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Activity of sulphate reducing bacteria in bentonite as a function of water availability

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Abstract

The activity of sulfate reducing bacteria (SRB) in bentonite was studied as a function of the water availability (liquid and various relative humidity (RH) values). The focus was on experiments with the commercially available *Pseudodesulfovibrio aespoeensis* (formerly known as *Desulfovibrio aespoeensis*) originally isolated from the Äspö Hard Rock Laboratory with further experiments using cell concentrates from a natural groundwater from the Äspö Hard Rock Laboratory boreholes KA3105A:3 and KA2511A:5. Additionally, one demonstration experiment was performed to investigate if the natural presence of SRB in bentonite could be activated. When gypsum, lactate (serving as both energy and carbon source), nutrients, and liquid water were added the SRB reduced sulfate to sulfide regardless of whether bentonite was present or not. This was observed as a black precipitate in the inoculated samples. However, in some cases, there were strong indications that the bentonite reacted with some or all formed sulfide, decreasing the amount of detected sulfide compared to experiments with no bentonite (series 3; samples S2 and S3). When replacing the liquid water with only moist air (up to 100 % RH) no sulfate reduction was observed. Hence, the conclusion from these experiments is that in order to have sulfate reduction, 100 % RH is insufficient to promote sulfate reduction; both liquid water and an energy source such as lactate in this case, need to be present. In some of the experiments, silver plates were added as a potential indicator of sulfide production. With SEM/EDS it was observed that the darkening was not necessarily an effect from sulfide production, but instead indicated minor remains of montmorillonite on the surface. However, detection of sulfide was possible when the background level of sulfide was compared with the plate from the experiment to a reference silver plate. Additionally, silver chloride crystals were found on the silver plate from the experiment, possibly this phase was formed during the storage of the sample in oxic conditions. The work with the silver plates should be regarded as method development of this kind of experiment.

Sammanfattning

Aktiviteten av sulfatreducerande bakterier (SRB) i bentonit studerades som en funktion av tillgången till vatten (vätska och olika relativa fuktigheter). Fokus var på experiment med den kommersiellt tillgängliga *Pseudodesulfovibrio aespoeensis* (tidigare kallad *Desulfovibrio aespoeensis*) som ursprungligen isolerades från Äspö HRL, men experiment utfördes också med en anrikning från ett naturligt grundvatten från Äspö laboratorieborrhålet KA3105A: 3. Dessutom utfördes ett demonstrationsexperiment i mätcylinder för att undersöka om den naturliga förekomsten av SRB i bentonit kunde aktiveras. När gips, laktat (som utgör både kol- och energikälla), näringsämnen och flytande vatten tillsattes kunde sulfatreduktion observeras oavsett om bentonit tillsatts eller ej.

I vissa fall fanns emellertid starka indikationer på att bentoniten reagerade med en del eller all sulfid som bildats, eftersom mängden bildad sulfid var lägre eller under detektionsgränsen i närvaro av bentonit (serie 3: prov S2 och S3).

Slutsatsen från denna studie är att det krävs både en energikälla (laktat i detta fall) och flytande vatten för att sulfatreduktion ska ske. Inte ens 100 % luftfuktighet var tillräckligt för att sulfid skulle bildas. I några av experimenten tillsattes silverplattor som en potentiell indikator för sulfidproduktion. Med SEM/EDS observerades att den mörka ytan som proverna fick i försöken inte nödvändigtvis var en effekt från sulfidproduktion, utan i stället kunde indikera andra saker, som rester av montmorillonit på ytan. Däremot var detektering av sulfid möjligt när bakgrunden av sulfid jämfördes mellan plattan från experimentet mot en referens. Dessutom hittades silverkloridkristaller på silverplattan från experimentet, vilket möjligen bildades under lagring av provet under aeroba förhållanden. Arbetet med silverplattorna ska ses som metodutveckling av försöksuppställningen.

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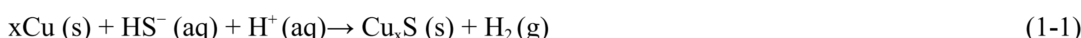
1 Introduction

Some bentonites contain gypsum ($CaSO_4 \times 2\ H_2O$), typically around or below 1 wt% (e.g. Karnland et al. 2006, Svensson et al. 2020). Gypsum is often observed in bentonite deposits as larger individual crystals or aggregates (Figure 1-1).

Sulfate reducing bacteria (SRB) are also present in the bentonite and in the surroundings within a repository environment. The SRB respire sulfate, such that they use the sulfur in sulfate as an electron acceptor. This results in the formation of sulfide ($S^{2-}/HS^-/H_2S$) that in turn may corrode the copper canisters used in the KBS-3 concept for high-level radioactive waste disposal (Myers and Cohen 1984). The reaction is summarized in Hallbeck (2014) as:



$$xCu\ (s) + HS^-\ (aq) + H^+\ (aq) \rightarrow Cu_xS\ (s) + H_2\ (g) \qquad (1\text{-}1)$$

In order to be active, microbes need water, nutrients, an electron donor and acceptor, an inorganic or organic carbon source and space. Water is crucial as bacteria use the water to take in food and to remove unwanted waste products. Although hydrogen is the preferred energy source for lithotrophic SRB, some SRB use e.g. lactate as both the carbon source and electron donor (e.g. Madigan et al. 2012). Those bacteria able to switch between an aerobic i.e. using O_2 as electron acceptor and anaerobic growth are called facultative anaerobes. Many SRB are strict anaerobes and thus, require reducing conditions to carry out anaerobic respiration. Typical terminal electron acceptors during anaerobic respiration include NO_3^-, Fe^{3+}, Mn^{4+}, SO_4^{2-}, CO_2, UO_2^{2+}, and AsO_4^{3-} and it is relatively common within certain groups of bacteria, such as the nitrate and iron reducers, that they can switch between different electron acceptors. Without the presence of an energy source (electron donor), the bacteria are unable to respire even if there are sufficient nutrients and electron acceptors. However, some bacteria are able to use the same molecule both as an electron donor and electron acceptor (termed 'fermentation') to obtain energy.

During water saturation, bentonite expands and builds up a swelling pressure such that once it reaches a pressure greater than approximately two MPa, microbes are expected to be inactive due to limited space and generally hostile conditions (Bengtsson and Pedersen, 2016, 2017). However, during the early period of the repository lifetime and before the bentonite is water saturated, there may be a window of opportunity when the bentonite is sufficiently moist for the RH to support SRB activity. This study aims at finding out if there is a moisture threshold limit for the bentonite when SRB may produce sulfide.

Figure 1-1. *Natural gypsum (~ 3 × 1 cm) on top of bentonite, Wyoming, U.S.A.*

2 Materials and methods

2.1 Experimental layout

2.1.1 Demonstration experiment in a glass cylinder

The demonstration experiment was conducted in a glass measuring cylinder containing non-sterilized MX-80 Wyoming bentonite presumably containing indigenous SRB and amended with lactate, nutrients, gypsum, and water. The cylinder was sealed with a rubber septum in order to make it as airtight as possible and thus, with time create oxygen free layers within the clay and making it favorable for sulfide production. The experiment was performed on a laboratory bench at oxic conditions at 20 °C.

2.1.2 Tube experiments in anaerobic glovebox

The tube experiments were conducted at 20 °C under oxygen free conditions in a N_2-atmosphere inside a MBraun 200B glovebox, generally the O_2 concentration was < 0.1 ppm according to the O_2 sensor, but was sometimes higher for some hours (up to 100 ppm O_2) when solutions were transferred into the glovebox. All equipment including instruments, containers, solutions and chemicals were sterilized or purchased sterile (except for the clay that was non-sterile), placed in the airlock overnight and then transferred into the anaerobic glovebox before starting the experiment. All chemicals were of analytical grade. All experimental series are listed in Table 2-1.

The bacterial strain chosen for the study was *Pseudodesulfovibrio aespoeensis* (formerly *Desulfovibrio aespoeensis*; DSM 10631) originally isolated from the Äspö Hard Rock Laboratory (HRL) (Motamedi and Pedersen 1998) that was purchased from DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen, www.dsmz.de). Among other considerations, *P. aespoeensis* was chosen as it is relatively easy to culture, well-studied, common in deep groundwaters, and able to use lactate as energy source instead of H_2. This species was used in series #1 to #4 and #6 to #8 (Table 2-1). The bacterium was delivered as freeze-dried cells contained within a glass ampoule. The ampoule was cracked inside the anaerobic glovebox and the pellet was dispersed in medium in order to revive the cells using a sterile pipette and poured into a serum bottle containing 100 mL medium (Tables 2-7 to 2-9). The serum bottle was sealed with a rubber septum and an aluminum cap, shaken, and left to adjust and grow for a couple of weeks inside the glove box before the initiation of the experiment. All pre-cultures used in series #1–#3 were prepared this way. The inocula were withdrawn from this serum bottle using a pipette with sterile tip and added to the experiments. The same pre-culture was used for experimental series #1 and #2. New bacteria were purchased for #3 and thus a fresh pre-culture was prepared.

Table 2-1. Overview of experiments conducted 2017–2019. The duration of the individual experiments was between 3 and 4 months.

Series	Organization	Setup	Time
#1	SKB	Type 1 (dual test tube design, see Figure 2-1) with *P. aespoeensis*	2017
#2	SKB	Type 2, similar design as Type 1 but using 250 mL glass jars containing glass beakers with ten fold more bentonite than in the glass test tubes in Type 1	2017
#3	SKB	Type 1	2018
#4	SKB-LNU	Type 1 with increased mass of bentonite	2018
#5	SKB-LNU	Type 1 with increased mass of bentonite and microbes from groundwater (no addition of purchased *P. aespoeensis*)	2018
#6	SKB-LNU	Type 1 with increased mass of bentonite and different RH, long-term series	2019
#7	SKB-LNU	Type 1 with increased mass of bentonite and the addition of elemental sulfur instead of gypsum	2019
#8	SKB-LNU	Type 1 with increased mass of bentonite and the addition of sodium sulfite instead of gypsum	2019
#9	SKB-LNU	Type 1 with increased mass of bentonite, addition of elemental sulfur instead of gypsum, and microbes from groundwater (no addition of purchased *P. aespoeensis*)	2019
#10	SKB-LNU	Type 1 with increased mass of bentonite, addition of sodium sulfite instead of gypsum, and microbes from groundwater (no addition of purchased *P. aespoeensis*)	2019

For series #1 and #3, medium recipe listed in Tables 2-7 to 2-9 (medium I) was used. The composition of the medium used for series #4 and #6 to #8 is listed in Tables 2-10 to 2-12 (medium II). In all cases, the cells were pre-cultured for a couple of weeks to make sure that viable cells were introduced in the experiment. For series #2, the inoculum was taken from the same pre-culture as #1 some months later.

P. aespoeensis served as a model organism in this study. However, this strain represents only a small proportion of the indigenous microbial community in deep groundwaters (Hubalek et al. 2016, Lopez-Fernandez et al. 2018a, b). Therefore, in series #5, #9, and #10, cell concentrates of a natural groundwater microbial community from the Äspö HRL boreholes KA3105A:3 and KA2511A:5 were used, as described later.

The strategy was to have one reaction tube containing the bentonite, microbes, lactate, gypsum, and nutrients that was placed inside a larger tube such that both tubes shared the same atmosphere. In the outer tube, water or a saturated salt solution was present to set the RH. A pure water solution gives the highest possible RH in the system, while saturated salt solutions of various salts give rise to lower RH's (Figure 2-1). As it was unknown whether there was a potential threshold RH promoting microbial growth, two different salts were chosen to investigate this empirically. A small amount of copper sulfate was added in the outer solution as an indicator of formed hydrogen sulfide by the following reaction:

$$Cu^{2+}\,(aq) + HS^{-}(aq) \rightarrow CuS\,(s) + H^{+}(aq) \qquad (2\text{-}1)$$

This reaction is extremely fast and the formed CuS is easily visually observed as a brownish black precipitate, while the blue copper solution slowly desaturates in color (for details see Svensson et al. 2017). The inner tube was a test tube made of glass with a volume of 5 mL and the outer tube was a Falcon 50 mL polypropylene conical centrifuge tube (Corning Life Science Inc).

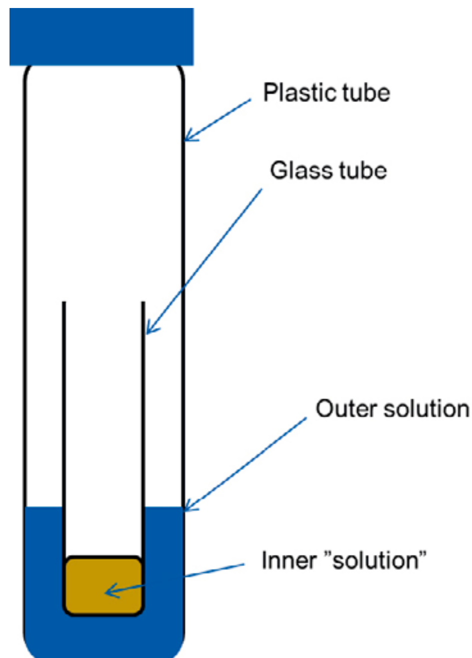


Figure 2-1. *Experimental setup type 1: an inner glass tube without lid contained within a larger polypropylene tube (Falcon tube) with 15 mL aquatic solution with or without salt addition. The outer solution sets the RH and contains divalent copper ions to detect sulfide.*

In series #1 to 3 (Tables 2-2 to 2-3) the positive controls had at least semi-optimal conditions for *P. aespoeensis* as they had access to growth medium amended with gypsum, lactate, macronutrients, nutrient broth, and reductants (resazurin and cysteine-HCl) in order to obtain the appropriate anaerobic conditions (< 0.1 ppm O_2) plus non-compacted bentonite. This positive control was used to check the viability of the microbes with the current experimental set-up and the given laboratory conditions provided. Vitamins and trace elements were excluded in the medium (medium I) used in series #1 to #3. The first series (#1) was a pilot experiment to test if the method worked (Table 2-2) and had similar set of experimental conditions as series #3 (Table 2-3), the second series (#2) was an attempt to increase the scale of the experiment to improve the detection limit of the formed sulfide, however, this series failed. Series #3 worked as intended, i.e. the positive controls showed microbial growth.

The experimental setup of series #4 to #10 equaled the setup from series #1 (type 1, see Figure 2-1) but more bentonite was used (2.5 g instead of 2.0 g; Table 2-4).

Previous studies of deep groundwaters show that sulfide is not only produced by SRB using the whole pathway from sulfate to sulfide. Instead, microorganisms that use alternative sulfur compounds are also present such as sulfur- and sulfite-reducers (Wu et al. 2016, Anantharaman et al. 2018) and therefore, can be relevant sources of sulfide production. Thus, elemental sulfur was added as an electron acceptor instead of gypsum in series #7 and #9 and sulfite was added for series #8 and #10.

Table 2-2. Detailed experimental conditions for series #1. Positive controls were used, i.e. with conditions favorable for the inoculum *P. aespoeensis*. In addition, a control with non-sterile clay was used. All samples with addition of excess liquid i.e. 5 mL medium counted as positive controls. Lower amounts of liquid insufficient to make the bentonite moist.

			Inner tube				Outer tube		
No.	Replicates	~RH (%)[a]	Bentonite (g)	Gypsum (mg)	*P. aespo*[c]	Sterile medium (mL)	Salt[d]	$CuSO_4$ (mg)	Deionized water (mL)
A1	2	100	-	60	+	5	-	100	15
A2	2	100	2	60	-	5	-	100	15
A3	2	100	2	60	+	0.5	-	100	15
A4	2	11[b]	2	60	+	0.5	LiCl	100	15
A5	2	76[b]	2	60	+	0.5	NaCl	100	15
A6	2	97[b]	2	60	+	0.5	K_2SO_4	100	15

[a] Relative humidity. [b] Choudhury et al. (2011). [c] 100 µL inoculum from culture in late log phase. [d] Supersaturated.

Table 2-3. Detailed experimental conditions for series #3. Positive controls were used, i.e. with conditions favorable for the inoculum *P. aespoeensis*. In addition, a control with non-sterile clay was used. All samples with addition of excess liquid i.e. 5 mL medium counted as positive controls.

			Inner tube				Outer tube		
No.	Replicates	~RH (%)[a]	Bentonite (g)	Gypsum (mg)	*P. aespo*[c]	Sterile medium (mL)	Salt[d]	$CuSO_4$ (mg)	Deionized water (mL)
S1	2	100	-	60	+	5	-	100	15
S2	2	100	-	60	+	5	-	100	15
S3	2	100	2	60	+	5	-	100	15
S4	2	100	2	60	+	0.5	-	100	15
S5	2	97[b]	2	60	+	0.5	K_2SO_4	100	15
S6	2	76[b]	2	60	+	0.5	NaCl	100	15

[a] Relative humidity. [b] Choudhury et al. (2011). [c] 100 µL inoculum from culture in late log phase. [d] Supersaturated.

The experimental conditions for series #4 to #10 were slightly modified as shown in Tables 2-4 to 2-6. In addition, a more complex medium (medium II) was used than in the previous series (see 2.2). The complete composition of the medium II is listed in Tables 2-10 to 2-12.

A new *P. aespoeensis* culture was purchased from DSMZ for series #4 and #6 to #8. Similar to pre-culturing procedures for series #1 to #3, the glass ampoule with the cell pellet was cracked inside the anaerobic glovebox and the pellet was suspended in medium (Tables 2-10 to 2-12). The cell suspension was added to a serum bottle with 20 mL medium. Then, the serum bottle was sealed with a rubber septum and an aluminum cap, and left to grow for two to four weeks inside the anaerobic glovebox before the initiation of the experiment.

For series #4, the inner tube as illustrated in Figure 2-1 contained combinations of bentonite, gypsum, *P. aespoeensis*, medium, and a silver-plate as an indicator for sulfide production (Table 2-4). The idea was that AgS would precipitate if sulfide was produced. The actual cell number of the *P. aespoeensis* inoculum was analyzed via light microscopy and a counting chamber (Neubauer Improved, Marienfeld). To check for sulfide production derived from the sterile medium or non-sterilized bentonite, an uninoculated control (T4) was also included. Physiological NaCl solution (0.85 %) was added to the inner tubes for treatments T1 and T2 instead of the medium added for series #1 and #3 (Tables 2-2 to 2-3) in order to avoid the influence of the medium itself as it contains substrates and nutrients. The outer tube contained 15 mL of 50 mM $CuSO_4$ solution for all treatments. All treatments were set up with four replicates.

Series #5 (Table 2-5) was initiated using a cell concentrate of a natural groundwater microbial community to test the effect of entire community composition on sulfide production in relation to RH. Therefore, groundwater from Äspö HRL boreholes KA3105A:3 and KA2511A:5 were sampled anoxically. Groundwater from these boreholes contain a higher concentration of sulfide compared to other boreholes (0.15 and 0.16 mg L^{-1}, respectively; Lopez-Fernandez et al. 2018a) suggesting a high biological sulfate reduction rate. Microbial cells from approximately 1 liter of groundwater were concentrated via centrifugation with Spin-X® UF concentrator tubes (Corning, Inc.) under anaerobic conditions according to the manufacturer's instructions. The cell concentrate (for details see Section 2.2) was washed twice with the medium (Tables 2-10 to 2-12) to avoid the introduction of sulfide from the groundwater. The treatments were inoculated with this groundwater cell concentrate (theoretical cell number after cell concentration: 6×10^6 cells mL^{-1} for both boreholes; Lopez-Fernandez et al. 2018a) into medium described in Tables 2-10 to 2-12. To verify the cell number, a subsample of the cell concentrate was fixed with 1 % formaldehyde solution and stored at 6 °C. The fixed sample was filtered on a Nuclepore™ black polycarbonate membrane filter (0.22 µm; GE Healthcare Whatman™), stained with SYBR Green I (Invitrogen™), and analyzed by epifluorescence microscopy (Olympus BX50). To check for chemical sulfide production derived from the groundwater or the media, controls of cell-free groundwater (0.1 µm filtered; T4) and sterile media (T5) were included (Table 2-5).

Series #6 was a long-term series and inoculated with *P. aespoeensis* (Table 2-6). In this series, two additional treatments were included that contained saturated salt solutions of K_2SO_4 (T4) and NaCl (T5) in the outer tube creating a RH of 97 % and 76 %, respectively. For each treatment, a silver plate was added to one replicate. This long-term series also included two controls (T7 and T8) with sterilized bentonite (autoclaved three-times at 121 °C for 20 min with cooling down overnight in between), one inoculated with cells in media, and one with sterile media. These controls were included to differentiate between the effects of the microorganisms potentially present in the bentonite versus the microorganism derived from the inoculum. All treatments were set up with six replicates.

Series #7 was set up as series #4 (Table 2-4), but elemental sulfur (14 mg) was added instead of gypsum. Sulfur was sterilized by autoclaving three-times at 100 °C for 20 min with cooling overnight in between. Furthermore, no silver plates were added to this series. Similarly, for series #8, sulfite (sodium sulfite, 55 mg) was added instead of gypsum and the series was set up as series #4 (Table 2-4), but without silver plates. Sodium sulfite was sterilized by autoclaving (121 °C for 20 min). Series #9 and #10 were set up as series #5 (Table 2-5) with elemental sulfur (14 mg) and sulfite (sodium sulfite, 55 mg) instead of gypsum, respectively.

Series #4, #5, #7 to #10 were terminated after almost 4 months (118 days) whereas the long-term series #6 was terminated after one year.

Table 2-4. Experimental details for the different treatments of series #4.

Sample	Replicates	Inner tube					Outer tube	
		Bentonite (g)	Gypsum (g)	*P. aespo.* cells in medium (mL)	Sterile liquid (mL)	Ag-plate	$CuSO_4$	Deionized water (mL)
T1	2 × 2[a]	-	0.075	0.5	4.5[b]		+	15
T1	1	-	0.075	0.5	4.5[b]	+	+	15
T2	2 × 2[a]	2.5	0.075	0.5	4.5[b]		+	15
T2	1	2.5	0.075	0.5	4.5[b]	+	+	15
T3	2 × 2[a]	2.5	0.075	0.5	-		+	15
T3	1	2.5	0.075	0.5	-	+	+	15
T4 (control)	2 × 2[a]	2.5	0.075	-	0.5[c]		+	15
T4 (control)	1	2.5	0.075	-	0.5[c]	+	+	15

[a] Treatments were set up with four replicates. [b] Physiological NaCl salt solution. [c] Medium II.

Table 2-5. Experimental details for the different treatments of series #5.

Sample	Replicates	Inner tube				Outer tube	
		Bentonite (g)	Gypsum (g)	Natural cells in medium (mL)	Sterile liquid (mL)	$CuSO_4$	Deionized water (mL)
T1	2 × 2[a]	-	0.075	0.5	4.5[b]	+	15
T2	2 × 2[a]	2.5	0.075	0.5	4.5[b]	+	15
T3	2 × 2[a]	2.5	0.075	0.5	-	+	15
T4 (control)	2 × 2[a]	2.5	0.075	-	0.5[c]	+	15
T5 (control)	2 × 2[a]	2.5	0.075	-	0.5[d]	+	15

[a] Treatments were set up with four replicates. [b] Physiological NaCl salt solution. [c] Sterile groundwater. [d] Medium II.

Table 2-6. Experimental details for the different treatments of series #6.

Sample	Replicates	Inner tube					Outer tube	
		Bentonite (g)	Gypsum (g)	*P. aespo.* cells in medium (mL)	Sterile liquid (mL)	Ag-plate[e]	Salt	Deionized water (mL)
T1	3 × 2[a]	-	0.075	0.5	4.5[c]	+	$CuSO_4$	15
T2	3 × 2[a]	2.5	0.075	0.5	4.5[c]	+	$CuSO_4$	15
T3	3 × 2[a]	2.5	0.075	0.5	-	+	$CuSO_4$	15
T4	3 × 2[a]	2.5	0.075	0.5	-	+	$CuSO_4/K_2SO_4$	15
T5	3 × 2[a]	2.5	0.075	0.5	-	+	$CuSO_4$/NaCl	15
T6 (control)	3 × 2[a]	2.5	0.075	-	0.5[d]	+	$CuSO_4$	15
T7 (control)	3 × 2[a]	2.5[b]	0.075	0.5	-	+	$CuSO_4$	15
T8 (control)	3 × 2[a]	2.5[b]	0.075	-	0.5[d]	+	$CuSO_4$	15

[a] Treatments were set up with six replicates. [b] Sterile bentonite was used. [c] Physiological NaCl salt solution. [d] Medium II.
[e] One Ag-plate in one replicate was used for each treatment.

2.2 Medium types

Medium I was used in series #1 to #3 and was modified from Hallbeck and Pedersen (2008) and the components are listed in Tables 2-7 to 2-9. The modifications included:

1. no adjustment of pH,
2. no trace elements and vitamins were added, and
3. H_2 and CO_2 were not added to the headspace.

Medium I

Table 2-7. Composition of basal medium (A).

Component	Concentration (g L^{-1})
NaCl	7
$CaCl_2 \times 2\ H_2O$	1
KCl	0.67
NH_4Cl	1
KH_2PO_4	0.15
$MgCl_2 \times 6\ H_2O$	0.5
$MgSO_4 \times 7\ H_2O$	3.0

Table 2-8. Solution (B).

Component	Amount (mL)
Basal medium (A)	913 (860 in the recipe)
Na-resazurin[a]	2
Cysteine-HCl[a]	10
$NaHCO_3$[a]	60
Na-lactate (50 %)	5
Nutrient broth[a]	10

[a] Amount from the stock solutions in Table 2-9.

Table 2-9. Stock solutions (all prepared separately and added to Solution (B)).

Component	Concentration (g L^{-1})
Na-resazurin	0.5
Cysteine-HCl	50
$NaHCO_3$[a]	84
Nutrient broth[b]	50

[a] Preparation was done without lid due to extensive gas formation. [b] Standard Nutrient Broth for Microbiology, Sigma-Aldrich.

The medium was autoclaved at 12 °C for 20 min, bubbled with N_2-gas while cooling in a water bath for about 2 h, and then placed in the airlock of the anaerobic glovebox overnight together with all other equipment needed for the inoculation.

For series #4 to #10, the DSMZ medium 193 (Tables 2-10 to 2-12) was used as the previously used medium showed some precipitation. The medium (medium II) was prepared according to the DSMZ procedures with some modifications . The modifications included:

1. using Cysteine-HCl instead of Na_2S to exclude precipitation with the Cu^{2+} solution in the outer tubes which could cause a false positive result
2. exclusion of the vitamin solution (solution E) as the previous experiment showed that the culture also grows without this addition.

Medium II

Table 2-10. Components of the complete SRB medium (see DSMZ medium 193; modifications are in italics). All DSMZ media are described in detail on www.dsmz.de.

Component	Amount
Solution A:	
Na_2SO_4	3.00 g
KH_2PO_4	0.20 g
NH_4Cl	0.30 g
NaCl	7.00 g
$MgCl_2 \times 6\ H_2O$	1.30 g
KCl	0.50 g
$CaCl_2 \times 2\ H_2O$	0.15 g
Selenite-tungstate solution	1.00 mL
Na-resazurin solution (0.1 % wt/vol)	0.50 mL
Distilled water	930.00 mL
Solution B:	
Trace element solution SL-10	1.00 mL
Solution C:	
$NaHCO_3$	4.00 g
Distilled water	50.00 mL
Solution D:	
Na-L-lactate	2.5 g
Yeast extract[a]	1 g
Distilled water	10 mL
Solution E:	
Vitamin solution	-
Solution F:	
Cysteine-HCl × H_2O	*0.50 g*
Distilled water	10.00 mL

[a] Standard yeast extract for microbiology, Sigma-Aldrich

Table 2-11. Components of the selenite-tungstate solution (see DSMZ medium 385).

Components	Amount
NaOH	0.5 g
$Na_2SeO_3 \times 5\ H_2O$	3.0 mg
$Na_2WO_4 \times 2\ H_2O$	4.0 mg
Distilled water	1 000.0 mL

Table 2-12. Components of the trace element solution SL-10 (see DSMZ medium 320).

Component	Amount
HCl (25 %; 7.7 M)	10.00 mL
$FeCl_2 \times 4\ H_2O$	1.50 g
$ZnCl_2$	70.00 mg
$MnCl_2 \times 4\ H_2O$	100.00 mg
H_3BO_3	6.00 mg
$CoCl_2 \times 6\ H_2O$	190.00 mg
$CuCl_2 \times 2\ H_2O$	2.00 mg
$NiCl_2 \times 6\ H_2O$	24.00 mg
$Na_2MoO_4 \times 2\ H_2O$	36.00 mg
Distilled water	990.00 mL

All media components (solutions A, B, C, D, and F) were prepared separately. Solutions A, B, and C were autoclaved at 121 °C for 20 min while solutions D and F were sterilized by filtration (0.1 µm). The complete medium was purged with N_2-gas for approximately 45 min until almost all oxygen had been removed making the medium almost completely decolorized. The final pH was checked and adjusted to 7.1–7.4 with sterile 5 M HCl.

2.3 End-point measurements

Cu^{2+} determination (spectrophotometric)

For series #4 to #10, the concentration of Cu^{2+} in the outer solution was analyzed after the experiments were terminated to indirectly determine the amount of sulfide formed due to CuS precipitation. The Cu^{2+} concentration was directly measured with a spectrophotometer (UV-2600, UV-VIS spectrophotometer, Shimadzu). For this, the absorption maximum of a 50 mM $CuSO_4$ solution was determined by measuring a spectrum from 500–1 000 nm giving a maximum at approximately at 810 nm and thus, this wavelength was used for the Cu^{2+} concentration measurement. For all measurements, a calibration curve with Cu^{2+} concentrations ranging from 0 to 50 mM was recorded.

After terminating the experimental series, the CuS precipitation attached to the outside of the inner tube was scratched off with a spatula and the inner tube was removed from the outer tube. The outer tube was centrifuged at 3 000 × g for 20 min (Allegra™ 25R Centrifuge, Beckman Coulter™). Then the supernatant was directly analyzed with a spectrophotometer at 810 nm.

CuS determination (gravimetric)

After the spectrophotometric Cu^{2+} determination, the remaining $CuSO_4$ solution was carefully poured through filter paper (weighed before), the remaining precipitation was scratched off the tube wall, and the filter paper was dried at 40 °C overnight. The dry filter papers with the precipitated CuS were weighed again and the difference represented the weight of CuS. This method is named 'filter method' and was applied for series #4, #5, #7, #8 and the detection limit determination. Due to difficulties and inaccuracy, a second method for CuS determination was tested, called the 'tray method'. For this method, the CuS precipitates in the outer tube were scratched off the tube wall. Then the $CuSO_4$ solution with the precipitates was poured in an aluminium tray (weighed before). If necessary, the tube was flushed with MilliQ water to ensure the transfer of all precipitates to the tray. After most of the precipitates settled in the tray, the excess solution was carefully removed with a Pasteur pipette. The trays with the wet CuS precipitates were dried at 60 °C overnight and weighed again. The weight difference represented the weight of CuS. This method was used for series #6, #9, and #10.

The results of the spectrophotometric and both gravimetric methods were compared and discussed.

Analysis of silver plates

Silver plates were added in some of the experiments to evaluate if it could be a technique for detecting formed sulfide as a complement the Cu^{2+}(aq)-solution that requires gaseous H_2S for the detection. Not all silver plates used in the study were analyzed, as the initial results indicated that it did not work. However, a compilation of the results is reported in Chapter 4.

2.4 Determination of the sulfide detection limit

The sulfide detection limit was determined for the experimental setup (Figure 2-1). Therefore, a sulfide stock solution ($Na_2S \times 9\ H_2O$) with a calculated concentration of 10 mM was prepared with oxygen-free MilliQ ultrapure water (adjusted to pH 10 with NaOH) in the anaerobic glovebox. From the stock solution, a 1:10 dilution series was made in the range of 0.001 to 10 mM sulfide (calculated concentration) with the pH-adjusted water. In addition, a negative control with pH-adjusted water was included. Then, 5 mL of each dilution was added to 10 mL of $CuSO_4$ solution (final concentration = 50 mM and final volume = 15 mL, as in the experiments). Each dilution step was measured

in triplicate. The detection limit was verified visually (precipitation), by spectrophotometric Cu^{2+} concentration measurement, and by the gravimetric CuS 'filter method' (see 2.3). The precise concentration of the sulfide stock solution was measured by the methylene blue method (Fisher 1883) at the chemistry lab at Äspö HRL.

2.5 Powder X-ray diffraction (XRD)

In order to verify the nature of the formed copper sulfide and to have a look at the bentonites crystalline composition (mineralogy) XRD was used.

The equipment used was a Panalytical XPert diffractometer using a Cu X-ray tube and a PIXcel detector. A programmable divergence slit was used, and data was typically collected in the interval of 4–80 or 4–100 degrees two theta.

The samples were milled in an agate mortar prior to the measurement, and if needed dried at 60 °C. If the sample amount was low a zero background silicon substrate was used as a sample holder, otherwise, backloaded standard sample holders were used.

2.6 Scanning electron microscopy (SEM)

A Hitachi TM4000 Plus SEM with a Bruker SCU EDS detector (energy dispersive spectroscopy for elemental analysis) was used to study the silver plates used in some of the experiments. The silver plates were cleaned using water, dried, and mounted on conductive tape prior to the insertion to the microscope. Standard settings from the manufacturer optimized for EDS were used.

3 Results and discussion

3.1 Demonstration experiment in a glass cylinder

In the demonstration experiment, it was shown that the indigenous SRB present in a natural bentonite were activated if favorable conditions occur. After approximately 2 months, gas formation was visually observed as a pocket approximately 5 mm above the added gypsum layer in the clay. After 4 months, a $CuSO_4$ solution was added through the septum to the top of the cylinder, instantaneously forming a dark CuS precipitate (Figure 3-1).

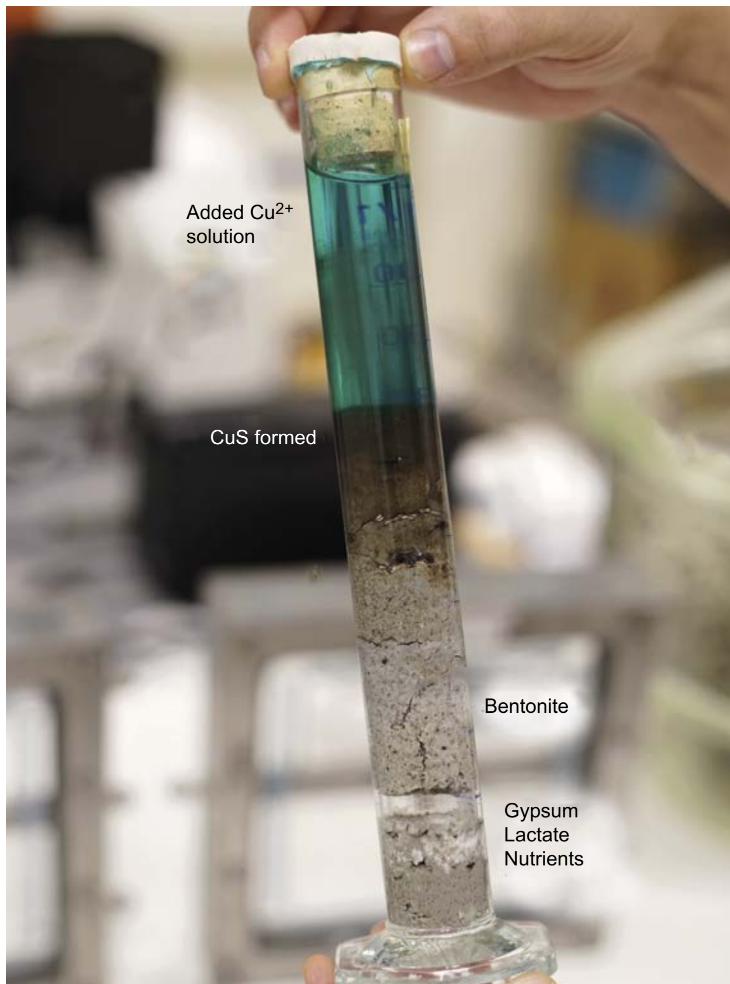


Figure 3-1. *Demonstration experiment showing that addition of gypsum, lactate, nutrients, and liquid water to Wyoming bentonite activates naturally present SRB. Sulfide was detected by addition of a blue copper sulfate solution that resulted in black precipitates in the clay. The picture was taken approximately 11 months after the start of the experiment.*

3.2 Tube and jar experiments in anaerobic glovebox (series #1 and #2)

Series #1 worked as intended. Sulfide was detected in the positive control samples (Table 2-2, A1) as CuS on the outer surface of the inner glass tube as indicated by the arrow in Figure 3-2. Sulfide was formed with or without bentonite but only when liquid water was available to the gypsum-lactate-SRB mixture. In the experiments using no liquid in the inner tube, no sulfide was detected independent of the outer solution (deionized water or saturated salt solution). In addition, no sulfide was detected in the solid material from series #1 by an external laboratory (ALS Scandinavia, www.alsglobal.se) even when CuS was formed in the outer solution.

Series #2 was similar but used larger containers replacing the smaller test tubes in #1. The rationale for the larger jars in #2 was to obtain more sample mass in order to reach above the detection limits of sulfide for analysis. The control samples were unsuccessful in #2 and these data are not further described. The likely cause of the problems was that the anaerobic glovebox did not function optimally during this experiment and that the oxygen levels inhibited SRB from forming hydrogen sulfide. Another explanation could be that there were no viable microbes in the inoculum. Since this series failed, there are no results to present.

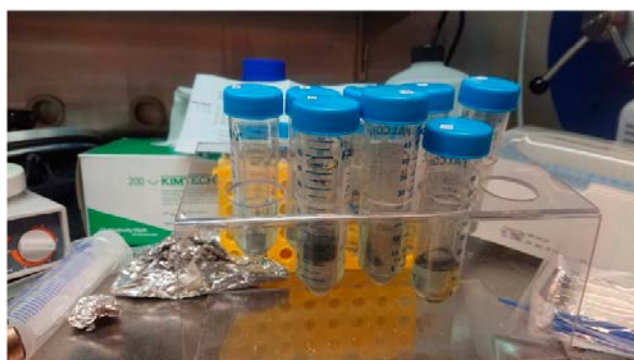


Figure 3-2. *Tube experiments in anaerobic glovebox. An outer tube containing the inner tube (top) and a set of tubes placed in a rack (bottom) in the anaerobic glovebox with* N_2*-atmosphere.*

3.3 Tube experiments in anaerobic glovebox (series #3)

Series #3 was identical to #1 in design, it worked as intended, and the outcome was very similar to series #1. Sulfide was detected in the positive control samples with bacteria and 5 mL medium as CuS on the outer surface of the inner glass tube on the S1 and S2 samples (Table 3-1) when no bentonite was used. On the outside of one S2 tube approximately 5 mg of CuS was formed.

In S3 in which bentonite was added, sulfide was only observed in one of the duplicates (Figure 3-3). On the outside of one S3 tube, approximately 0.5 mg of CuS was formed while no CuS formed on the other S3 tube. However, microbial activity was seen in both of the duplicates as a dark region in the wet bentonite that was not present at the start of the experiment (Figure 3-3). The CuS phase was identified as covellite using powder X-ray diffraction (Figure 3-4).

Table 3-1. Summary of the results from series #3. Duration: 3 months.

			Inner tube				Outer tube		
No.	CuS formation	~RH (%)[a]	Bentonite (g)	Gypsum (mg)	*P. aespo.*[c]	Sterile medium (mL)	Salt[d]	$CuSO_4$ (mg)	Deionized water (mL)
S1	Yes	100	-	60	+	5	-	100	15
S2	Yes	100	-	60	+	5	-	100	15
S3	Yes in one of the two replicates	100	2	60	+	5	-	100	15
S4	no	100	2	60	+	0.5	-	100	15
S5	no	97[b]	2	60	+	0.5	K_2SO_4	100	15
S6	no	76[b]	2	60	+	0.5	NaCl	100	15

[a] Relative humidity. [b] Choudhury et al. (2011), [c] 100 µL inoculum from culture in late log phase, [d] Supersaturated.

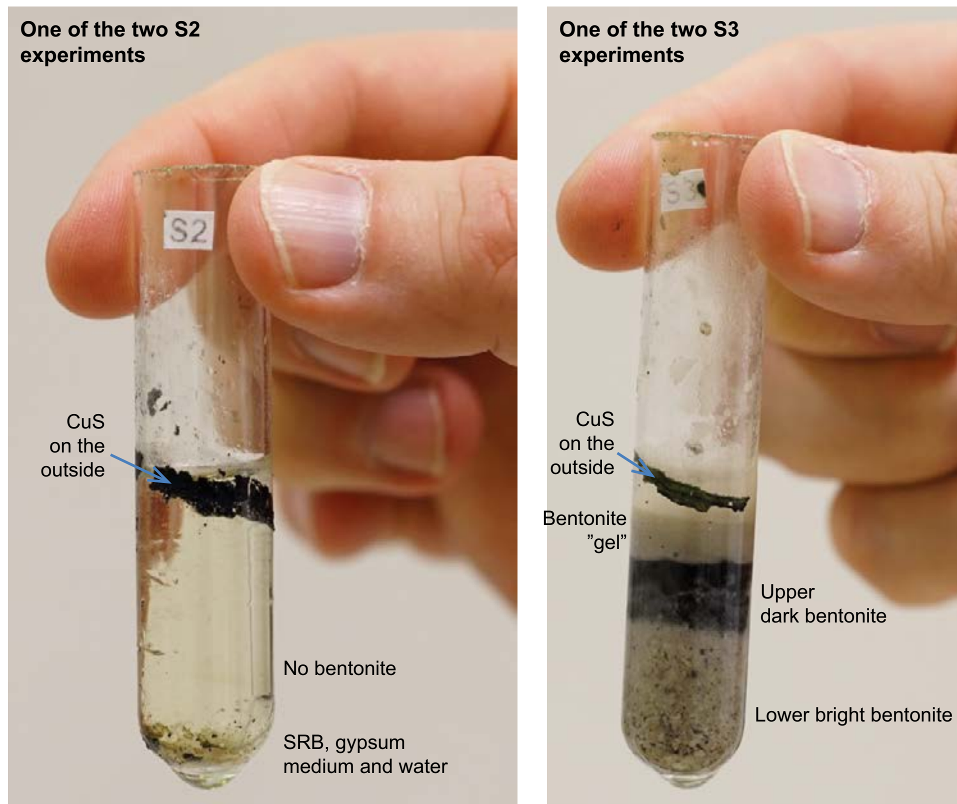


Figure 3-3. *Two inner tubes from Series #3. To the left: visual observation of copper sulfide formed on the outside of the inner tube by the reaction of divalent copper ions with hydrogen sulfide in the S2 tube. To the right: copper sulfide formed on the outside of the inner tube of the S3 tube shown. Additionally a dark section in the bentonite formed with expected microbiological activity.*

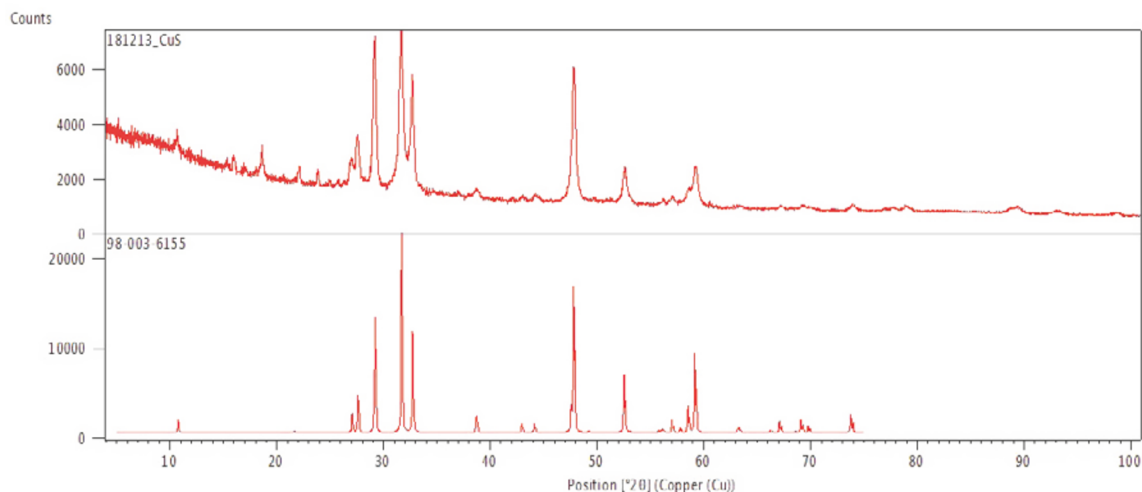


Figure 3-4. *Powder X-ray diffractograms (XRD). Top: S2 (series #3) CuS sample confirming the covellite (CuS) phase. Bottom: Calculated pattern of covellite using Panalytical High Score software and database.*

In S4–S6 (Table 2-3), only 0.5 mL liquid was added in the inner tube and no sulfide formation was detected. However, the uppermost 2–3 mm of the bentonite had a somewhat darker tone, which was expected to originate from the higher water content in the clay from the high RH of the gas phase.

A possible explanation for the smaller amount of CuS formed in S3 (compared to S1 and S2) is that the wet bentonite reacted with the some of the produced sulfide. Removal of sulfide by bentonite has been observed by Svensson et al. (2017) and the cause may be oxidation of the sulfide by redox active Fe(III) in the montmorillonite. No sulfide could be detected in S4–S6 (Table 3-1), regardless whether the outer solution contained pure or salt water. These results indicated that there was no threshold moisture limit and that SRB need liquid water to form sulfide from the added gypsum and lactate, at least during the time scale of two months. Other explanations could be that sulfide was already formed and precipitated in the bentonite or that sulfide formation was extremely slow under these conditions. However, during unsaturated conditions the bentonite is very porous and is not expected to trap hydrogen sulfide gas as effectively as a wet bentonite gel that has relatively low gas permeability. Hence, the lack of CuS-formation in these experiments likely reflects an absence of the sulfate reduction reaction. The pH of the bentonite-water solution is expected to be fairly alkaline that favors HS^-(aq) over H_2S (g). However, the reaction forming CuS in the outer solution is extremely fast and CuS is very stable, this drives the equilibria from HS^- ð H_2S ð CuS.

The bright and dark bentonite volumes of samples S2 and S3 were dried and ground (Figures 3-3 and 3-5) and no significant difference could be seen in the XRD patterns (Figure 3-6). The differences in the 001 reflections arise most likely from uneven drying of the clays, and both clays show comparable amounts of gypsum present (based on the relative intensity of the main gypsum reflection and the 4.5 Å montmorillonite reflection).

Figure 3-5. *Dried and ground bentonite from S3 tube from Series #3. To the left: the dark bentonite with expected microbial matter. To the right: bright bentonite from the bottom of the tube.*

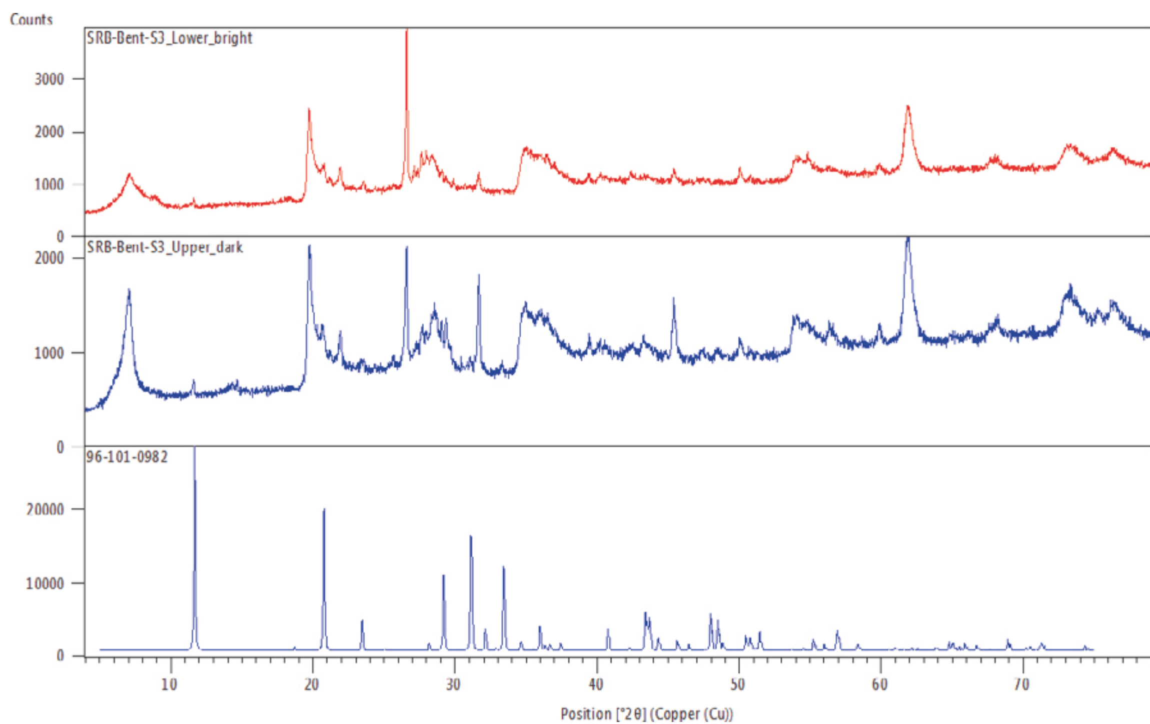


Figure 3-6. *Powder X-ray diffractograms (XRD) of S3 (series #3) bentonite samples. Top diagram is from the bright bentonite, the middle diagram is from the dark bentonite, and the bottom is a calculated diffraction pattern of gypsum for comparison.*

3.4 Tube experiments in anaerobic glovebox with *P. aespoeensis* and groundwater cells (series #4 and #5)

For series #4 that was performed with *P. aespoeensis* (inoculum 1×10^6 cells mL^{-1}), sulfide production (black precipitate in outer $CuSO_4$ solution and in bentonite in inner tube) was observed only for the T2 treatment with bentonite and excess liquid (Table 3-2). The Cu^{2+} concentration in the outer solution of T2 treatment tubes decreased by 1.2 mM compared to the T4 control treatment (Figure 3-7). CuS precipitates were below detection limit. There was no evidence of sulfide formation in the treatment T1 with excess liquid but without bentonite (Table 3-2) as found in series #3 (Table 3-1, S1 and S2). This might be due to the different liquid composition in both experiments – while the whole liquid consisted of sterile media in series #3, in series #4 the amount of media was kept constant for all treatments (0.5 mL) and the excess liquid consisted of physiological NaCl solution that did not contain substrates or nutrients. The additional amount of substrates and nutrients in series #3 might have stimulated microbial growth and activity compared to series #4. As in series #3, the no liquid water treatment T3 and the control (T4) did not show any sulfide production (Table 3-2).

In series #4, a silver plate that was placed directly in the bentonite (inner tube) was tested as a more sensitive sulfide formation indicator. However, the silver plates in almost all treatments (except T1) turned blackish (Figure 3-8). The $CuSO_4$ solution in the outer tube did not show any precipitation at this time point. The empty layer is likely due to gas formation.

Series #5 with the groundwater cell concentrates was started with inocula of approximately 7×10^4 cells mL^{-1} for both Äspö HRL KA3105A:3 and KA2511A:5 boreholes. However, the results differed between the two boreholes. For KA3105A:3, a slight sulfide production was observed in the outer tube as well as gas formation for the excess liquid treatment T2 (Table 3-2). The decrease in Cu^{2+} concentration in the outer solution was 1.4 mM compared to the T4 and T5 control treatments (Figure 3-7), whereas CuS weight was below detection limit (Figure 3-9). In contrast, there was a strong sulfide production observed for KA2511A:5 in T2 (outer and inner tube) and also in T1 (outer tube) (Table 3-2). The Cu^{2+} concentration in the outer solution decreased by 11.3 and 3.7 mM in T2 and T1, respectively compared to the control treatment T4 and T5 (Figure 3-9). CuS weight was 4.5 mg for the T2 treatment. No sulfide production was observed in the no liquid treatment T3 or the control treatments T4 and T5 for both borehole cell concentrates.

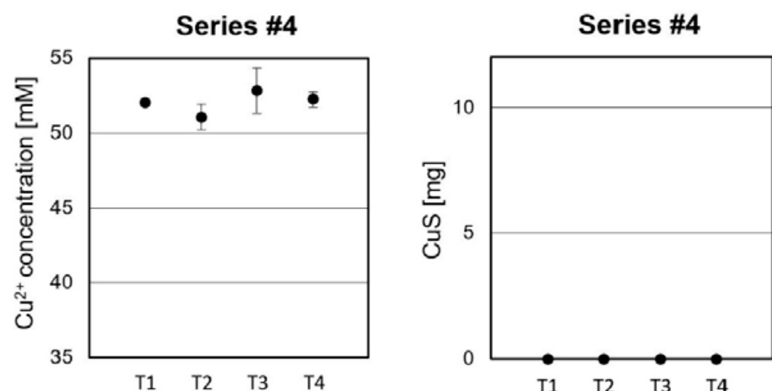


Figure 3-7*. Cu^{2+} concentrations (left; spectrophotometric method) and CuS weight (right; 'filter method') measured at the end of series #4 for the T1 to T4 treatments. Error bars indicate the standard deviation (n = 4).*

Figure 3-8. *Darkening of the Ag-plate emplaced in the top part of the bentonite clay.*

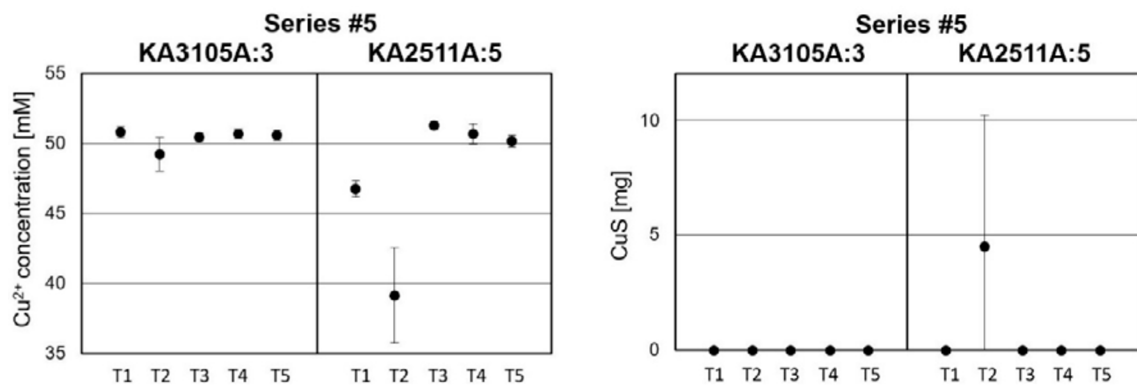


Figure 3-9. *Cu^{2+} concentrations (left; spectrophotometric method) and CuS weight (right; 'filter method') measured at the end of series #5 for the treatments T1 to T5. Error bars indicate the standard deviation (n = 4).*

The first evidence of sulfide production was observed after approximately one month for *P. aespoeensis* and the cell concentrate from borehole KA2511A:5, whereas it took almost two months for KA3105A:3. Similarly, the amount of sulfide formed during the experiment differed between the pure strain and the natural microbial cells as well as between the two boreholes. The cell numbers of the inocula were similar for the two boreholes, but their microbial community compositions differ as shown by a Beta diversity (Bray-Curtis dissimilarity) of 0.6 (Lopez-Fernandez et al. 2018a). This shows that the speed and amount of sulfide production probably depend on the composition of the groundwater microbial communities. In addition, the natural microbial groundwater community might have a longer reactivation time as compared to the already actively growing culture of *P. aespoeensis* indicating that the metabolic state of the microbial cells might also influence the sulfide production rate. Furthermore, the natural microbial community consists of populations with a range of growth strategies compared to the pure culture of sulfate reducing *P. aespoeensis* (Wu et al. 2016). Therefore, despite starting the experiments with similar total cell numbers, the proportion of SRB was likely lower for the groundwater enrichment and can require more time for sulfide production to become evident.

Table 3-2. Visual observations (precipitation, gas formation, and silver plate) for series #4 and #5: – negative, (+) slightly positive, + positive, ++ strongly positive, NA not applicable.

Series	Treatment	Precipitation (outer tube)	Precipitation (inner tube)	Gas formation	Ag plate color change
#4	T1	-	-	NA	-
	T2	+	+	+	+
	T3	-	-	-	+
	T4 (control)	-	-	-	+
#5 (KA3105A:3)	T1	(+)[a]	-	NA	NA
	T2	(+)	-	+	NA
	T3	-	-	-	NA
	T4 (control)	-	-	-	NA
	T5 (control)	-	-	-	NA
#5 (KA2511A:5)	T1	++	-	NA	NA
	T2	++	++	+	NA
	T3	-	-	-	NA
	T4 (control)	-	-	-	NA
	T5 (control)	-	-	-	NA

[a] Positive for one replicate out of four.

3.5 Long-term tube experiments in anaerobic glovebox (series #6)

The long-term series (#6) with *P. aespoeensis* (inoculum 1×10^6 cells mL^{-1}) ran for one year and included treatments with different liquid content and RH (Table 2-6). Sulfide production was only observed for the excess liquid treatment T2 in the outer and inner tube (Table 3-3). The Cu^{2+} concentration in the outer solution decreased by 2.2 mM in T2 compared to the control treatments T6 to T8 (Figure 3-10). The CuS weight was 1.8 mg for the T2 treatment. The Cu^{2+} concentrations for treatments T4 and T5 seem to be also reduced but the addition of another salt to set the RH (i.e. potassium sulfate and sodium chloride, respectively; Table 2-6) changed the color of the solution and therefore, biased the spectrophotometric measurement. Nevertheless, the visual observation clearly showed no formation of a black precipitates, neither in the outer nor in the inner tube (Table 3-2). This series was set up with one silver plate in one replicate for each treatment. The color of all silver plates (except for treatment T1 without bentonite) turned from a shiny surface to blackish.

There was no evidence of sulfide formation in the treatments T3 to T5 with varying RH or in the control treatments T6 to T8 (Table 3-3).

Overall, the results are in line with series #4 and even after a longer experiment duration, sulfide production seems to only occur in the presence of excess water (and not a high RH). For the positive treatment (T2), the sulfide production reaches a steady state after approximately one to two months, potentially as the substrate and/or nutrients are depleted and were not refilled (media). However, microbial sulfide production in a repository environment might continue to be supported by a very low but continuous supply of substrates and nutrients with groundwater inflow.

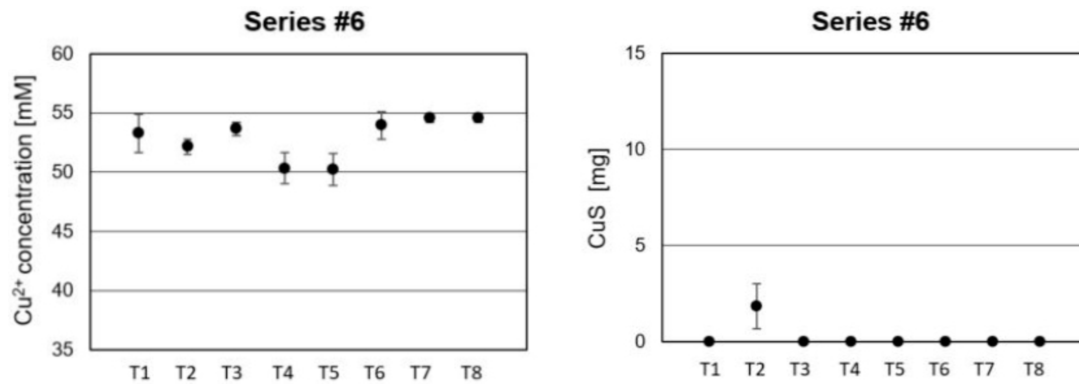


Figure 3-10*. Cu^{2+} concentrations (left; spectrophotometric method) and CuS weight (right; 'tray method') measured at the end of series #6 for the treatments T1 to T8. Error bars indicate the standard deviation (n = 6).*

Table 3-3. Visual observations (precipitation, gas formation, and silver plate) for series #6 after seven months of incubation: – negative, (+) slightly positive, + positive, ++ strongly positive.

Series	Treatment	Precipitation (outer tube)	Precipitation (inner tube)	Gas formation	Ag plate color change
#6	T1	-	-	-	-
	T2	+	+	+	+
	T3	-	-	-	+
	T4	-	-	-	+
	T5	-	-	-	+
	T6 (control)	-	-	-	+
	T7 (control)	-	-	-	+
	T8 (control)	-	-	-	+

3.6 Tube experiments in anaerobic glovebox with *P. aespoeensis* and elemental sulfur and sulfite (series #7 and #8)

As sulfate is not the only potential electron acceptor that can result in sulfide production, series #7 and #8 investigated *P. aespoeensis* growth (inoculum 2×10^5 cells mL^{-1}) with alternative electron acceptors. For series #7, elemental sulfur served as electron acceptor (instead of gypsum). Sulfide production was observed in both excess liquid treatments T1 (without bentonite) and T2 (with bentonite) (Table 3-4). However, the precipitation was less pronounced in the T1 treatment and the Cu^{2+} concentration in the outer solution decreased by 0.7 and 2.9 mM in T1 and T2, respectively compared to the control treatment T4 (Figure 3-11). CuS weight was below detection limit for all treatments.

In addition, series #8 tested *P. aespoeensis* growth with sulfite as electron acceptor. In this experiment, sulfide production was only observed in treatment T2 but was more pronounced compared to series #7 with a decrease in Cu^{2+} concentration in the outer solution of 7.5 mM compared to the control treatment T4 (Figure 3-11).

No sulfide production was observed in the no liquid treatment T3 or the control treatments T4 for series #7 and #8 (Table 3-4).

Both series #7 and #8 show that *P. aespoeensis* was able to use alternative electron acceptors such as elemental sulfur and sulfite in the presence of water. The sulfide production from these alternative acceptors was likely to be higher than compared to sulfate reduction as in the highly oligotrophic conditions (Lopez-Fernandez et al. 2018a) elemental sulfur and sulfite only need six and two electrons, respectively compared to eight electrons for complete reduction of sulfate to sulfide.

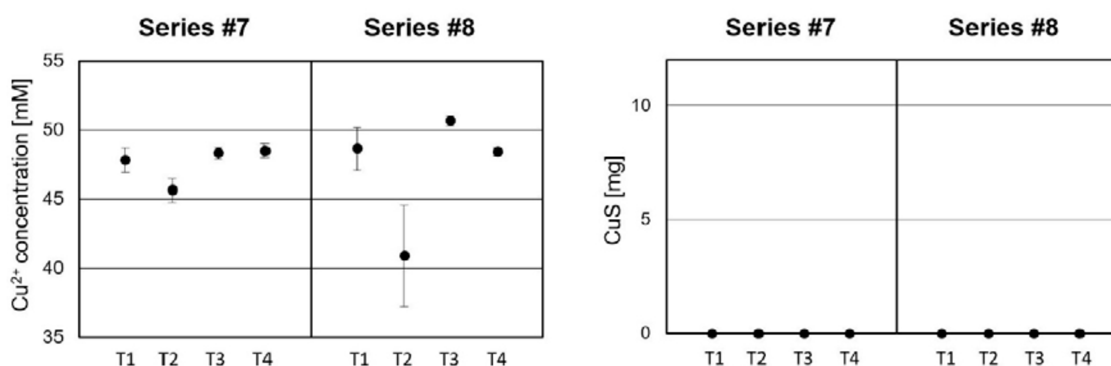


***Figure 3-11**. Cu^{2+} concentrations (left; spectrophotometric method) and CuS weight (right; 'filter method') measured at the end of series #7 and #8 for the treatments T1 to T4. Error bars indicate the standard deviation (n = 4).*

Table 3-4. Visual observations (precipitation, gas formation, and silver plate) for series #7 and #8: – negative, (+) slightly positive, + positive, ++ strongly positive, NA not applicable.

Series	Treatment	Precipitation (outer tube)	Precipitation (inner tube)	Gas formation	Ag plate color change
#7 (S^0)	T1	(+)	(+)[a]	NA	NA
	T2	+	+	+	NA
	T3	-	-	-	NA
	T4 (control)	-	-	-	NA
#8 (SO_3^{2-})	T1	-	-	-	NA
	T2	+	++	+	NA
	T3	-	-	-	NA
	T4 (control)	-	-	-	NA

[a] Positive for one replicate out of four.

3.7 Tube experiments in anaerobic glovebox with groundwater cells and elemental sulfur and sulfite (series #9 and #10)

In series #9 and #10, the ability of using alternative electron acceptors such as elemental sulfur (#9) and sulfite (#10) for sulfide production was investigated for natural microbial communities from groundwaters at Äspö HRL (boreholes KA3105A:3 and KA2511A:5). Despite that the cell number of the inocula were below detection limit for both groundwaters (i.e. $< 1 \times 10^4$ cells mL^{-1}), the results showed that the cell concentration method worked. For elemental sulfur (series #9), sulfide production was observed for both boreholes in treatments T1 (without bentonite) and T2 (with bentonite), but was less pronounced for KA3105A:3 (Table 3-5). The Cu^{2+} concentration in the outer solution decreased by 5.2 mM in T2 compared to the control treatments T4 and T5 for KA3105A:3 and by 2.3 and 5.6 mM in treatments T1 and T2, respectively for KA2511A:5 (Figure 3-12). The CuS weight was 0.75 mg for treatment T1 (KA3105A:3). For KA2511A:5, the CuS weights were 3.8 mg and 4.5 for the treatments T1 and T2, respectively.

Little sulfide production (except for precipitation in the outer tube for one replicate in T1) was observed for sulfite as electron acceptor (series #10) for cells derived from borehole KA3105A:3 that contrasted with strong sulfide production for the T1 and T2 treatments with KA2511A:5 borehole water. For KA2511A:5, the Cu^{2+} concentration in the outer solution decreased by 4.2 and 4.3 mM and the CuS weights were 4.5 mg and 7.0 mg for the treatments T1 and T2, respectively (Figure 3-13).

Finally, no sulfide production was observed in the no liquid treatment T3 or the control treatments T4 and T5 for both electron acceptors and both boreholes (Table 3-5).

These data matched a metagenomic study where genomes of both elemental sulfur and sulfite reducing microbes were reconstructed from the Äspö HRL groundwaters (Wu et al. 2016). The results also showed that the ability of using alternative electron acceptors depends on water availability and that in the presence of liquid water, sulfide production depends on and varies with the microbial community composition (see 3.4).

Table 3-5. Visual observations (precipitation, gas formation, and silver plate) for series #9 (S^0) and #10 (SO_3^{2-}): – negative, (+) slightly positive, + positive, ++ strongly positive, NA not applicable.

Series	Treatment	Precipitation (outer tube)	Precipitation (inner tube)	Gas formation	Ag plate color change
#9 (KA3105A:3)	T1	(+)	+[a]	NA	NA
	T2	+	+	+	NA
	T3	-	-	-	NA
	T4 (control)	-	-	-	NA
	T5 (control)	-	-	-	NA
#9 (KA2511A:5)	T1	+[b]	+[a]	NA	NA
	T2	++	+	+	NA
	T3	-	-	-	NA
	T4 (control)	-	-	-	NA
	T5 (control)	-	-	-	NA
#10 (KA3105A:3)	T1	+[a]	-	NA	NA
	T2	-	-	(+)	NA
	T3	-	-	-	NA
	T4 (control)	-	-	-	NA
	T5 (control)	-	-	-	NA
#10 (KA2511A:5)	T1	++[b]	-	NA	NA
	T2	++	++	+	NA
	T3	-	-	-	NA
	T4 (control)	-	-	-	NA
	T5 (control)	-	-	-	NA

[a] Positive for one replicate out of four. [b] Positive for three replicates out of four.

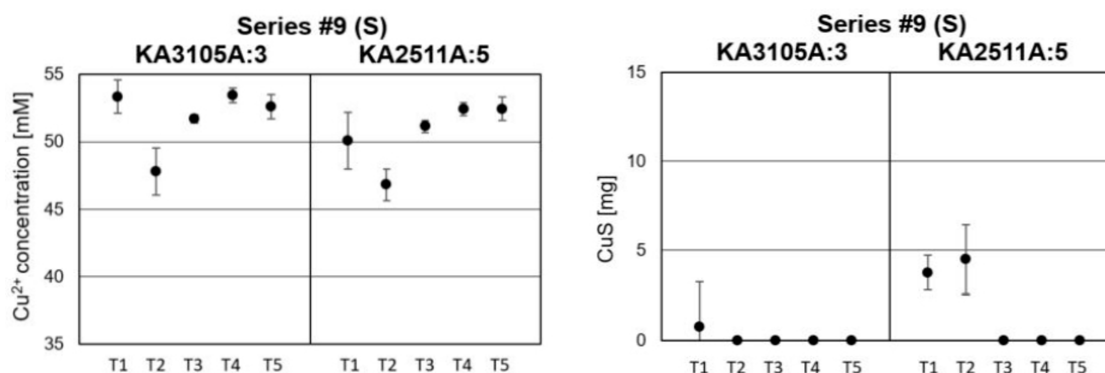


***Figure 3-12**. Cu^{2+} concentrations (left; spectrophotometric method) and CuS weight (right; 'tray method') measured at the end of series #9 for the treatments T1 to T5. Error bars indicate the standard deviation (n = 4).*

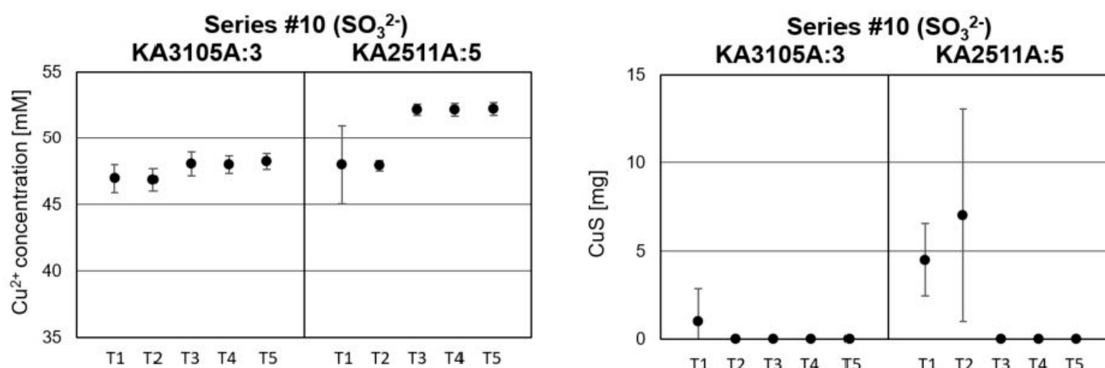


***Figure 3-13**. Cu^{2+} concentrations (left; spectrophotometric method) and CuS weight (right; 'tray method') measured at the end of series #10 for the treatments T1 to T5. Error bars indicate the standard deviation (n = 4).*

3.8 Determination of the sulfide detection limit

In a tube experiment in the anaerobic glovebox, the detection limit of the experimental setup was determined. Different concentrations of sulfide solution ranging from 0 to 1 000 µM were added to $CuSO_4$ solution (as in the outer tube of the experimental series #4 to #10). The lowest concentration that led to a clearly visible precipitation was 100 µM sulfide (Figure 3-14). This observation was in line with the spectrophotometric measurement. The Cu^{2+} concentration was constant for an addition of 0 to 10 µM sulfide and dropped between 10 and 100 µM by 0.4 mM (Figure 3-15). However, for the CuS method there was an increase by 0.7 mg for 10 µM sulfide, but the CuS weight was below detection limit for 100 µM sulfide. For 1 000 µM sulfide the weight was 5 mg.

***Figure 3-14**. Detection limit experiment: The tubes contain $CuSO_4$ solution and different concentrations of sulfide solution. The numbers (0 to 1 000) represent the concentration of the added sulfide solution in µM. With increasing sulfide concentration a pronounced precipitation (CuS) and decolorization of the $CuSO_4$ solution was observed.*

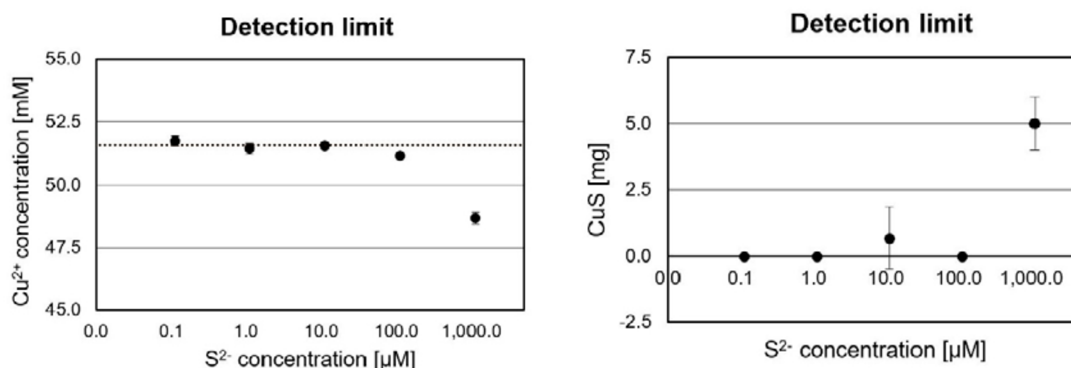


***Figure 3-15**. Cu^{2+} concentrations (left; spectrophotometric method) and CuS weight (right; 'filter method') plotted against the S^{2-} concentration for the detection limit experiment. Error bars indicate the standard deviation (n = 3). The dotted line represents the Cu^{2+} concentrations of the negative control.*

3.9 Comparison of spectrophotometric Cu^{2+} and CuS determination

The results of series #4 to #10 and the detection limit experiment showed that the spectrophotometric Cu^{2+} determination method was more precise (lower standard deviation) and had a higher sensitivity compared to the CuS weight method. This might be partly related to the protocol used for the CuS method and the experimental design that made it very difficult to remove the CuS precipitate from the outer tube because it was strongly attached to the tube wall. However, the results for the gravimetric 'tray method' show that this method seemed to be an improvement in comparison to the 'filter method' because the data matches better to the results of the spectrophotometric measurement.

4 Evaluation of silver plates with SEM/EDS

The silver plates were added as a technique for potentially detecting formed sulfide as a complement the $Cu^{2+}_{(aq)}$-solution that requires gaseous H_2S for the detection.

The silver plates were shiny prior to the experiment, while afterwards they typically looked dull and darker (Figure 4-1).

The dark spots or regions were initially assumed to be sulfide but were confirmed to be a clay-type material and/or oxides high in Si, Al, and O while low in S (Figure 4-2b). This was most likely mainly montmorillonite fixed or attached to the silver surface. This observation made us falsely to draw the initial conclusion that the plates were unsuitable for observation of sulfide production and hence, they were not further analyzed for most of the remaining time of the project. For this reason, not all silver plates used in the study were analyzed with SEM/EDS. However, when comparing a reference plate with the exposed silver plate from treatment T2 of series #4 it was clear that the background level of sulfur on the plate from the experiment was higher than the reference (Figure 4-2a). This was interpreted as S attached to the silver surface. However, the form of the sulfur phase from this data alone was unclear, but was most likely a silver sulfide. Additionally, in some parts (e.g. Figure 4-2), enrichment of chloride was seen on the EDS maps. Upon higher magnification (400–2 500 fold), cubic crystals were observed (Figure 4-2c) that EDS analysis confirmed to be AgCl. The sample was stored outside of the glovebox for approximately six months prior to SEM/EDS analysis and it cannot be ruled out that the oxidation of silver occurred during storage. Hence, the use of silver plates for detection of sulfide production seems possible if evaluated with a chemical method such as SEM/EDS. Visual inspection of the silver plates is not enough, as the darkening of the silver plate is not necessarily a sign of sulfide production, but can also be due to other factors.

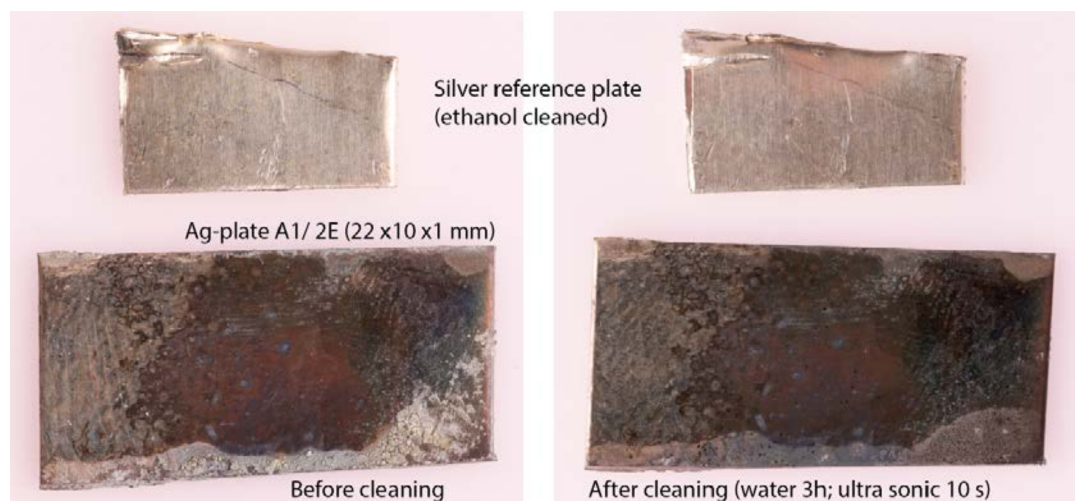


Figure 4-1. *Visual appearance of investigated silver plates before and after cleaning in water and some minor ultra-sonic treatment.*

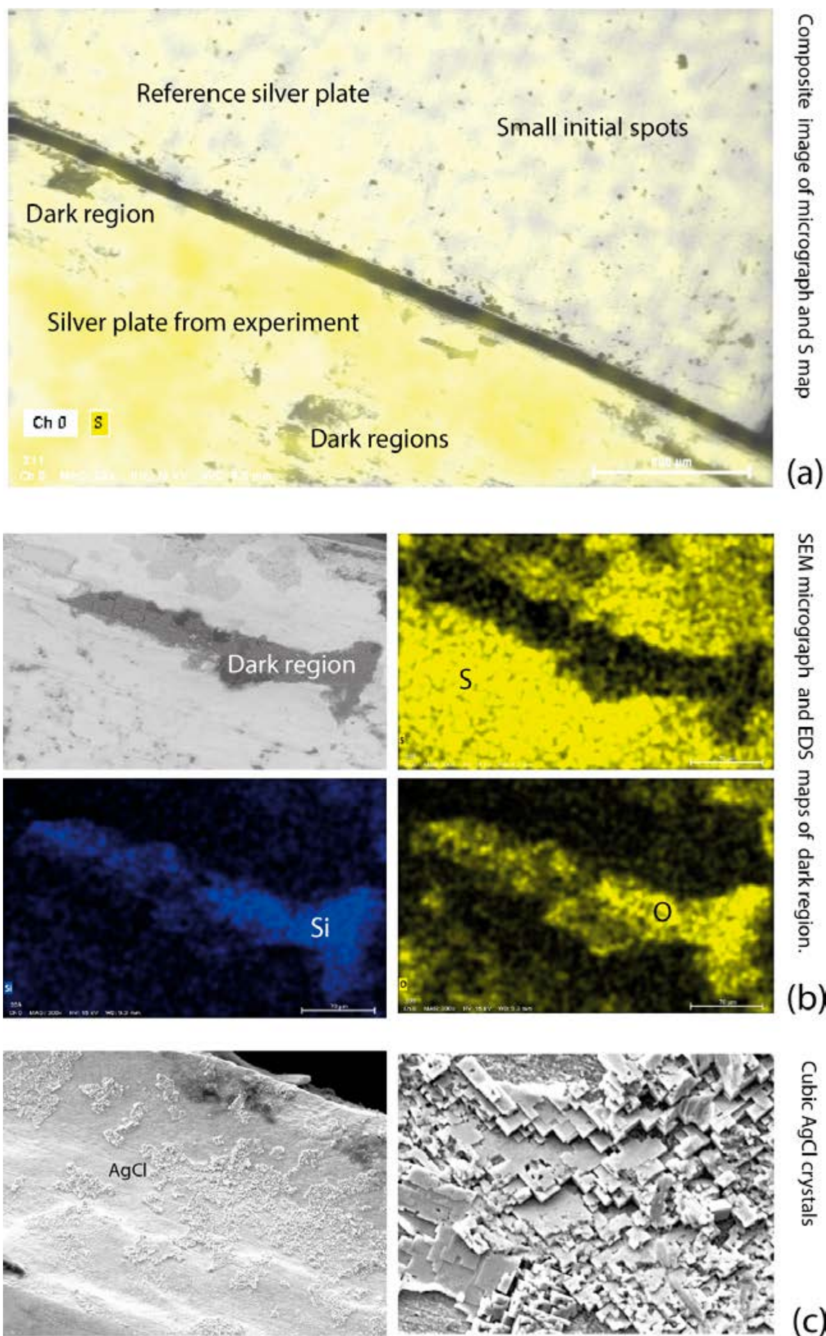


Figure 4-2. *SEM/EDS investigation of silver plate from series #4/T2 (a) Elemental map of sulfur (S) comparing the reference silver plate to plate from experiment. Scale bar is 900 µm. (b) Micrograph and EDS maps of dark region. Scale bar is 70 µm. (c) silver chloride crystals on the surface of the series #4/T2 silver plate at 400 (left) and 2 500× (right) magnification.*

5 Conclusions

Microbiological reduction of sulfate to sulfide (hydrogen sulfide) was detected when the pure and mixed microbial cultures had access to liquid water (in addition to gypsum, lactate, nutrients, and no swelling pressure); while no sulfide production was detected when the microbes were restricted to a RH of 75–100 %. However, it cannot be ruled out that sulfate reduction may occur when microbes are in a high RH environment without access to liquid water, although at a very low reaction rate, making it undetectable at the time scale used in this study.

The natural microbial community enrichments also produced sulfide, but sometimes at a lower rate than the *P. aespoeensis* pure culture. This was likely due to community members with alternative growth strategies (such as nitrate reduction and fermentation) lowering the number of SRB added to the experiment that also competed for electron donor. These results match data from culture free, 'omics' community reconstructions (Wu et al. 2016).

This study focuses on lactate as an organic carbon and energy source. However, it does not investigate the Äspö HRL populations known to be present and active that are able to oxidize alternative electron donors such as hydrogen gas (Lopez-Fernandez et al. 2019). It is possible that these populations are more tolerant to low relative water humidity than the heterotrophic species enriched here (Stone et al. 2016).

In the demonstration experiment that was conducted in a glass cylinder, it was possible to activate indigenous SRB in Wyoming bentonite by the addition of lactate and gypsum. As gypsum is naturally present in the bentonite, evidently these microbes have not converted the gypsum to sulfides even though the bentonite has been wet many times through history. This may be due to that the present SRB are restricted by the lack of available energy sources in the bentonite and hence, sulfate reduction will not occur unless e.g. lactate or any other energy source is added.

The detection of sulfide was negatively affected by the bentonite present, as bentonite by itself interacts with sulfide.

In one of the samples without bentonite (series #3; S2), 5 mg CuS was formed while with bentonite 0–0.5 mg CuS was formed (series #3; S3 samples). This effect is not expected to have had a significant impact on the dry bentonite samples that were only in contact with high RH, as the dry bentonite has a high porosity allowing any $H_2S(g)$ formed to escape more easily compared to a wet dense bentonite gel. Sulfide formation was mainly studied using a gas trap for formed $H_2S(g)$. However, the use of silver plates was introduced in later experiments possibly also allowing detection of liquid phase sulfide such as dissolved sulfide. However, this method needs to be verified.

In some of the experiments, silver plates were also added as a potential indicator of sulfide production. Silver sulfide was expected to be seen by the darkening of the plates. However, with SEM/EDS it was observed that the darkening was not necessarily an effect from sulfide production, but instead indicated minor remains of montmorillonite on the surface. Hence, it was more problematic that first expected, but still detection of sulfide was possible when the background level of sulfide was compared between the plate from the experiment to a reference silver plate. Additionally, silver chloride crystals were found on the silver plate from the experiment, possibly this phase was formed during the storage of the sample with access to some oxygen.

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