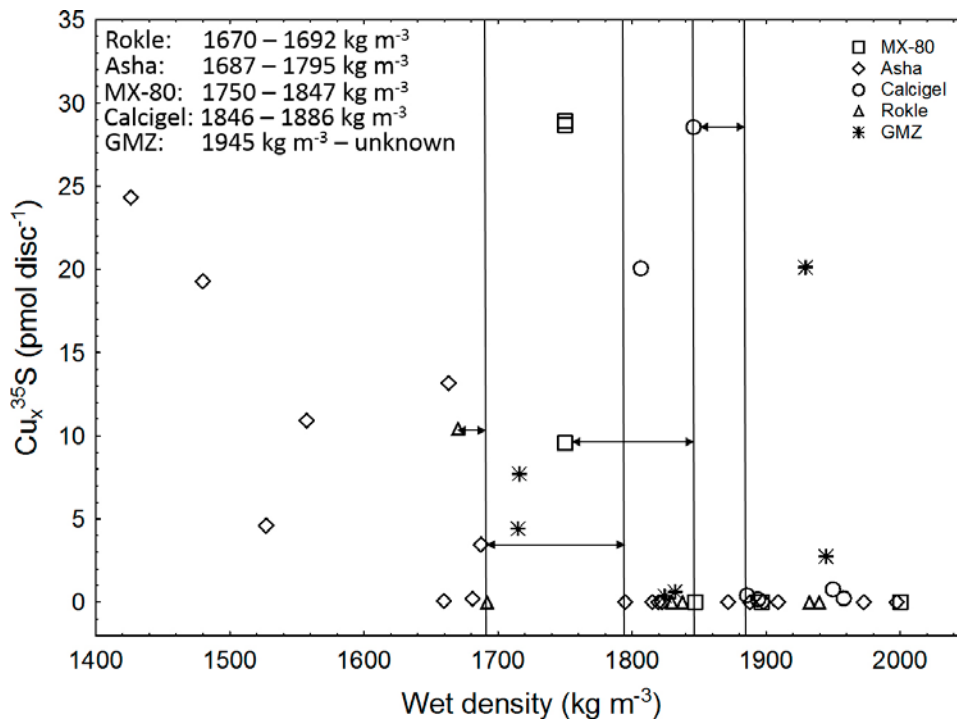
Figure 4-1. *Accumulated $Cu_2^{35}S$ on copper discs (pmol) over wet density. The respective intervals where sulphide-production shifts from high to low are indicated with arrows. The corresponding analysed wet densitiy intervals are inserted, for GMZ all tested wet densites shows high sulphide production. Data for Asha, MX-80 and Calcigel from Bengtsson et al. (2017)*

4.2.1 Heterogeneous distribution and growth of bacteria in the studied clays

The presence of colony growth in the GMZ clay, visible as black spots of iron sulphide (Figure 3-4) is typical for bacterial growth. Bacteria grow by division and one cell can rapidly, over less than a day, increase to billions of cells if growth conditions are optimal. Although the clays were ground to small particles, there may be irregularities in grain size and impurities present that locally, in the micrometre scale, can offer better growth conditions than the bulk of the clay cores. The test cell 44 is an example where colonies of SPB grew and produced much more copper sulphide locally in spots (Figure 3-6), than what was found on any other Rokle copper disc. In the same position, there was a much larger production of acetate than what can be explained by lactate oxidation. The colony appearances in both GMZ and Rokle suggests that bacterial growth in compacted bentonite clay is heterogeneously distributed.

4.3 Bacterial lactate consumption and acetate production

4.3.1 Lactate consumption

The only source of lactate was the added lactate. Lactate could not be detected in the clays at start of the experiments (Figure 2-4). Lactate was evenly distributed in all test cells after 30 days (Figure 3-5 and Figure 3-7) which shows that lactate was relatively rapidly transported to all analysed positions in the clay cores. The average values given in Table 3-4 consequently reflect the whole clay cores well. More than 90 % of the added lactate was consumed in all GMZ test cells. For Rokle, there was a small but significant consumption of lactate in all test cells except for test cells 41 and 47. The consumption of lactate decreased with increasing wet density for the GMZ clay cores while for Rokle, the two 1 750 kg m^{-3} clay cores with added SPB showed a significantly higher lactate consumption than did the two higher densities (Figure 4-2). The consumption of lactate agrees with a consumption of sulphate which suggests that some or all "missing" lactate was consumed by SPB.

4.3.2 Acetate production

Just as for lactate, acetate was detected in approximately similar values for all analysed positions in each test cell although the values tended to be slightly higher at the top and bottom clay layer compared to the other analysed positions. Acetate could not be detected in the clays at start of the experiments (Figure 2-5 and adjacent text) which shows that all analysed acetate must have been produced during the incubation of the clay cores. This production appears to have occurred all over the clay core at approximately equal rates at all positions. The added SPB were all incomplete lactate oxidizers that expel acetate that is not further metabolised. When the consumed amounts of lactate are compared with the produced amounts of acetate, several of the Rokle test cells had more acetate than what can be explained by the amount of consumed lactate. There must, consequently have been some other carbon source available that could be degraded to acetate. The acetate production was much larger in the GMZ clay cores than what can be explained by the added lactate (Figure 4-3). Consequently, there must have been more organic carbon available in this clay than the added lactate (Marshall et al. 2015). The acetate production was present at all tested wet densities which attests that bacteria can be active in wet densities up to at least 1 950 kg m^{-3}. Previously, it was found that SPB could actively produce sulphide in Boom clay at a wet density of 2 000 kg m^{-3} (Bengtsson and Pedersen 2016). Wet density, *per see*, obviously does not cut off bacterial activity in compacted clays, but there is a confirmed mitigating effect from increasing wet density that varies over clay type.

Acetogenic bacteria are a diverse group of strictly anaerobic bacteria that play an important part in the global carbon cycle by their production of acetate. Most members also show an outstanding metabolic flexibility for utilizing a vast variety of different substrates, including lactate, carbohydrates and alcohols (Schuchmann and Müller 2016). Metabolic flexibility is a key ability of acetogens to compete in ecosystems and might explain the almost-ubiquitous distribution of acetogenic bacteria in anoxic environments such as the clay cores in the experiments described here. Previously, cultivable acetogens were found in large numbers in various bentonite clays (Svensson et al. 2011) and they are reported to occur in deep Forsmark groundwater at occasionally large numbers (Hallbeck and Pedersen 2012). In the experiments reported here, acetogens must have competed with SPB over the lactate in the clays but also over other organic carbon compounds in the clays. High-resolution analysis of the diversity of organic carbon in bentonite clays, and quantification of specific compounds is needed to fully explain the observed acetate production (Marshall et al. 2015, Marshall and Simpson 2014). Different clays may have different composition and amounts of organic carbon.
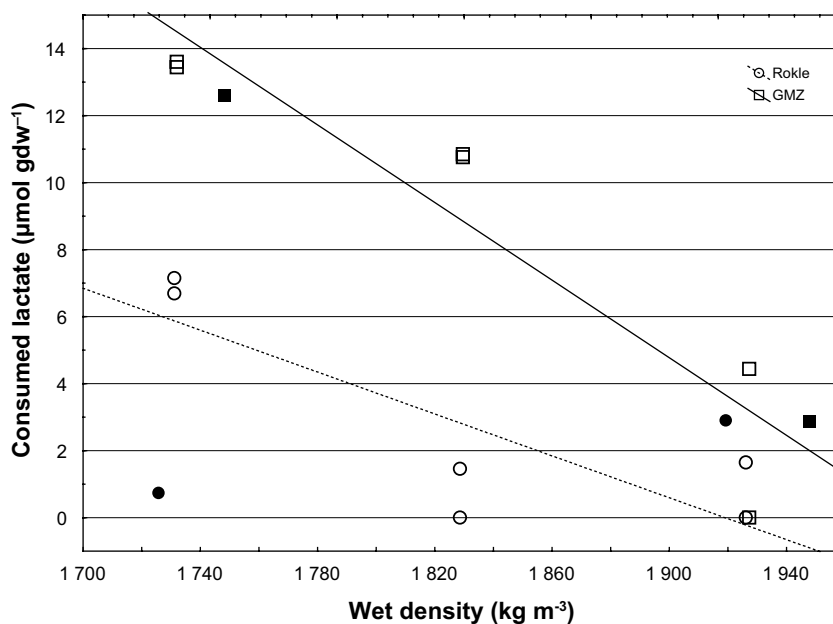

Figure 4-2. *The average amounts of consumed lactate in test cells over wet density (from Table 3-4). The solid line shows linear fit for GMZ and the dashed linear fit for Rokle. Solid symbols indicate control test cells without bacterial addition.*
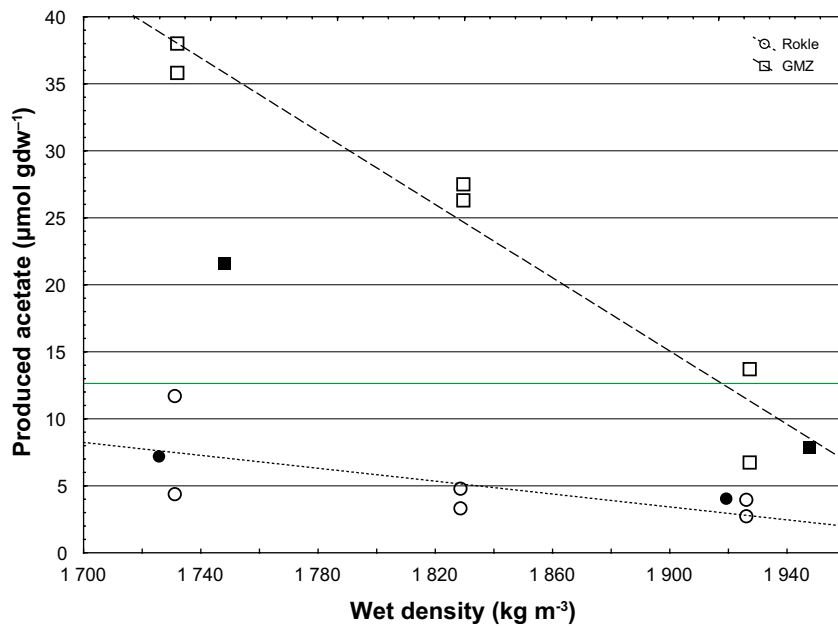
Figure 4-3. *The average amounts of produced acetate in test cells over wet density. The dashed lines show linear fit for each clay according to legend. Solid symbols indicate control test cell without bacterial addition. The green horizontal line indicates the average level of added lactate for all test cells.*

The lactate consumption and the acetate production mainly occurred during the first 33 days, although there was some variation from test cell to test cell. This is not surprising. Bacteria are opportunists and will always rapidly respond with growth and metabolism when good growth conditions prevail. Thus, acetate production may have occurred very soon after water saturation of the tested clays until all utilizable carbon is metabolized to acetate, by acetogens and SPB. Acetate can be further metabolized to carbon dioxide by other bacteria present in the clays.

The MPN method was designed only for SPB to analyse cultivability over a range of wet densities. The results showed presence of SPB at all tested wet densities. Acetogens were not cultivated, but the production of more acetate than what could be explained by oxidation of lactate to acetate at all wet densities indicates that acetogens were active at all wet densities as well.

The metabolic activity of SPB and acetogens appeared to slow down with increasing wet density as judged by the amounts of consumed lactate and produced sulphide and acetate over density, most obvious for the GMZ clay. A longer experimental time than the 78 days applied here, will show if the observed sulphide and acetate production rates eventually will have consumed all organic carbon available for such production at high densities.

5 Conclusions

- Sulphide-producing bacteria could be cultivated from all tested wet densities (with or without bacterial addition) and there were significant numbers of inherent sulphide-producing bacteria in both tested clays.

- A cut-off in sulphide-producing activity by SPB was observed over increasing wet density at discrete wet density intervals that differed from clay to clay.

- The organic carbon in the clays could be utilised by acetogens, and likely also by sulphide-producing bacteria, with concomitant production of acetate.

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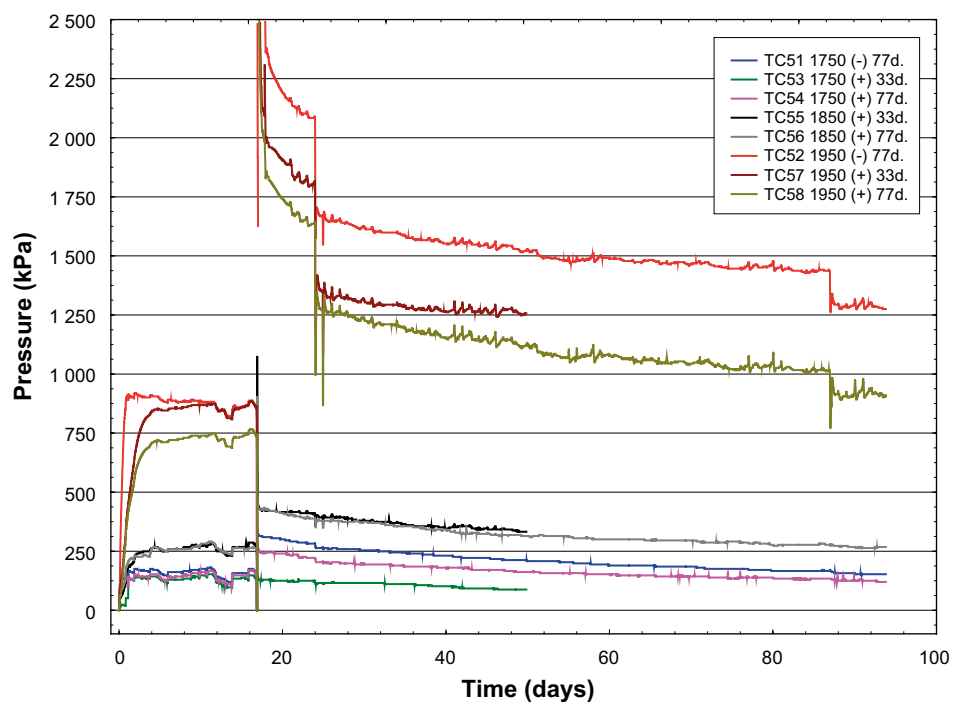
Recorded pressures, Rokle
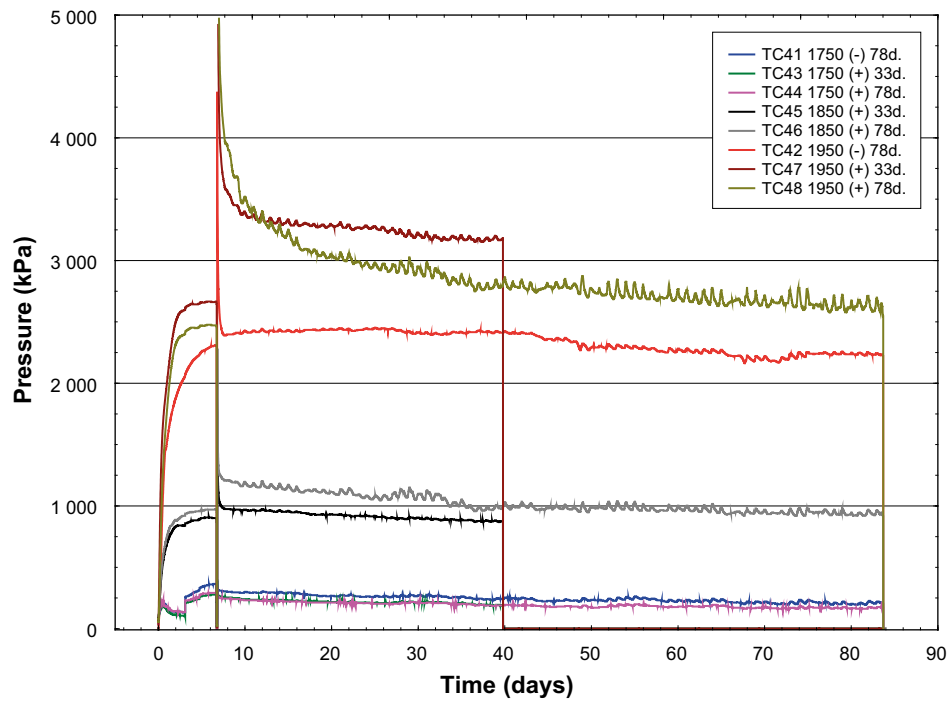
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