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Bacterial activity in compacted bentonites

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Contents

Publishable Summary

Bentonite clay will be used as a buffer material in engineered barrier systems (EBS) which will contain, protect and surround nuclear waste canisters in geological disposal concepts. The dissimilatory reduction of sulphate, thiosulphate and sulphur to sulphide by sulphide-producing bacteria (SPB) is a main concern for the safety case of a geological disposal since sulphide is a corrosive agent for metal waste canisters. Bacterial activity is generally measured by the turn-over of one or several metabolic products such as ATP or in the SPB case, the production of sulphide. Bacterial viability (or presence) on the other hand does not imply that the bacteria must be active *in vivo* or *in situ*, it only states that they are able to become activated when a suitable environment presents itself. It has been hypothesised that cut-off bentonite density thresholds exist above which all bacterial sulphide-producing activity stops or is inhibited to a such a level that it can be regarded as negligible. This report discusses if that hypothesis can be considered true or if more variables other than clay density determine bacterial activity in bentonites. The clays analysed and discussed in this report have been investigated previously. This deliverable summarizes previously performed work and new work performed within MIND.

Six different bentonites of interest for geological disposal concepts have been studied: Wyoming Volclay MX-80 (USA), Asha (India), Calcigel (Germany), GMZ (Gaomiaozi, China, Rokle (Czech Republic) and FEBEX clay (Switzerland). In addition, Boom Clay (Belgium) has been studied as a reference to a non-swelling clay. These clays had a varying element and mineral composition. In particular, the amount of montmorillonite varied from 66% to 88% and the iron content, analysed as Fe_2O_3 varied from 3.3% to 13.4%. Analysis of mobility and reactivity of sulphide with the clays showed an immobilisation effect that can reduce the mass of sulphide that corrode metal canisters over repository life times. However, the concomitant reduction of ferric iron may be problematic due to the destabilizing effect of ferrous iron on dioctahedral smectites such as montmorillonites. Further, a new method for the analysis of natural organic matter in the clays showed a great diversity of organic compounds in the clays. Alcohols, esters, ketones, aldehydes, fatty acids, alkanes, and more were detected. These compounds can serve as electron donors and sources of carbon for growth of bacteria in the clays.

Sulphide-producing bacteria could be cultivated from all clay samples and the numbers decreased over wet density for some but not all tested clays. The range of saturated wet densities studied was from 1400 to 2000 kg m^{-3}. Experiments analysing bacterial sulphide-production showed that there were intervals where the measurable accumulation of copper sulphide for each clay changes from significant to below detection. For two clays, these density ranges are yet to be determined, sulphide was produced at all studied densities. It is therefore concluded that density alone does not control bacterial activity in clays. Other variables must be studied as well.

The relation between water saturated clays at varying wet density and bacterial sulphide-producing activity is well studied. However, wet density is just a value of the total amount of clay and water. That value does not reflect the conditions in a compacted clay where several variables of importance for bacterial life can be of importance, such as clay type, pH, temperature, transport conditions, water content, pressure, pore space and pore water composition. These variables need further attention for a full understanding of what conditions control bacterial activity in compacted bentonite clays.

Significant acetate formation from natural organic matter present in the clays was detected in the studied bentonites. This production occurred at all wet densities and suggests that bacterial

activity, *per see*, was possible also at densities where sulphide-production could not be detected. Acetate is known to induce stress-corrosion cracking of copper and other metals and the possible formation of acetate should, therefore, be further investigated.

1 Introduction

Bentonite clay will be used as a buffer material in engineered barrier systems (EBS) which will contain, protect and surround nuclear waste canisters in geological disposal concepts. The concepts rely on the swelling capabilities of the bentonite clay when it becomes water saturated as one of the main protective features. To reach the desired swelling pressure of >5 MPa a clay dry density of >1600 kg m^{-3} is generally required. Depending on the minerology of the specific bentonite type and the groundwater composition, where salinity is an important factor, different swelling pressures are produced at the same wet density. A high density is believed to have an inhibiting influence on bacterial activity of the natural bacterial populations in the bentonite clays, meaning that growth will stop and metabolic activity will cease, but present bacteria will not necessarily die. The dissimilatory reduction of sulphate, thiosulphate and sulphur to sulphide by sulphide-producing bacteria (SPB) is a main concern for the safety case of a geological disposal since sulphide is a corrosive agent for metal waste canisters. Bacterial activity is generally measured by the turn-over of one or several metabolic products such as ATP or in the SPB case, the production of sulphide (Figure 1-1). Bacterial viability (or presence) on the other hand does not imply that the bacteria must be active *in vivo* or *in situ*, it only states that they are able to become activated when a suitable environment presents itself (Figure 1-2). Dormant bacteria can, for instance, survive for millions of years in subseafloor sediments (Jorgensen and Boetius 2007). Analyses such as most probable number (MPN) is a perfect example on methods that measure bacterial viability. To keep consensus of the terminology used to describe degrees of bacterial metabolic levels a list with definitions follows here with the words and meanings used in this report.

1. Activity: bacteria are active and have an ongoing metabolism (Figure 1-1).
2. Presence: bacteria exists in the sample or at the sampled site and they are present as live or dead cells (Figure 1-2).
3. Viability: bacteria are alive and active or exists as dormant cells which have the power to become active when presented with a favourable environment (Figure 1-2).
4. Cultivability: bacteria can be cultivated in tubes with liquid media or on agar plates (Figure 1-2).

It has been hypothesised that cut-off bentonite density thresholds exist above which all bacterial sulphide-producing activity stops or is inhibited to a such a level that it can be regarded as negligible. This report discusses if that hypothesis can be considered true or if more variables other than clay density determine bacterial activity in bentonites.

The clays analysed and discussed in this report have been investigated previously. This deliverable summarizes previously performed work and new work performed within MIND. Six different commercial available bentonites of interest for geological disposal concepts have been studied. Sulphide producing activity was first studied using Volclay Volclay MX-80 (Pedersen 2010). Thereafter Wyoming Volclay MX-80 (USA), Ashapura (India) and Calcigel (Germany) was investigated (Bengtsson and Pedersen 2017). The clays GMZ (Gaomiaozi, China) and Rokle (Czech Republic) was investigated recently (Bengtsson et al. 2017a) as was FEBEX clay (Switzerland) (Bengtsson et al. 2017b) and in addition Boom Clay (Belgium) has be studied as a reference to a non-swelling clay (Bengtsson and Pedersen 2016). Finally, to fully understand the mobility and reactivity of sulphide in bentonite clays, investigations were done using suspensions of bentonite clays and sulphide (Pedersen et al. 2017).

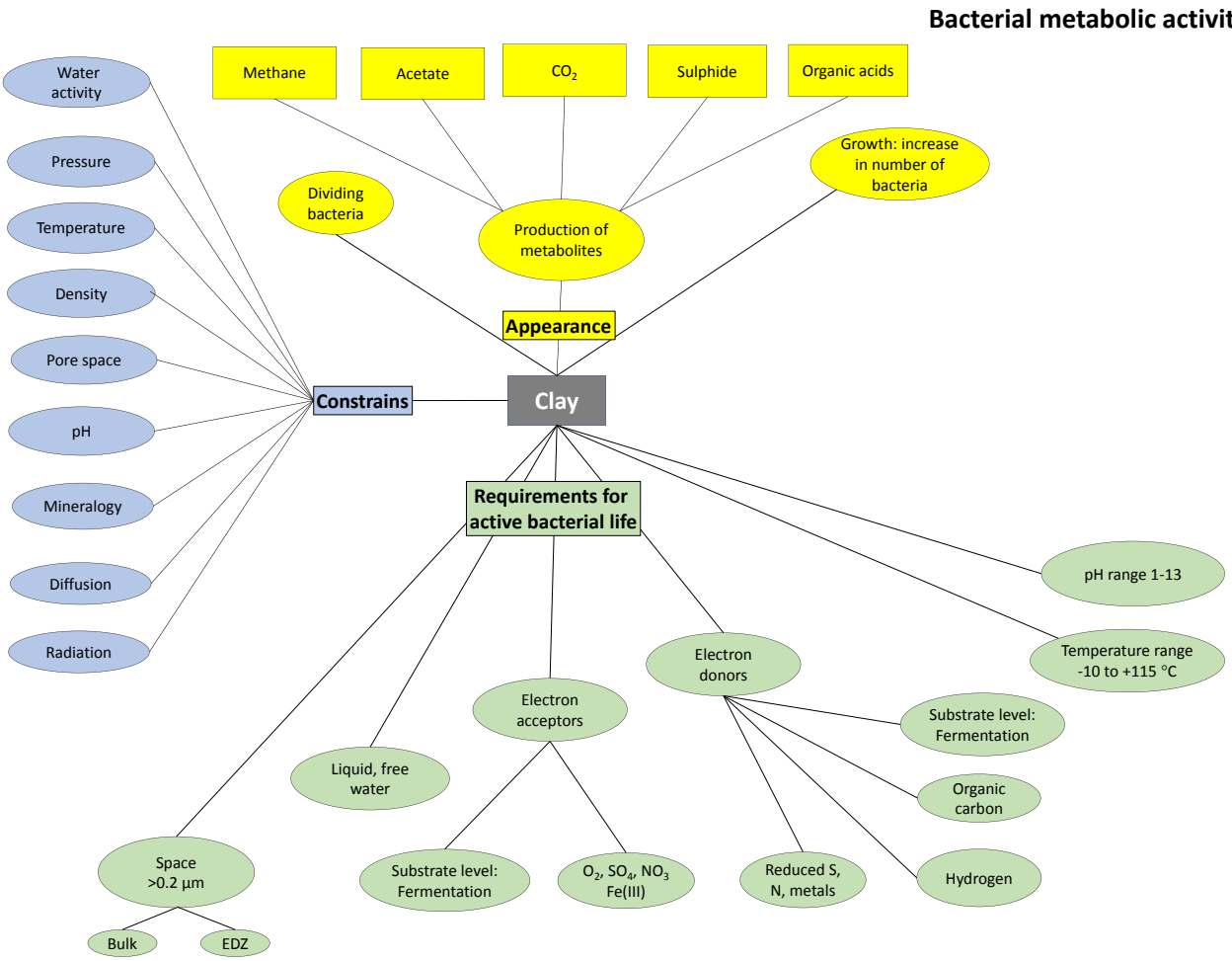

Figure 1-1. Examples of metabolites that can be used to detect appearance of metabolic activity are marked yellow, requirements for bacterial life are marked green, and possible constrains for bacterial life in compacted clays are marked blue.

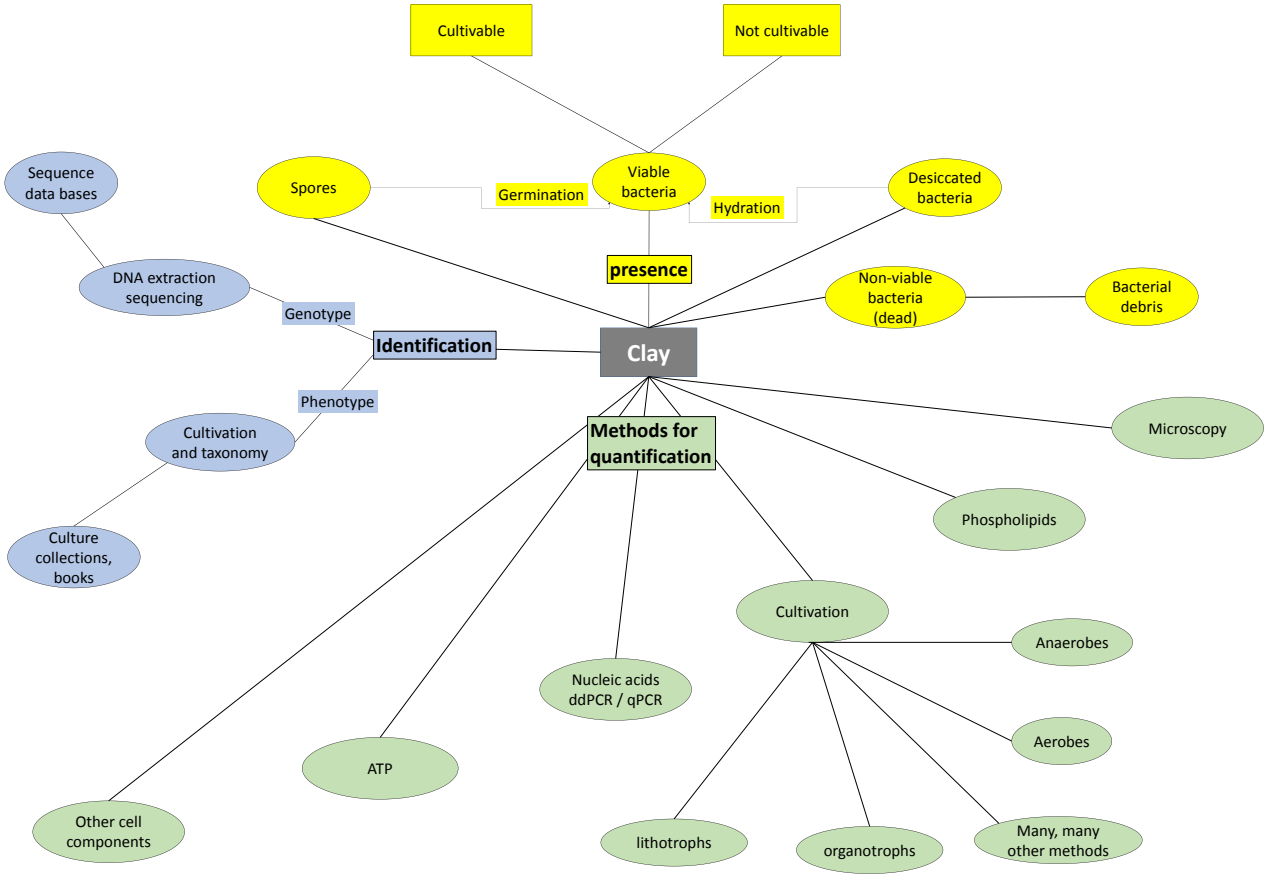

Figure 1-2. Methods for quantification of bacteria in clays are marked green, appearance of bacteria in yellow and methods for identification are marked blue.

2 Investigated clays

2.1 Clay types

Six different bentonites of interest for geological disposal concepts have been studied: Wyoming Volclay MX-80 (USA), Asha (India), Calcigel (Germany), GMZ (Gaomiaozi, China, Rokle (Czech Republic) and FEBEX clay (Switzerland). In addition Boom Clay (Belgium) has be studied as a reference to a non-swelling clay (Bengtsson and Pedersen 2016).

2.2 Mineral and element composition of the studied clays

The mineral compostion was analysed previously (Table 2-1). All bentonite clays have a high content of montmorillonite and minor amounts of various other clay minerals. Volclay MX-80 and Asha had most montmorillonite followed by Rokle, GMZ and Calcigel. FEBEX is reported to have >88% montmorillonite (Bengtsson et al. 2017b).

The element composition was analysed with inductively coupled plasma sector field mass spectrometry (ICP-SFMS) after two different treatments. First, the clays were leached with 7 M HNO_3 and the leachates were analysed with ICP-SFMS (ALS Scandinavica, Luleå, Sweden). Second the clays were sintered (1000 °C) and therafter dissolved in diluted nitric acid (ALS Scandinavica, Luleå, Sweden). The leaching method shows elements that potentially can be leached to the pore water while the sinter method report the total amount of elements. However, the diversity of analysed elements was larger for the leching method compared to the sinter method.

The Asha and Rokle bentonites contained more heavy elements such as Cd, Co, Cr, Cu and V than the other clays (Table 2-2). The Asha and Rokle bentonites also contained much more iron (analysed as Fe_2O_3) compared with the other clays (Table 2-3).

Table 2-1. Average results from the XRD analyses of mineral compositions of the Volclay MX-80 (n=6), (Karnland 2010), Asha (n=>5) (Karnland 2010; Sandén et al. 2014), Calcigel (n=2) (Herbert and Moog 2002), Rokle (Karnland et al. 2006) and GMZ (Delage et al. 2016), FEBEX (Bengtsson et al. 2017b). – = No data

Component	Asha	Volclay MX-80	Rokle	GMZ	Calcigel
Montmorillonite	82	81	69.4	75.4	66
Muscovite	1.9	3.4	2.8	–	14
Plagioclase	0.82	3.5	0.2	–	3
Pyrite	0.66	0.6	1.1	–	0
Quartz	1.2	3.0	2.5	11.7	8.2
Other	13.4	8.5	24	12.9	8.8

Table 2-2. The element composion of the bentonite materials expressed as weight percent of major element oxides of dry mass after leaching in 7 M HNO_3 and analysis on ICP-SFMS; DM = dry mass

		Clay						
Element	Unit	Asha	Volclay MX-80	Rokle	GMZ	Calcigel	FEBEX	Boom Clay
DM	%	91.5	91.7	84.2	91.8	91.2	83.4	97.2
As	mg/kg DM	<3	8.46	<3	4.00	4.88	5.00	9.18
Ba	mg/kg DM	54.5	34.3	122	60.9	93.2	31.2	44.8
Be	mg/kg DM	0.56	1.53	1.38	6.64	1.99	1.96	0.718
Cd	mg/kg DM	0.211	0.27	<0.1	<0.1	0.118	<0.1	<0.1
Co	mg/kg DM	50.3	1.34	31.3	0.756	7.18	1.10	9.73
Cr	mg/kg DM	71.4	0.26	52.8	1.82	12.8	2.04	32.8
Cu	mg/kg DM	114	3.10	68.8	6.79	9.58	4.57	22.0
Fe	mg/kg DM	42500	4520	51400	4070	12400	2310	17600
Hg	mg/kg DM	<1	<1	<1	<1	<1	<1	<1
Mn	mg/kg DM	725	102	694	28.7	647	235	74.3
Ni	mg/kg DM	57.4	3.16	49.1	1.48	16.3	3.70	34
P	mg/kg DM	281	160	1400	125	218	128	178
Pb	mg/kg DM	1.33	43.3	3.73	3.05	24.3	14.2	19.0
Sr	mg/kg DM	220	203	94.0	307	41.5	148	62.5
V	mg/kg DM	60.9	0.403	23.7	1.10	2.83	1.19	20.8
Zn	mg/kg DM	139	80.3	61.6	4.87	51.7	7.99	67.2

Table 2-3. The element composion of the bentonite materials expressed as weight percent of major element oxides of dry mass after sintering and analysis on ICP-SFMS. LOI denotes the percent mass loss due to sintering; DM = dry mass.

		Clay				
Element	**Unit**	**Asha**	**Volclay MX-80**	**Rokle**	**GMZ**	**Calcigel**
DM	%	91.4	91.9	83.9	91.8	91.3
SiO_2	% DM	45.5	61.2	45.3	64.9	56.6
Al_2O_3	% DM	16.9	18.4	13.6	13.4	17.8
CaO	% DM	2.85	1.15	2.10	0.841	1.69
Fe_2O_3	% DM	13.0	4.15	13.4	3.31	6.11
K_2O	% DM	0.128	0.599	0.584	0.605	1.55
MgO	% DM	2.34	2.02	3.40	2.90	2.92
MnO	% DM	0.112	0.0146	0.0921	0.0368	0.085
Na_2O	% DM	1.30	1.56	1.21	1.50	0.251
P_2O_5	% DM	0.0954	0.0541	0.364	0.037	0.0531
TiO_2	% DM	1.03	0.148	2.69	0.124	0.402
Sum	**% DM**	**83.3**	**89.3**	**82.7**	**87.7**	**87.5**
LOI 1000°C	% DM	9.5	5.9	9.1	4.6	7
Ba	mg/kg DM	95.5	273	234	251	312
Be	mg/kg DM	<0.5	1.51	2.03	6.41	3.21
Co	mg/kg DM	74.2	<5	53.6	<5	10.1
Cr	mg/kg DM	239	<10	333	18.7	41.6
Mo	mg/kg DM	<5	<5	<5	<5	<5
Nb	mg/kg DM	10.9	28.1	80.6	53.1	20.7
Ni	mg/kg DM	111	<10	165	<10	39.3
Sc	mg/kg DM	48.1	4.59	23.4	8.35	16.1
Sr	mg/kg DM	244	254	163	348	79.7
V	mg/kg DM	203	9.1	196	15.8	47.1
W	mg/kg DM	0.416	0.421	1.27	1.94	1.44
Y	mg/kg DM	81	39.4	23.3	48.6	41.8
Zr	mg/kg DM	92.5	199	298	96.9	234

3 Mobility and reactivity of sulphide in bentonite clays

3.1 Main results

In a study of the mobility and reactivity of sulphide in bentonite clays, sulphide was found to reduce ferric iron in montmorillonite type bentonites denoted Asha, Volclay MX-80 and Calcigel under the formation of elemental sulphur, ferrous iron and iron sulphide (Pedersen et al. 2017). 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. In addition, the effective diffusion coefficients for sulphide in Asha bentonite, compacted to saturated wet densities of 1750 kg m^{-3} and 2000 kg m^{-3}, was determined to 2.74×10^{-11} m^2 s^{-1} and 6.60×10^{-12} m^2 s^{-1}, respectively. The found immobilisation effect can reduce the mass of sulphide that corrode metal canisters over repository life times, but the concomitant reduction of ferric iron may be problematic due to the destabilizing effect of ferrous iron on dioctahedral smectites such as montmorillonites.

3.2 Implications for engineered barriers

Sulphide may originate from metabolic activity of SPB in the geosphere, buffers, backfill and in organic wastes. All three clays immobilised significant amounts of the added Na_2S up to at most 90 μmole H_2S (g clay)$^{-1}$ at the highest load of Na_2S. This immobilisation effect can reduce the mass of sulphide that reacts with metal canisters over repository life times which may influence the longevity of metal canisters. There was a clear retardation in transport of sulphide also at a low load of ≤0.45 μmole H_2S (g clay)$^{-1}$ in the diffusion experiment which suggests that the immobilisation capacity of the clays spans over a large range of sulphide amounts. This immobilisation capacity does not include the immobilisation of sulphide as FeS. The immobilisation capacity may, therefore, be larger than indicated above, in buffers with pH above 7. Sulphide will, consequently, not initially migrate as a non-reactive monovalent anion in engineered clay barriers. The transport of sulphide in bentonite buffers should be modelled as reactive until all ferric iron available to react with sulphide is reduced to ferrous iron. Further, the oxidation of sulphide to sulphur in bentonite EBS will change the concentration gradient of sulphide in groundwater adjacent to the barrier. This oxidation will induce a diffusive transport of sulphide towards the clay until reactive ferric iron is depleted and available ferrous iron has formed FeS. Thereafter, the bentonite will act only as a diffusion barrier and the metal canisters will be the only sink for sulphide.

The immobilisation of sulphide by bentonites in EBS will retard the transport of sulphide towards metal canisters, but reduction of structural ferric iron may be problematic due to the destabilizing effect of ferrous iron on dioctahedral smectites (Bradbury et al. 2014; Lantenois et al. 2005; Soltermann 2014). The sulphide concentrations applied in this work were higher than what is generally found in geological environments intended for radioactive waste repositories and is, therefore, not fully realistic. The incorporation of SPB in experiments with clays as done elsewhere (Bengtsson and Pedersen 2016; Bengtsson and Pedersen 2017; Liu et al. 2012; Stone et al. 2016), can provide more realistic experimental conditions for detailed analysis of the influence of sulphide from SPB on the stability of bentonite in engineered barriers.

4 Organic content of the clays

4.1 Methods

Clay samples were analysed granulated in dry condition. On dry samples, there was no risk that the extraction would be affected by non-miscible water-solvent phase formation. The extraction was performed using a Soxhlet extractor (Jensen 2007) and ethyl acetate as solvent. The choice of ethyl acetate (Rathburn, Genetec, Gothenburg, Sweden) as solvent was made on the presumption that the clay samples contains both polar and non-polar organic compounds and that the extraction from the dry samples will be most efficient using a solvent that is miscible/has affinity for both polar and non-polar molecules.

The mass of each sample used for the extraction was 35 g. The sample was placed into a cellulose thimble (Hahnemühle Dassel, Germany) and extracted using 80 mL ethyl acetate for 4 hours. The number of repeated extraction cycles was approximately 16. The collected extracts were evaporated using N_2 to a final volume of 2 mL. The extract was collected in 2 ml glass vials intended for analysis of trace organics equipped with a PTFE-lined red rubber seal (Genetec, Gothenburg, Sweden).

As a correction for possible present impurities in solvents and timbles as well as for possible contamination from sample handling, blank samples were prepared. These were in the form of empty Soxhlet timbles extracted using exactly the same procedure as for the clay samples. Also repeated injections of the pure solvent were made.

The samples were analysed on GC-MS (Gas Chromatography - Mass Spectrometry) The instrument used was an Agilent 7090B chromatograph (Agilent, California, USA) connected to an Agilent 240 Ion-Trap (Agilent, Palo Alto, USA) operating in internal ionization mode. The gas chromatograph was equipped with a programmed-temperature vaporization injector (MMI, Gerstel, Mülheim, Germany) and the separating column used was a VF-5ms, 30m × 0.25mm × 0.25 μm (Agilent, Middelburg, the Netherlands). The injector was operated in the temperature range of 60-350 ºC and the chromatograph oven was programmed in the range of 35-340 ºC using helium as a carrier gas. Acquisition in the ion-trap was in the range 38-400 atomic mass units (amu). The samples were analysed in numerical order with blank samples between every clay sample. Analysis of all samples and corresponding blanks was made as one full sequence run overnight.

For evaluation the chromatogram from a blank sample, prepared as described above, was overlaid on the sample chromatogram. Then the whole sample chromatogram was evaluated using library search of the obtained component spectra in the NIST 14 mass spectral library. In some cases, no exact matching compounds are given.

4.2 Results

The analysis showed a large diversity of organic compounds in the clays (Table 4-1). Rokle and GMZ had the largest diversity follow by Asha, Volclay MX-80 and Calcigel.

In all four samples, straight and branched alkanes were found. Carboxylic acids (fatty acids) of the corresponding alkanes were also detected, but at significantly lower levels than the alkanes. Glycerol was detected in all samples. A foul odour was present from the extract of the MX-80 clay.

Apart from the substances found in all samples there were individual differences between the clays as well. The following groups of substances were characteristic. Calcigel: Traces of borneol-related derivatives, a rigid cyclic compound. Febex: Derivatives of sugar (ribose)-related compounds as well as succinates, aromatic alcohols and larger cyclic and branched ketones and carboxylic acids. MX-80: Alcohols and aldehydes of lower molecular weight, derivatives of pyrrole (probably causing the odour) as well as steroles and larger polyaromatic substances (PAH). The analysis is not quantitative, but when comparing the total signal in the chromatograms the general trend in total amount of organic matter was the same as the trend in colour of the extract. Lowest amount in Asha and highest in MX-80.

The origin of these compounds is likely a combination of compounds presents before mining and compounds introduced during mining and processing. Alkanes were found in all clays and likely originate from diesel fuel. TNT was found in GMZ and could have been introduced during blasting. The borneol-related derivatives may have been present before mining and could originate from ancient forests.

Table 4-1. Output from gas chromatography mass spectroscopy analysis of organic compounds extracted from bentonite clays. CAS no., Chemical Abstract Service number.

Organic Compound	CAS no.
Asha	
alkanes	
alkenes	
phthalates	
Hexanoic acid	142-62-1
Benzyl alkohol	100-51-6
Cyclododecanol	1502-05-2
Nonanoic acid	112-05-0
[2-(2-Butoxyethoxy)ethyl] acetate	124-17-4
Hexadecanoic acid	57-10-3
Octadecanoic acid	57-11-4
Volclay MX-80	
alkanes	
alkenes	
phthalates	
steroles	
Hexanoic acid	142-62-1
Octanal	124-13-0
2-Nonen-1-ol	2104-79-6
3-Ethyl-4-methylpyrrole-2,5-dione	20189-42-8
Fluorene	86-73-7
Benzo(a)pyrene	192-97-2
Rokle	
alkanes	
alkenes	
alcohols	

aldehydes	
carboxyl acid esters	
phthalates	
Styrene	100-42-5
Hexanoic acid	142-62-1
4-Chlorophenyl benzoate	2005-08-05
Naphthalene	91-20-3
1,2-Benzisothiazole	272-16-2
2-methylnaphthalene	91-57-6
1-methylnaphthalene	90-12-0
Triacetine	102-76-1
Vanilin	121-33-5
methylnaphthalene	
Nonanoic acid	112-05-0
Decanoic acid	334-48-5
t-Butylhydroquinone	1948-33-0
Undecanoic acid	112-37-8
Benzophenone	119-61-9
2(3H)-Benzothiazolone	934-34-9
Tridecanoic acid	638-53-9
Phenanthrene	85-01-8
Tetradecanoic acid	544-63-8
Phenanthrene	85-01-8
6,10,14-Trimethylpentadecan-2-one	502-69-2
phenanthrene derivatives	
Butyl citrate	77-94-1
Dehydroabietic acid	1740-19-8

GMZ

alkanes	
alkenes	
chloroalcanes	
aldehydes	
phenolderivatives	
carboxylic acids	
carboxyl acid esters	
phthalates	
n-Propyl acetate	109-60-4
Furfural	1998-01-01
2-n-Butylacrolein	1070-66-2
1-Hexanol	111-27-3
2-Heptanone	110-43-0
Cyclohexanone	108-94-1
2-chloro-3-methyl-1-phenylbutan-1-one	78706-77-1
Phenol	108-95-2
Heptanoic acid	111-14-8
Hexyl acetate	142-92-7
2-Nonen-1-ol	31502-14-4

(+)-2-Bornanone	464-49-3
endo-Borneol	507-70-0
toluic acid ester	
.alpha.-Terpineol	98-55-5
2,3,4-Trifluorobenzoic acid, tridec-2-ynyl ester	-
Vanilin	121-33-5
2,6-Di-tert-butyl-p-benzoquinone	719-22-2
TNT	118-96-7
Bayer 28,589	728-40-5
7,9-Di-tert-butyl-1-oxaspiro(4,5)deca-6,9-diene-2,8-dione	82304-66-3
Cholestane	481-21-0

FEBEX

alkanes	
alkenes	
phthalates	
cyclic compounds including different alkyl groups	
Hexanoic acid	142-62-1
Benzyl alkohol	100-51-6
Heptanoic acid	111-14-8
Decamethylcyclopentasiloxane	541-02-6
Cyclohexanol, 2,3-dimethyl-	1502-24-5
Nonanoic acid	112-05-0
Decanoic acid	334-48-5
Vanillin	121-33-5
Cyclododecane	294-62-2
Dodecanoic acid	143-07-7
Benzophenone	119-61-9
Succinic acid, 2-(2-chlorophenoxy)ethyl ethyl ester	-
Tridecanoic acid	638-53-9
Hexadecanoic acid	112-80-1
Octadecanoic acid	57-11-4

Calcigel

alkanes	
alkenes	
Hexanoic acid	142-62-1
(+)-2-Bornanone	464-49-3
Nonanoic acid	112-05-0
2,2-Dimethoxy-2-phenylacetophenone	24650-42-8
7,9-Di-tert-butyl-1- oxaspiro(4,5)deca-6,9- diene-2,8-dione	82304-66-3
2-Ethylhexyl hydrogen adipate	4337-65-9
4-Methyl-2-pentadecyl-1,3-dioxane	54950-57-1

5 Presence and activity of sulphide-producing bacteria

5.1 Methods

5.1.1 Test cells

The methods and equipment have been described in detail previously (Bengtsson and Pedersen 2016; Bengtsson and Pedersen 2017) and are only briefly summarized here. Cylindrical test cells made of titanium were used in the experiments (Figure 5-1). 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 with a salt solution. 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 opposite clay core side 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 radioactivity of $Cu_2{}^{35}S$ that had formed on the copper discs was located and quantified using electronic autoradiography (See example in Figure 5-2). Samples were taken from different layers of the bentonite core and analysed for distribution of ^{35}S, of sulphate, acetate, lactate and most probable number of cultivable SPB.

Favourable growth conditions were obtained by the addition of lactate which is a preferred carbon and energy source for most SPB. Lactate is metabolically oxidized to acetate by many sulphate-reducing bacteria upon growth. Hence, the experimental design offers two different methods of analyzing bacterial activity; the accumulation of $Cu_2{}^{35}S$ on the copper discs and the production of acetate, where the accumulation of $Cu_2{}^{35}S$ is specific to SPB and the production of acetate could be performed both by SPB and other natural types of bacteria in the clays. Since acetate was analysed on several different positions throughout the clay cores it also gave an understanding on where the bacterial activity took place. In addition, sulphide is generally believed to diffuse through bentonite like a regular ion but it has recently been shown that sulphide is partly immobilized in bentonite (see chapter 3) (Pedersen et al. 2017). If immobilization occurred, the observed levels of $Cu_2{}^{35}S$ accumulation might not be in direct correlation to the level of SPB activity since sulphide produced in the middle of the clay core possibly never reached the copper discs. Besides being a great complement to the $Cu_2{}^{35}S$ analysis where bacterial activity can be discovered even if it is masked by the sulphide immobilization effect, the analysis of produced acetate also showed if bacteria could use the found organic carbon sources (see chapter 4) in the clays other that the added lactate.

Test cells experiments have not been performed with the FEBEX bentonite. However, clay samples have been analysed for viability at different densities and water content levels after 18 years of incubation in a heated full-scale simulation of a geological disposal concept in granitic bedrock (Bengtsson et al. 2017b).

5.1.2 Acetate and lactate analysis

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 when added to Asha and Rokle bentonites (Figure 5-3 and Figure 5-4). The background values for lactate and acetate in 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}.

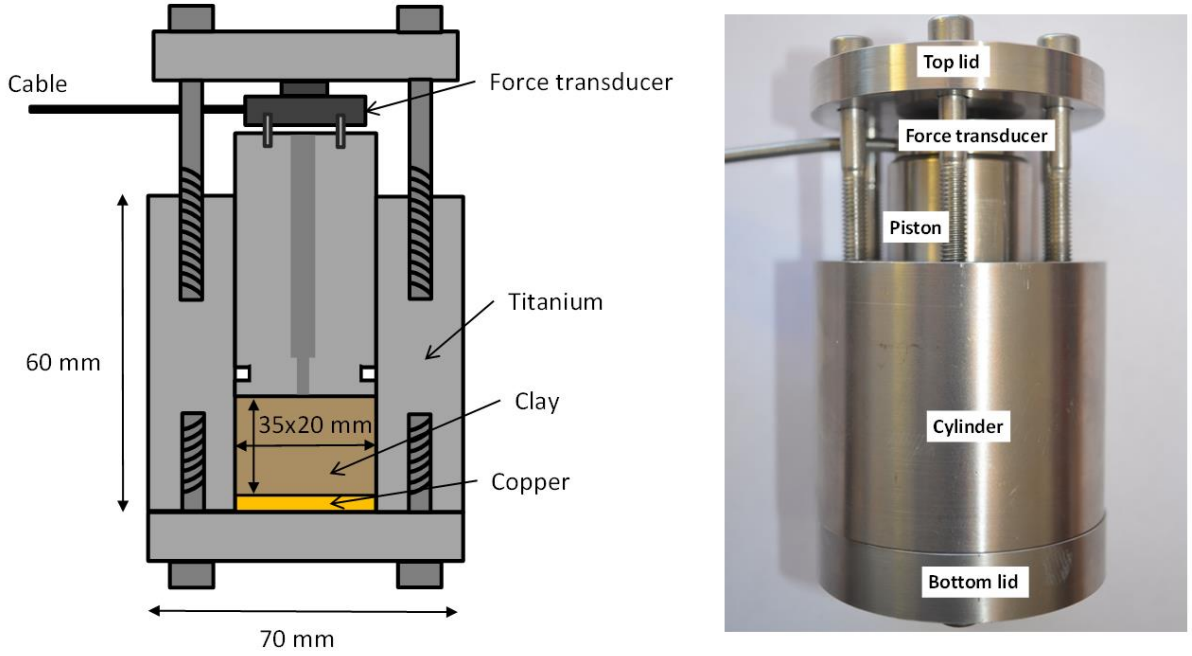

Figure 5-1. Left: A schematic cross section of a test cell. Right: An assembled test cell, spacers are not mounted.

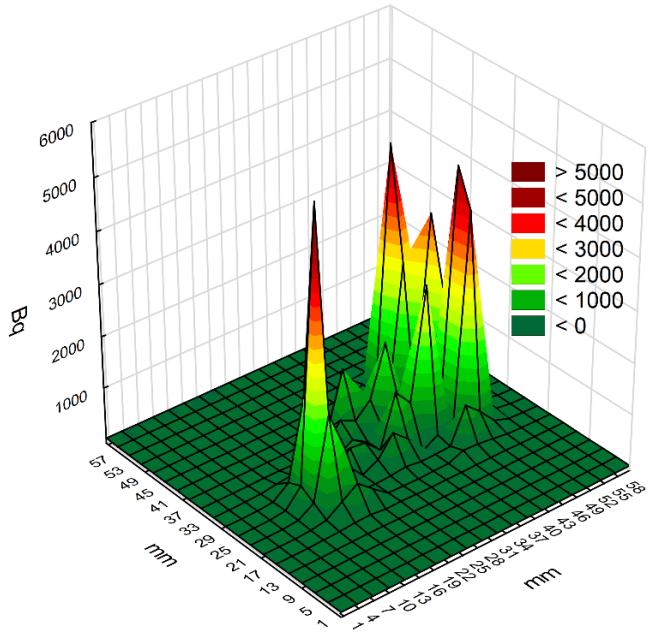

Figure 5-2. Surface radioactivity analysed on the copper disc of Rokle TC44 1750 (+) 78d (Bengtsson et al. 2017a).

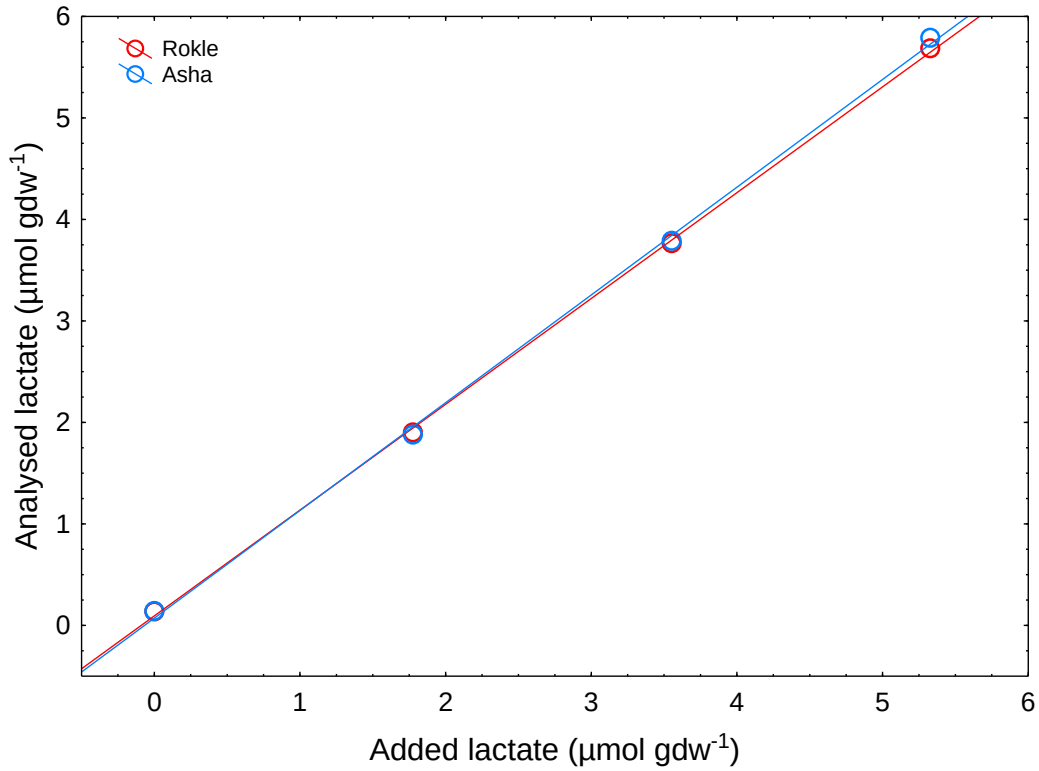

Figure 5-3. Added amounts of lactate to Rokle and Asha bentonite clays and the corresponding analysed amounts.

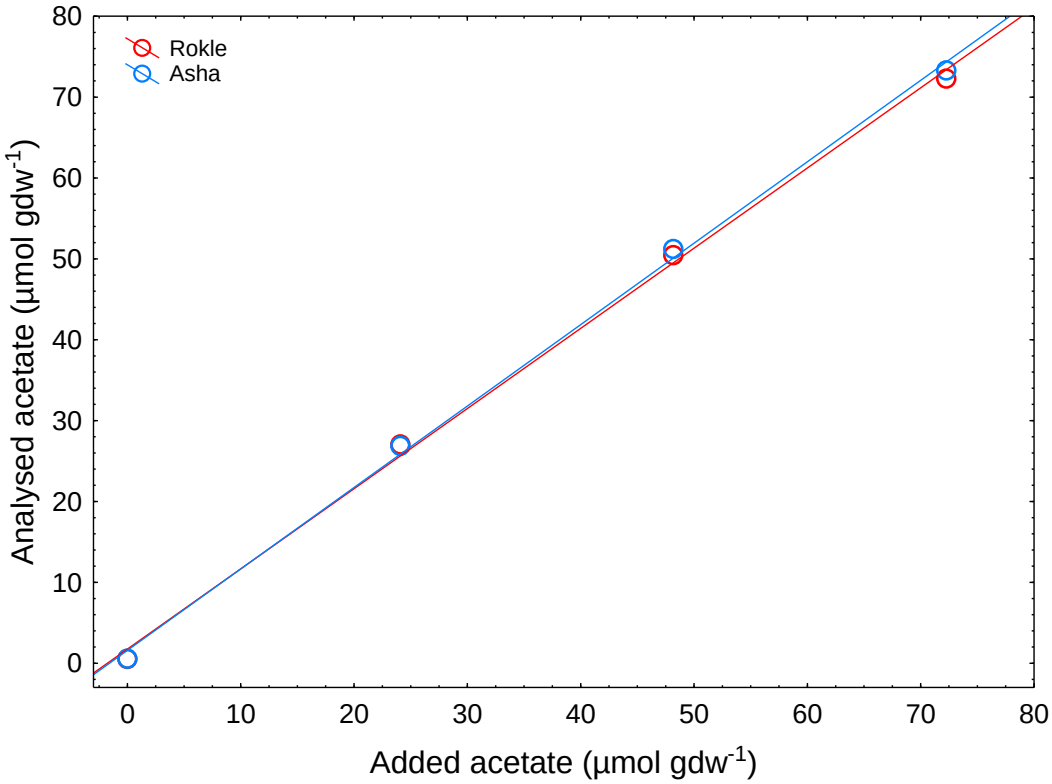

Figure 5-4. Added amounts of acetate to Rokle and Asha bentonite clays and the corresponding analysed amounts.

5.2 Clay density and cultivability of SPB

As shown in Figure 5-5, the overall cultivability of SPB cultivated from clay samples decreased over wet density. The far highest numbers reported as number per litre of pore water (>300 $\times 10^6$ MPN L^{-1}) were found below 1850 kg m^{-3} for samples of Asha and GMZ, both with and without initial bacterial addition. In addition, above 1850 kg m^{-3} there was predominantly samples with bacterial addition that could be cultivated. This may indicate that the inherent, cultivable SPB populations in the tested bentonites were not cultivable or killed during the experiment in wet densities over 1850 kg m^{-3}, although with some exceptions (Figure 5-5).

During sampling of the GMZ test cells, black spots were found in the profiles of the clay cores (Figure 5-6). The colour likely was due to FeS locally produced by SPB. The black spots rapidly disappeared when exposed to oxygen which agrees with that FeS is not stable in contact with O_2. Bacteria typically grow in colonies and the results with black spots confirm that growth of SPB in this clay, and likely also in the other clays, was heterogeneously distributed.

The different analysed clays had varying mineralogy with a large difference in content of toxic trace metals and iron for Asha and Rokle compared to the other clays. It has been previously suggested that iron-rich clays have bactericidal effects and they are sometimes used in medicinal treatment (Williams and Haydel 2010). Figure 5-7 shows that the intervals where the measurable accumulation of $Cu_2{}^{35}S$ for each clay changes from significant to below detection. For GMZ and Boom Clay the threshold densities are yet to be determined. Rokle is the clay with the largest iron content of the tested clay which interestingly also has the lowest threshold density. In fact, the density threshold of Rokle, Asha, Volclay MX-80 and Calcigel all fell approximately in the order of iron content from the lowest to the highest values of Fe_2O_3 (Table 2-3). For GMZ and Boom Clay, no cut-off density within the tested densities could be found. It is therefore concluded that density alone does not control bacterial activity in clays. Other variables must be studied as well.

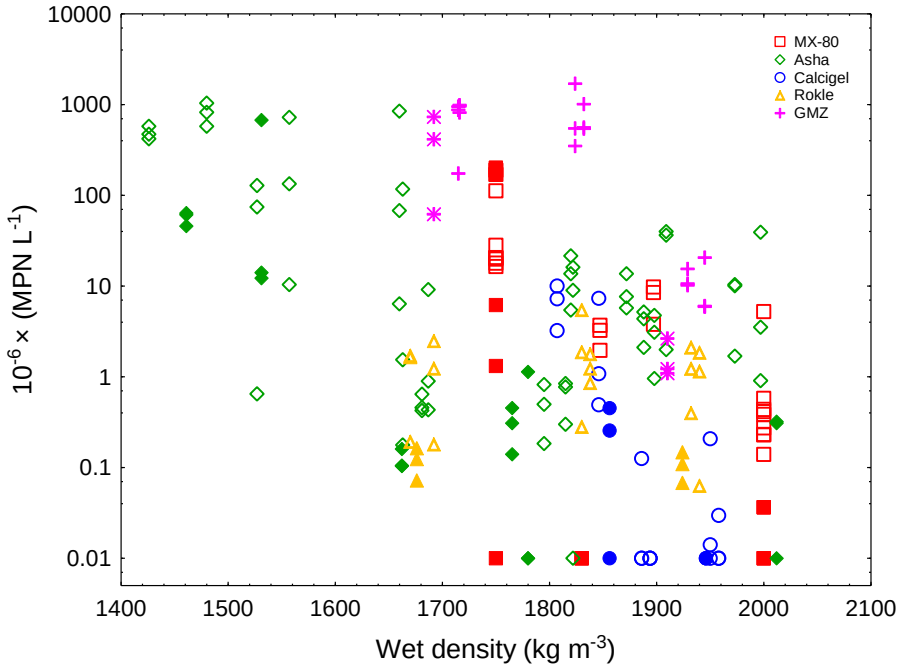

Figure 5-5. Number of cultivable cells (MPN) over wet density. Note that the y-axis is logarithmic. Control samples without bacterial addition are marked with filled symbols for each clay according to legend.

Figure 5-6. Black spots observed during sampling of test cell with GMZ bentonite.

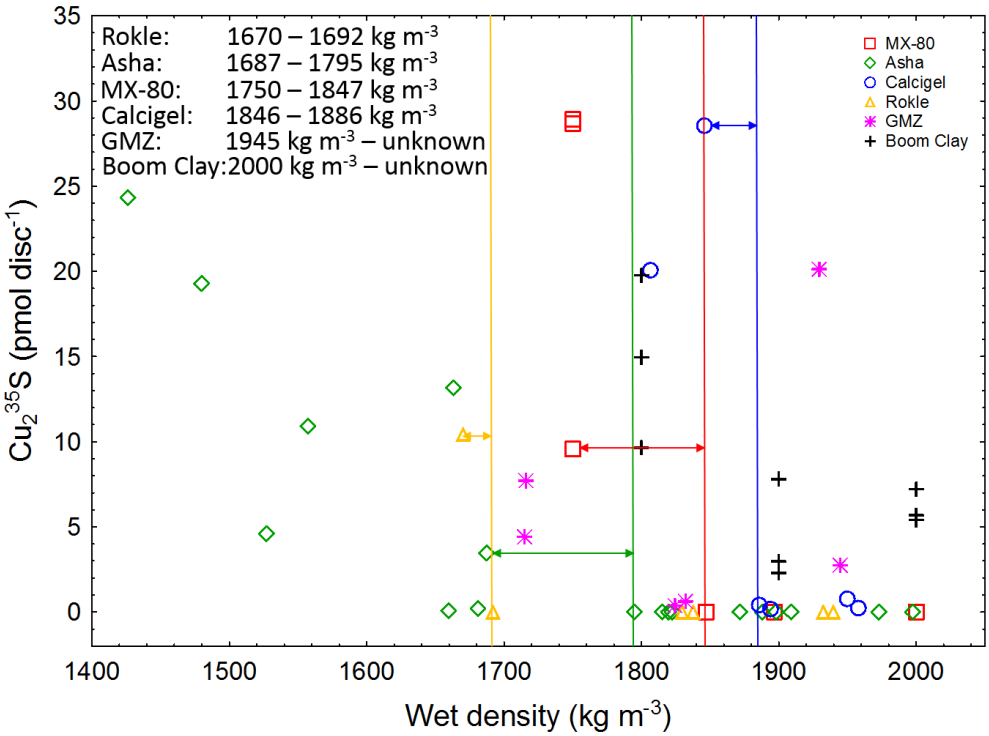

Figure 5-7. Accumulated $Cu_2{}^{35}S$ on copper discs over wet density for each tested clay according to legend. Vertical lines specify density intervals where sulphide-producing activity goes from positive to below detection.

5.3 Bacterial lactate consumption and acetate production

The methodology of analysing acetate and lactate was introduced on experiments with Rokle, Asha and GMZ. Detailed experimental lay-out are described in papers and reports by Bengtsson et al (2017a; 2017).

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

Rokle

The concentrations of leachable sulphate (For method, see Bengtsson and Pedersen 2017) 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 5-1). 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 5-8). 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 Rokle test cell TC41 without bacterial addition had not consumed any lactate but had still produced intermediate levels of acetate (Table 5-1). This formed acetate must then have been produced by bacterial degradation of organic matter in the clay. Rokle 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 with the aid of natural organic matter present in the Rokle clay.

TC44 had one very high value of acetate next to the copper surface (Figure 5-8) that was matched by the radioactivity on the disc (Figure 5-2). In addition, the MPN of SPB was 10 times higher in the position close to the copper disc compared to the MPN in the other 1750 kg m^{-3} test cells. This result argues for the heterogeneous growth of SPB in compacted bentonite also observed for GMZ (Figure 5-6).

GMZ

The concentrations of leachable sulphate were very low, at the limit of detection for the applied method (For method, see Bengtsson and Pedersen 2017). 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 radioactivity, 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 natural organic matter in the GMZ bentonite.

Table 5-1. 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. Each test cell experiment was given a unique number and a code with information of the planned bentonite wet density, addition of SPB and incubation time: TC =test cell, 1500 – 2000 = 1500 to 2000 kg m^{-3} bentonite wet density, (+/−) = with or without adding of SPB, d=days of incubation. (Detailed experiment lay-out is described by Bengtsson et al. 2017a)

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

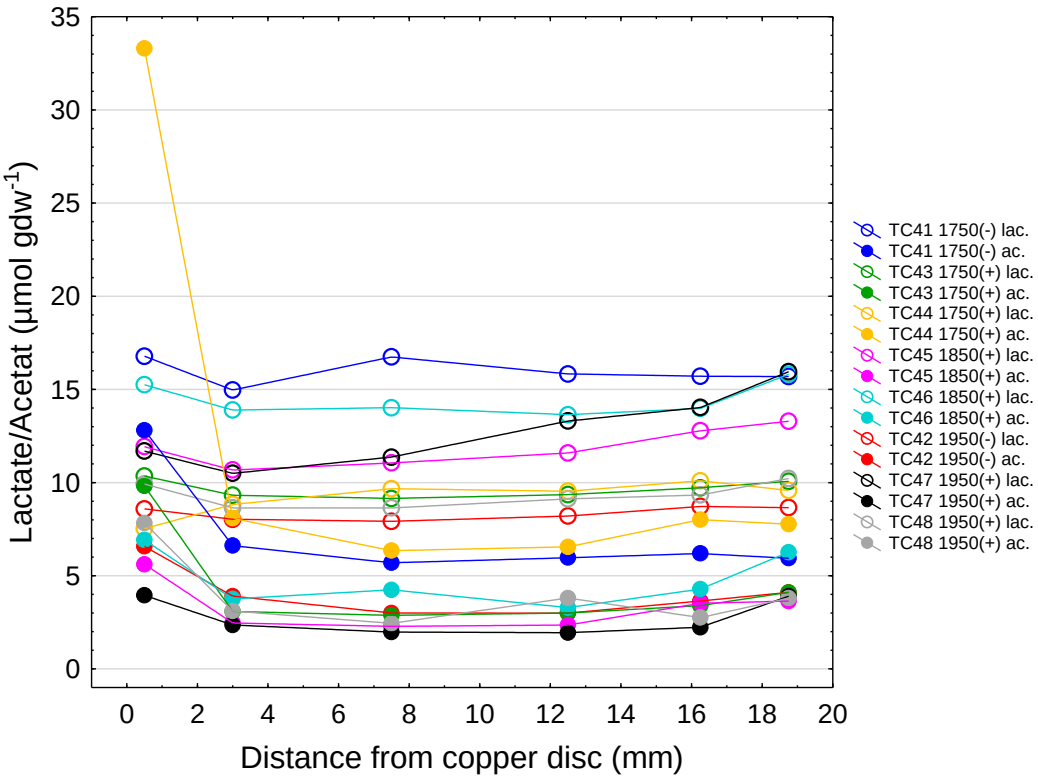

Figure 5-8. Amounts of lactate and acetate in profiles of Rokle bentonite cores for each test cell. Test cell number, bentonite density, addition of bacteria (+/−) and incubation time according to symbol description. lac. = lactate; ac. = acetate.

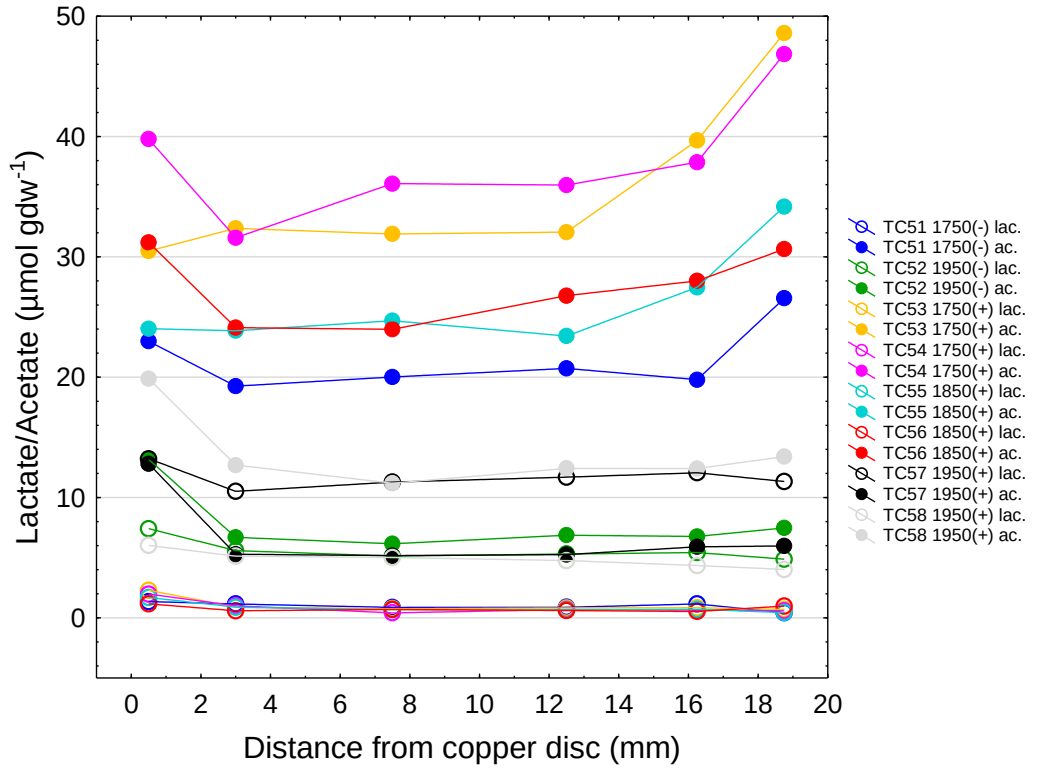

Figure 5-9. Amounts of lactate and acetate in profiles of GMZ bentonite cores for each test cell. Test cell number, bentonite density, addition of bacteria (+/−) and incubation time according to symbol description. lac. = lactate; ac. = acetate.

5.3.2 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 5-3). Lactate was evenly distributed in all test cells after 30 days (Figure 5-8 and Figure 5-9) which shows that lactate was relatively rapidly transported to all analysed positions in the clay cores. The average values given in Table 5-1 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 1750 kg m^{-3} clay cores with added SPB showed a significantly higher lactate consumption than did the two higher densities (Figure 5-10). The consumption of lactate agrees with a consumption of sulphate which suggests that some or all "missing" lactate was consumed by SPB.

5.3.3 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 5-4 and adjacent text) which shows that all analysed acetate must have been produced during the incubation of the clay cores (Figure 5-11). 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 have more acetate than what can be explained by the amount of consumed lactate (Figure 5-12). 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 5-12). Consequently, there must have been more natural organic matter available in this clay than the added lactate. The acetate production was present at all tested wet densities which attests that bacteria can be active in wet densities up to at least 1950 kg m^{-3}. Previously, it was found that SPB could actively produce sulphide in Boom clay at a wet density of 2000 kg m^{-3} (Bengtsson and Pedersen 2016). Wet density, *per see*, obviously does not cut off bacterial activity in compacted clays as was shown for GMZ, but there appears to exist a 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 Muller 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 and Olkiluoto groundwater at occasionally large numbers (Hallbeck and Pedersen 2012; Pedersen et al. 2008). In the experiments reported here, acetogens must have competed with SPB over the lactate in the clays but also over other present organic carbon compounds in the clays (Table 4-1). High-resolution analysis of the diversity of organic carbon in bentonite clays, and quantification of specific compounds attested presence of degradable organic carbon in the clays and can explain the observed acetate production. The different clays had different composition

and amounts of organic carbon. Organic carbon was previously demonstrated for Volclay MX-80 bentonite (Marshall et al. 2015).

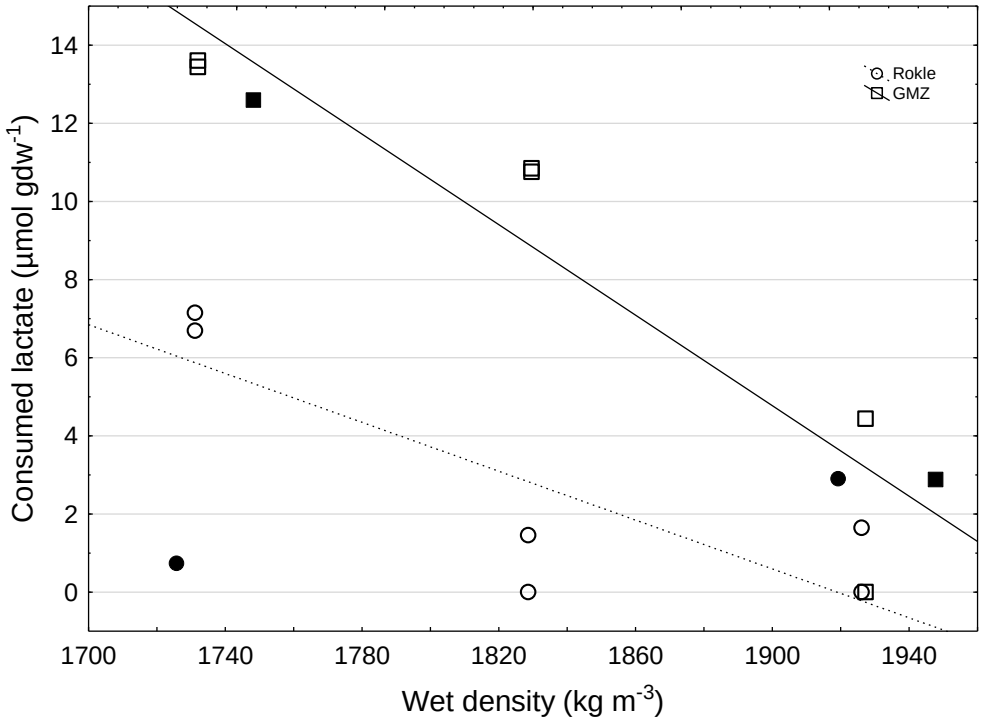

Figure 5-10. The average amounts of consumed lactate in test cells over wet density (from Table 5-1). The solid line shows linear fit for GMZ and the dashed linear fit for Rokle. Solid symbols indicate control test cells without bacterial addition.

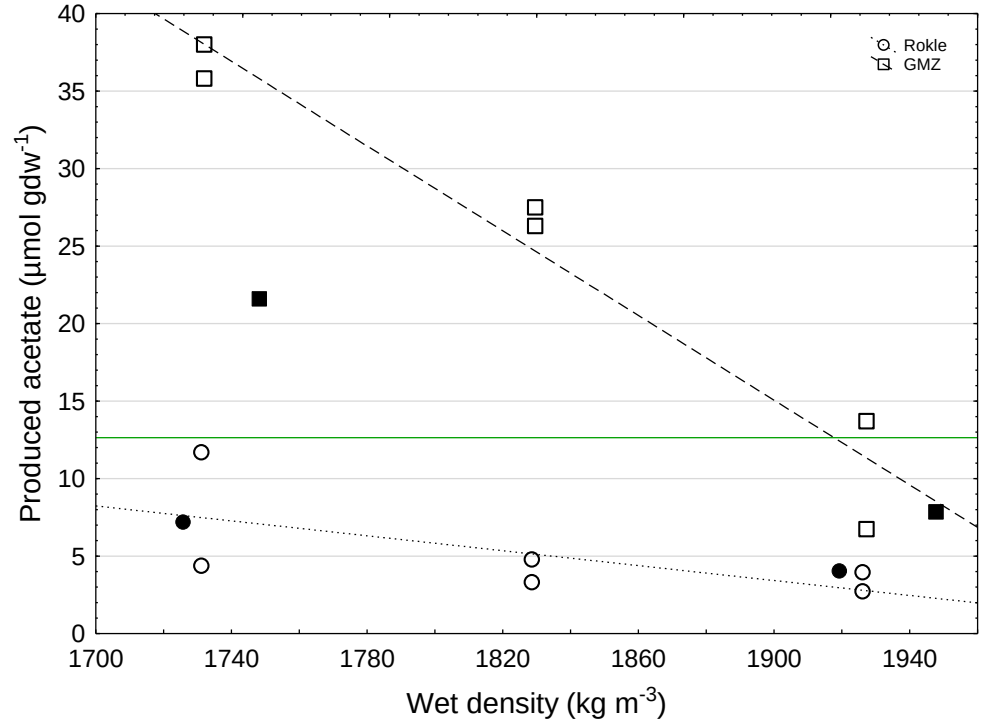

Figure 5-11. The average amounts of produced acetate in test cells over wet density. The dashed lines show linear fit for each clay according to legend. Solid symbols indicate control test cell without bacterial addition. The green horizontal line indicates the average level of added lactate for all 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 occur 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.

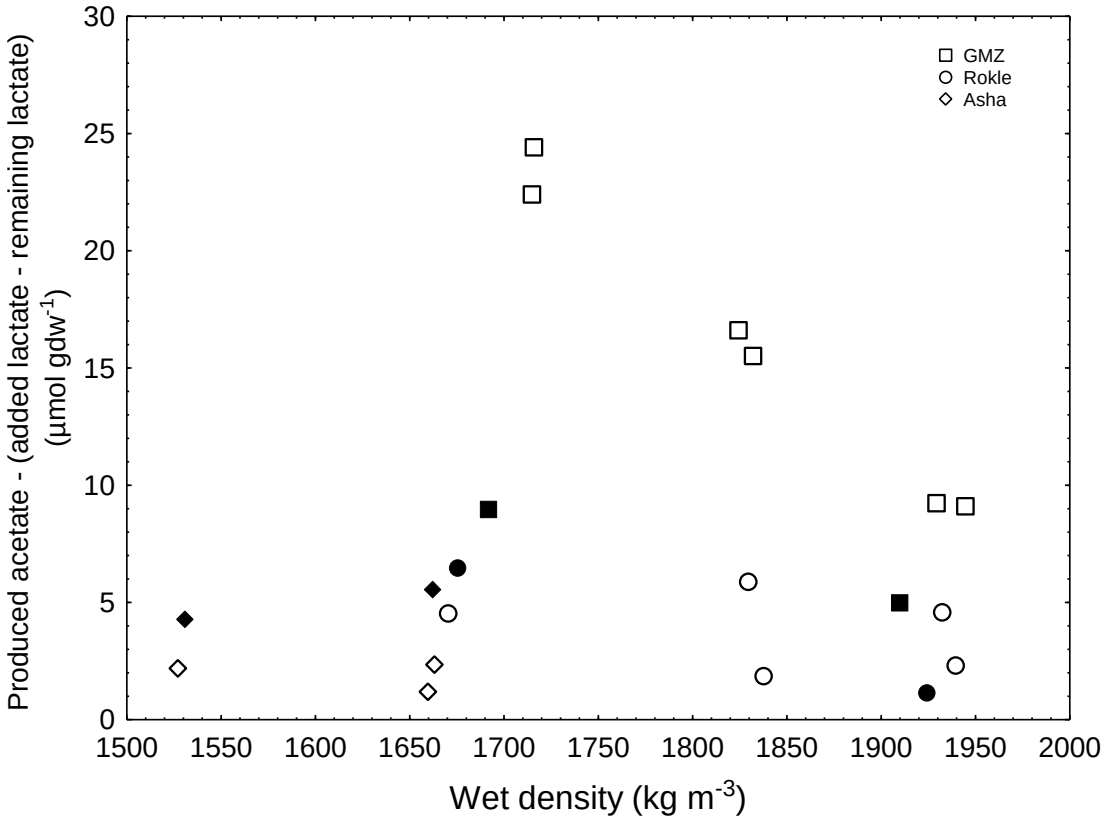

Figure 5-12. Produced acetate over wet density. The values in the graph have been subtracted with the amount of added lactate minus the amount of remaining lactate, meaning that the produced acetate in the graph is suggested to have originated from the analysed natural organic matter in the clays (see chapter 4).

6 Conclusions on variables that influence bacterial viability, cultivability, and activity in bentonite clays

6.1 Variables

Bacterial life, survival and activity will depend on several different variables in a buffer or backfill (Figure 6-1).

6.1.1 pH

The pH of most bentonite clays is slightly alkaline but still well within the range of what most bacteria can tolerate.

6.1.2 Temperature

In radioactive waster repository concepts, the maximum surface temperature of canisters may not exceed 90 °C in order to avoid formation of steam when water come in contact with the canister. In an earlier experiment, heat treatment at 120 °C for 15 h failed to kill inherent bacteria in the bentonite (Masurat et al. 2010). There was a heat treatment of the bentonite at 110 °C for 170 h in a follow up work (Bengtsson and Pedersen 2017) intended to sterilize the bentonite from bacteria; prolonged exposure to heat was expected to be efficient, but that was still not enough to kill off SPB in the bentonite because intensive sulphide-producing activity and large numbers of cultivable SPB were observed in MX-80. In addition, bentonite or rather montmorillonite, has a verified high affinity for water and the cell membrane of bacterial cells is water permeable. If a bacterial cell is surrounded by bentonite, it is possible that the water affinity of montmorillonite will extract water from the cell, leaving it in a desiccated state. The phenomenon of drying cells for prolonged disposal is well known and commonly used in microbiology (Gherna 1994). Slow desiccation can yield higher viability, after prolonged disposal, than can fast desiccation (Laroche and Gervais 2003; Potts 1994) and also increased heat resistance and viability for both spores and vegetative cells (Fine and Gervais 2005). Bacteria consequently have several mechanisms to survive prolonged periods of exposure to heat.

6.1.3 Diffusion

Transport of nutrients to, and metabolic products from bacteria will be diffusion limited due to the low porosity of buffers and backfill. The rates of diffusion will be very slow as was demonstrated for sulphide (see chapter 3). Bacterial activity will, consequently be diffusion limited in backfill and buffers when these EBS are fully water saturated. The only position not affected by diffusion barriers will be the interface between rock/aquifers/EDZ and buffer and backfill.

6.1.4 Pressure

The swelling pressure in the bentonite originates from separating flocs in the bentonite. This means that a mechanical pressure arises between the separating flocs, approximately equal to the swelling pressure. Even in low-density bentonites (1500 kg m^{-3}), a pore size in the nm range would theoretically not allow for bacterial existence unless the bacteria could withstand the mechanical pressure from the separating flocs (0.09 MPa at 1500 kg m^{-3}). Prokaryotic cells can compensate for the mechanical pressure in compacted bentonite by turgor pressure. Published data on turgor pressure in prokaryotic cells mention pressures between 0.08 MPa and 2 MPa

(Potts 1994). An upper limit of 2 MPa turgor pressure would mean that cell integrity is possible, though limited, at bentonite swelling pressures below 2 MPa. However, endospores can survive a much higher pressure.

6.1.5 Water content

Water is needed for active bacterial life and this water must be present externally because bacteria (except spores) cannot keep water inside their cell membranes that are freely permeable for water. Low water content in clays will inactivate or kill bacteria. However, as was discussed for temperature above, many bacteria survive desiccation and can be activated again when there is enough water.

6.1.6 Pore space

Bacteria are very small and if there are pores or other inhomogeneities in buffer and backfill with lower than planned pressures, local microbial activity will be possible. In addition, there will be interfaces between rock engineered disturbed zone (EDZ) and bentonite and between bentonite and canisters at which pressures may differ from the bulk of buffer and backfill.

6.1.7 Pore water composition

The pore water composition will vary with the type of bentonite and the composition of the saturating groundwater. Bentonites vary in composition with respect to elements (Table 2-2) and minerals (Table 2-3) and the type and content of natural organic matter (Table 4-1). The conditions for survival and activity of bacteria may, consequently vary significantly as inferred by the variation in the highest wet density at which sulphide production was detected above the detection limit for the method (Figure 5-7).

6.2 What need further attention?

The relation between water saturated clays at varying wet density and bacterial sulphide-producing activity is well studied. However, wet density is just a value of the total amount of clay and water. That value does not reflect the conditions in a compacted clay where several variables of importance for bacterial life can be of importance Figure 6-1). These variables need further attention for a full understanding of what conditions control bacterial activity in compacted bentonite clays.

The recently observed acetate formation from natural organic matter in three of the studied bentonites occurred at all wet densities suggests that bacterial activity, *per see*, was possible at densities where sulphide-production could be detected. Acetate is known to induce stress-corrosion cracking of copper and other metals (King et al. 2011) and must, therefore, be controlled.

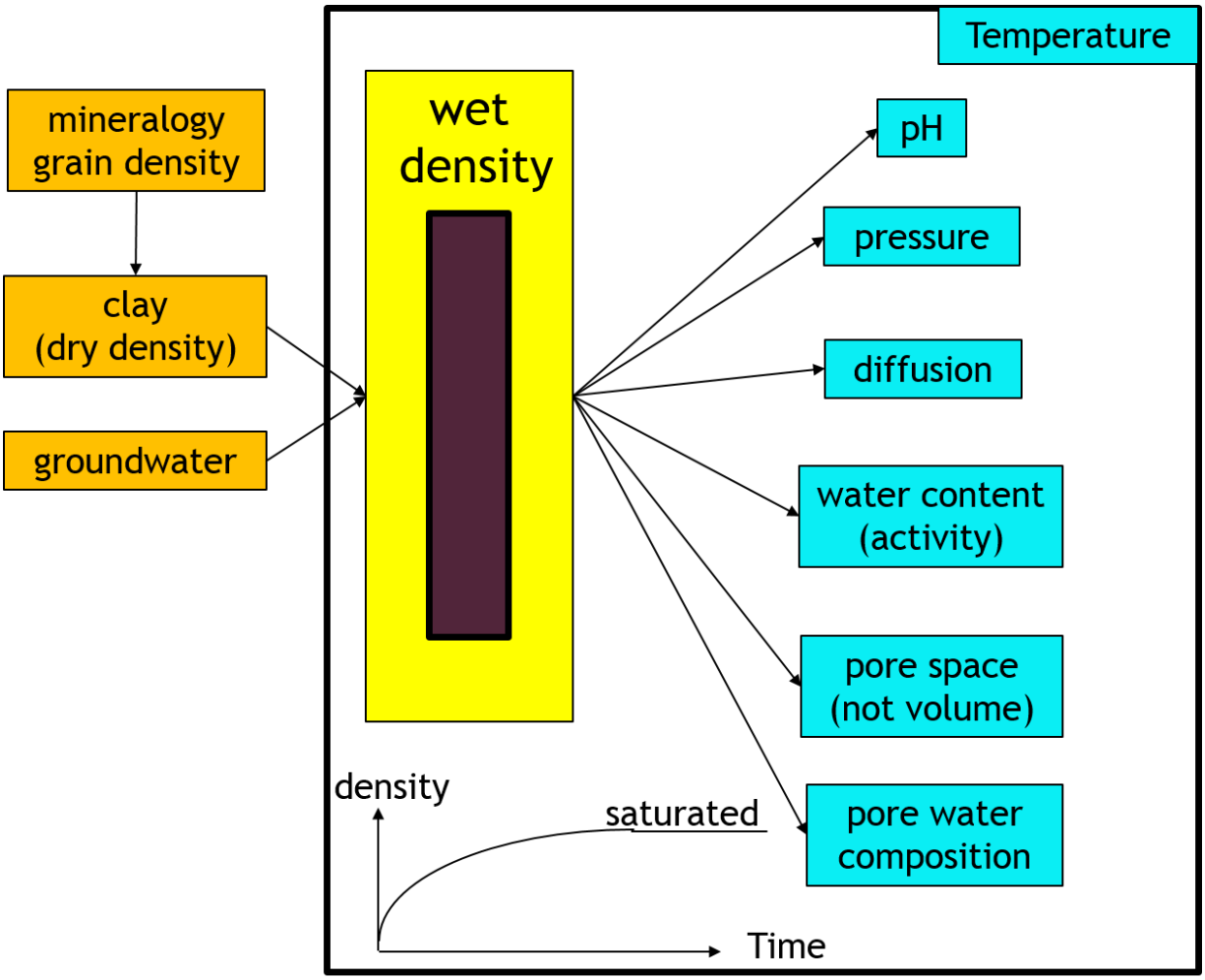

Figure 6-1. Variables influencing wet density, that upon water saturation give rise to suggested factors influencing bacterial viability, cultivability and activity (turquoise) except temperature, not coupled to wet density.

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