SKB

The effects of biofilm formation on deep groundwater chemistry and microbial sulfide production

Summary report

Andreas Bengtsson

Anders Blom

Björn Hallbeck

Trevor Taborowski

Linda Johansson

Jessica J. Morsing

Alexandra Chukharkina

Karsten Pedersen

SVENSK KÄRNBRÄNSLEHANTERING

The effects of biofilm formation on deep groundwater chemistry and microbial sulfide production

Summary report

Andreas Bengtsson, Anders Blom, Björn Hallbeck,
Trevor Taborowski, Linda Johansson, Jessica J. Morsing,
Alexandra Chukharkina, Karsten Pedersen

Microbial Analytics Sweden AB

Abstract

This project which included two parts with almost identical experimental setups were carried out during 2017 and 2018 (part 1 and part 2). The aim of the projects was to investigate effects of biofilm formation on deep groundwater chemistry and microbial sulfide production.

Flow Cell Field Circulation Systems (FCFCS's) that contained support for biofilm formation (crushed rock garnets) was compared to FCFCS's without biofilm support. All systems were filled with groundwater from borehole KFM03A:4, section 633.5–650 m in Forsmark that circulated in the systems for approximately two months. Sulfide, acetate, adenosine tri-phosphate (ATP), organic content, Eh, H_2 and nutrients, major components and metals were then analysed from the circulating water and the formed biofilm. Concentration of sulfide was analysed continuously during the circulation phase. To boost the systems, lactate (part 1) and acetate and H_2 (part 2) were added to the FCFCS's.

For part 1, the sulfide concentration in the FCFCS's with biofilm increased rapidly after the lactate addition while the system without biofilm remained at concentrations close to zero. At the end of the experiment approximately ten times more sulfide per liter groundwater were observed in the system with biofilm compared to the system without biofilm.

Less sulfate and lactate remained in the system with biofilm compared to the system without biofilm at the end of the experiment. However, more lactate was consumed than could be explained with the produced sulfide, indicating that other types of bacteria than SRB (sulfate-reducing bacteria) was metabolically active in the systems. The circulating water from both of the FCFCS's were similar in ATP content and extractable organic substances. The biofilm however had approximately 70 percent more ATP per unit and a completely different organic substance profile. The nutrients, major components and metals did not differ significantly between the original groundwater and after the experiment in the both systems except in turbidity and sulfate content where the system with biofilm was different from the groundwater and the system without biofilm.

For part 2, the sulfide concentration in the FCFCS's increased rapidly after the acetate and H_2 addition, however the increase was faster in the system with biofilm support compared to the system that contained only groundwater. At the end of the experiment approximately 0.65 mM more sulfide was observed in the system with biofilm compared to the system without biofilm.

Less H_2 and more ATP was observed in the system with biofilm compared to the system without biofilm at the end of the experiment. However, similar amounts of acetate were consumed in both systems, indicating that other types of bacteria than SRB (sulfate-reducing bacteria) were metabolically active in the systems (no bacterial phylogeny was done). The biofilm had significant amounts of ATP in the attached biomass and the circulating water of the same system had twice as much ATP as the FCFCS without biofilm support. In addition, the biofilm had a completely different organic substance profile, analysed with GC-MS, compared to the waters of the two FCFCS's. The nutrients, major components and metals did not differ significantly between the original groundwater and in the groundwater after the experiment in the both systems except for manganese, molybdenum and sulfate content.

Both experiments showed that microbial biofilm formation did not influence the surrounding water significantly in terms of organic substances or element and metals. The bacteria in the biofilm itself seemed also to be more metabolically active than the planktonic cells indicated by the high ATP content analysed from the biofilm support material and also the significantly elevated sulfide production.

Sammanfattning

Detta projekt som bestod av två delar med nästan identiska experimentella uppsättningar genomfördes under 2017 och 2018 (del 1 och del 2). Syftet med projekten var att undersöka effekterna av biofilmsbildning på vattenkemi för djupa grundvatten och mikrobiell sulfidproduktion.

Flödescellfältcirkulationssystem (FCFCS) som innehöll stöd för bildning av biofilm (krossat berg) jämfördes med FCFCS utan biofilmstöd. Alla system fylldes med grundvatten från borrhål KFM03A:4, sektion 633.5–650 m i Forsmark som cirkulerade i systemen i cirka två månader. Sulfid, acetat, adenosin-tri-fosfat (ATP), organiskt innehåll, Eh, H_2, näringsämnen, kemiska föreningar och metaller analyserades sedan från det cirkulerande vattnet och den bildade biofilmen. Koncentration av sulfid analyserades kontinuerligt under cirkulationsfasen. För att påskynda aktiviteten i systemen tillsattes laktat (del 1) och acetat och H_2 (del 2).

För del 1 ökade sulfidkoncentrationen i systemet med biofilm snabbt efter tillsatsen av laktat medan systemet utan biofilm förblev i koncentrationer nära noll. I slutet av experimentet observerades ungefär tio gånger mer sulfid per liter grundvatten i systemet med biofilm jämfört med systemet utan biofilm.

Mindre sulfat och laktat återstod i systemet med biofilm jämfört med systemet utan biofilm i slutet av experimentet. Emellertid konsumerades mer laktat än vad som kunde förklaras med mängden producerad sulfid, vilket indikerar att andra typer av bakterier än SRB (sulfatreducerande bakterier) var metaboliskt aktiva i systemen. Det cirkulerande vattnet från båda systemen hade både liknande ATP-innehåll och extraherbara organiska ämnen. Biofilmen hade dock ungefär 70 procent mer ATP per enhet och en helt annan organisk substansprofil. Näringsämnen, kemiska föreningar och metaller skilde sig inte väsentligt mellan det ursprungliga grundvattnet och det efter experimentet i något av systemen utom i grumlighet och sulfatinnehåll där systemet med biofilm skilde sig från grundvattnet och systemet utan biofilm.

För del 2 ökade sulfidkoncentrationen i systemen snabbt efter tillsats av acetat och H_2, men ökningen var snabbare i systemet med biofilmstöd jämfört med systemet som endast innehöll grundvatten. I slutet av experimentet observerades ungefär 0.65 mM mer sulfid i systemet med biofilm jämfört med systemet utan biofilm.

Mindre H_2 och mer ATP observerades i systemet med biofilm jämfört med systemet utan biofilm i slutet av experimentet. Emellertid konsumerades liknande mängder av acetat i båda systemen, vilket tyder på att andra typer av bakterier än SRB (sulfatreducerande bakterier) var metaboliskt aktiva i systemen (ingen bakteriefylogeni gjordes). Biofilmen hade betydande mängder ATP i den fastsittande biomassan och det cirkulerande vattnet i samma system hade dubbelt så mycket ATP som systemet utan biofilmstöd. Dessutom hade biofilmen en helt annan organisk substansprofil, analyserad med GC-MS, jämfört med vattnet i de två systemen. Näringsämnen, kemiska föreningar och metaller skilde sig inte nämnvärt mellan det ursprungliga grundvattnet och i grundvattnet efter experimentet i något av systemen med undantag för mangan, molybden och sulfatinnehåll.

Båda experimenten visade att mikrobiell biofilmbildning inte hade någon större påverkan på det omgivande vattnet vare sig beträffande organiska ämnen eller kemiska föreningar och metaller. Det höga ATP-innehållet som analyserades från biofilmsstödmaterialet tyder dock på att bakterierna i själva biofilmen var mer metaboliskt aktiva än de planktoniska cellerna. Ytterligare stöd för detta var den betydligt förhöjda sulfidproduktionen i flödescellerna med biofilmsstöd.

Contents

1 Introduction

Plans to dispose of spent nuclear fuel (SNF) waste contained in copper canisters (SKB 2010) in deep underground hard-rock repositories in the Fennoscandian Shield have prompted research into the diversity and activity of subterranean microorganisms (Pedersen 2001, 2014). Investigations of potential sites for these repositories have revealed diverse cultivable populations in all analysed groundwater samples from depths of a few meters down to approximately 1 000 m in Sweden (Hallbeck and Pedersen 2012) and Finland (Pedersen et al. 2008). O_2-, nitrate-, iron-, manganese-, and sulfate-reducing bacteria as well as acetogens and methanogens could be cultured in numbers ranging from a few cells mL^{-1} to more than 10^5 cells mL^{-1}. Evaluation of hydrogeochemical data pertaining, for example, to Eh, Mn^{2+}, Fe^{2+}, sulfide, and $d^{34}S$, from site investigations has suggested that the cultured microorganisms were actively metabolizing in their deep aquifers of origin (Hallbeck and Pedersen 2012, Pedersen et al. 2008). This is in line with earlier findings that the incorporation of ^{14}C-labelled CO_2, formate, acetate, lactate and glucose, and ^3H-labeled leucine indicated active autotrophic and heterotrophic metabolisms in the studied populations (Ekendahl and Pedersen 1994, Pedersen and Ekendahl 1992a, b).

It has repeatedly been shown that subterranean microorganisms rapidly attach to mineral surfaces and that attached microorganisms are at least as metabolically active as are planktonic microorganisms (Pedersen 2012, 2013, Pedersen and Ekendahl 1992a, b). Evidence for ancient, fossilised biofilms as well as active biofilms have been obtained from the Äspö Hard Rock Laboratory (HRL) (Jägevall et al. 2011, Pedersen et al. 1997). Because of the large surface to volume ratio in aquifers, the majority of subterranean microorganisms would be present as attached. For instance, from 4×10^3 up to 8×10^5 attached cells per planktonic cell were calculated for an aquifer with a mean width of 0.1 mm using data from borehole V2 in the Stripa mine (Pedersen and Ekendahl 1992b). While there were well developed methods for the analysis of numbers and diversity of planktonic microorganisms during the site investigations in Sweden (Hallbeck and Pedersen 2012, Pedersen et al. 2008), there was no information on numbers and diversity of attached microorganisms. Therefore, this study on how microbial biofilms affect deep groundwater chemistry was initiated which particularly focused on sulfide production was performed.

In this project water from borehole KFM03A:4, section 633.5–650 m, in Forsmark was flowed through and subsequently circulated in a Flow Cell Field Circulation System (FCFCS) with flow cells packed with support for biofilm formation. Another identical FCFCS without any biofilm support was also setup and gave the opportunity to compare how biofilm formation change ground-water chemistry, bacterial activity, sulfide production, redox potential and the composition of organic content in the systems

This report summarizes the results from two different experiments completed in 2017 and 2018, respectively. The subprojects were denoted as: The effects of biofilm formation on deep groundwater chemistry and microbial sulfide production – part 1 and 2, hereafter only denoted as part 1 and 2. In part 1, lactate was used to boost the microbial activity in the FCFCS's after a month of circulation. In part 2, hydrogen (H_2) and acetate were added instead to the FCFCS's to more realistically simulate final repository conditions. Hence, acetate and H_2 analysis were added to the analysis package, in all other regards the projects was carried out identically.

2 Material and method

In order to get an overview of the analyses performed in each of the two subprojects a list of the analyses is presented below. In short, the experimental design was identical between the two subprojects with the difference of bacterial growth additions. In part 1 lactate was used and in part 2, H_2 and acetate was used.

Table 2-1. Analyses performed in subproject 1 and 2.

Analysis	
Part 1	**Part 2**
Sulfide	Sulfide
Lactate	Acetate
ATP	ATP
Organic content	Organic content
Eh	Eh
Nutrients, major components and metals	Nutrients, major components and metals
Sulfate	H_2

2.1 Flow Cell Field Circulation Systems

Two identical Flow Cell Field Circulation Systems (FCFCS's) comprising four Flow Cells (FC's) each, a micropump (Micropump GAH series V21 J with a PEEK impeller; Labinett, Göteborg, Sweden), two pressure meters (S-11, 40 Bar 4-20 G1⁄2; WIKA – AB Svenska Industri Instrument, Göteborg, Sweden), a flow meter (Promag 50; Endress+Hauser Flowtech AG, Sollentuna, Sweden), and a 4-L expansion vessel were prepared for each of the two parts of the experiments (Figure 2-1).
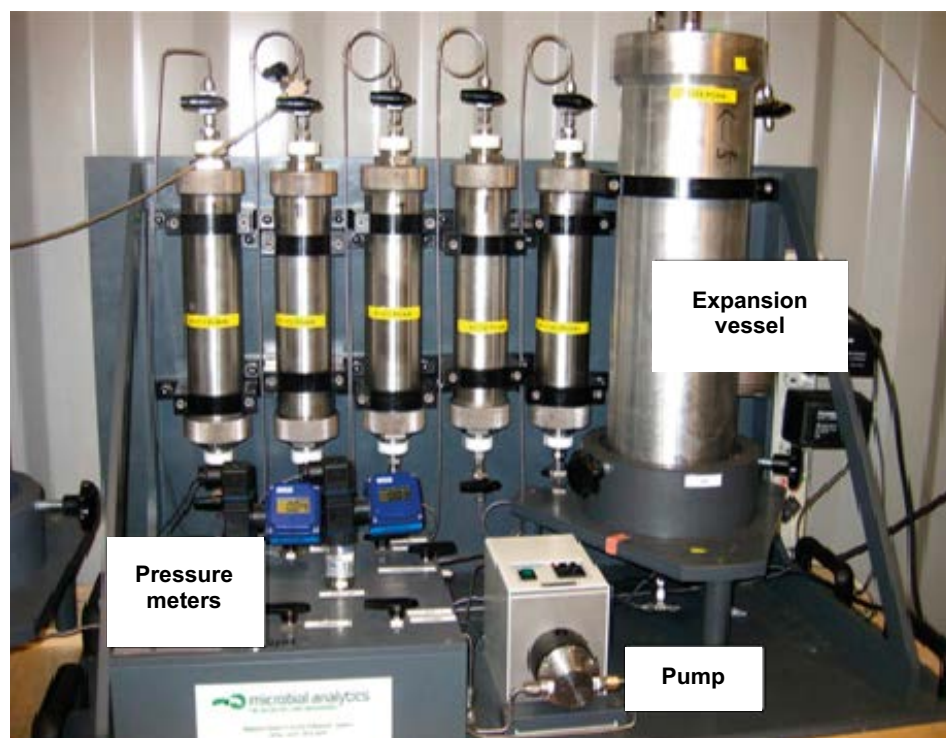
Figure 2-1. Flow cell field circulation system (FCFCS).

The FC's of one FCFCS was filled with support for biofilm formation. The FC's had a stainless-steel shell (length 300 mm, diameter 65 mm) and were lined with polyvinyldifluoride (PVDF) plastic (for details, see Pedersen 2005). Each flow cell had a 120-mm long PVDF insert with a 22×32 mm opening that supported 110 g of the crushed rock grains (2–4 mm diameter) offering a theoretical rock surface area of ~600 cm^2 per FC for microbial adhesion and biofilm development, assuming spherical rock grains with an average diameter of 3 mm (Figure 2-2). The rock grains were rinsed with sterile water and heat sterilized at 160 °C for 5 h. Three flow stabilizers at each end of the insert ensured even distribution and a slow laminar flow of water through each flow cell (Pedersen 1982).

For part 1, the FCFCS's were installed on 29 August 2017 and circulated with groundwater from borehole KFM03A:4, section 633.5–650 m for 30 d at a flow rate from the aquifer of 100 mL min^{-1}. For part 2, the FCFCS with support for biofilm formation was installed on 12 June 2018 and circulated with groundwater from borehole KFM03A:4, section 633.5–650 m for 24 d at a flow rate from the aquifer of 100 mL min^{-1}.

The other FCFCS's had identical FC inserts but without crushed rock (empty) and was filled with groundwater from the borehole at the day of disassembly from the drillhole for respective experiment. All FCFCS's were transported from Forsmark to Micans laboratory in Mölnlycke.

In the laboratory the circulation through the FCFCS's were continued by pumping the water with a flow rate of 25 mL min^{-1} in a closed loop via the 4L-expansion vessel filled with groundwater from borehole KFM03A:4.

2.2   Addition of lactate and hydrogen/acetate

For part 1, after 33 days of circulation in the laboratory, 10 mL of water were sampled from both FCFCS and analysed for sulfide. No measurable concentration of sulfide could be detected in any of the two systems. To speed up the bacterial processes in the systems but still retain the original question of how biofilm formation affects the groundwater chemistry 10 mM of lactate was added to both systems. The systems were then kept with a flow rate of 25 mL min^{-1} for an additional 41 days.
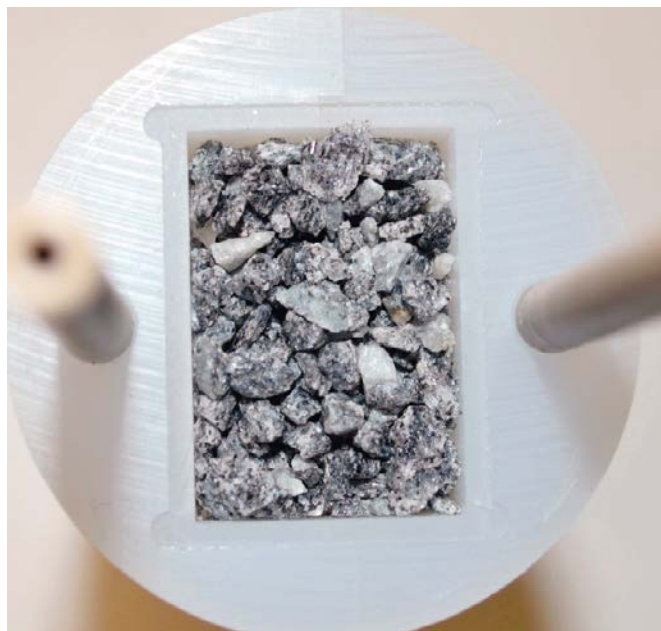
Figure 2-2. *Flow cell insert with crushed rock.*

For part 2, directly upon arrival, 10 mL of a sterile acetate solution was injected in to the circulation which resulted in a final concentration of 3.4 mM in both systems. In addition, approximately 10 mM of H_2 was added to both systems by connecting a gastight 500 mL stainless-steel vessel containing H_2 in a total pressure of 3 bars to the circulation. The water circulation in both systems were then started with a flow rate of 25 mL min^{-1} for 55 days under 6 bar total pressure. The pressure was added in order to dissolve the added H_2 in the circulating water.

2.3   Sulfide analysis

Sulfide was measured at several points after lactate and H_2/acetate addition. The colorimetric methylene blue method with an uncertainty of ± 17 % was used to analyse concentrations of sulfide (Swedish Standard Method SIS 028115). This method will measure both dissolved sulfide and colloidal FeS. Sulfide was precipitated with zinc acetate and will become blue when there is sulfide in the sample. The blue colour is proportional to the sulfide concentration which was analysed according to the manufacturer's instructions using a Genesys 10UV spectrophotometer (Thermo Fisher Scientific) for detection.

2.4   Sulfate analysis

Sulfate concentrations were determined of the water in both FCFCS's using the SulfaVer 4 method (HACH method no. 8051, programme 680, HACH Lange AB) according to the manufacturer's instructions. The uncertainty of the measurement is 10 % of a 30 mg L^{-1} sample according to the manufacturer and absorbance was measured on a HACH spectrophotometer.

2.5   Lactate analysis

Lactate concentrations from the circulating water in the FCFCS's were determined with the enzymatic UV method (kit no. 10139084035; Boehringer Mannheim/R-Biopharm AG, Darmstadt, Germany) using a Genesys 10UV spectrophotometer (Thermo Fisher Scientific) for detection.

2.6   Acetate analysis

Acetate concentrations from the circulating water in the FCFCS's were determined with the enzymatic UV method (kit no. 10148261035; Boehringer Mannheim/R-Biopharm AG, Darmstadt, Germany) using a Genesys 10UV spectrophotometer (Thermo Fisher Scientific) for detection.

2.7   ATP-analysis

Total ATP in living cells was analysed from crushed rock and from water in the FCFCS's using an enzymatic method (Biomass Kit HS Prod. No. 266–311, BioThema, Handen, Sweden). This ATP biomass method has been evaluated for use with Fennoscandian groundwater, including Olkiluoto groundwater, and the results were published (Eydal and Pedersen 2007).

For ATP-analysis of biofilm, 0.9 mL of ATP extraction solution (BioThema, Handen, Sweden) was added to an Eppendorf tube containing 1.00 g of biofilm support material. The tube was vortexed vigorously for 20 min in order to release the biofilm from the support material.

A 4.0 mL, 12-mm-diameter polypropylene tube (no. 68.752; Sarstedt, Landskrona, Sweden) was filled with 400 µL of the ATP kit reagent HS (BioThema, Handen, Sweden) and inserted into an FB12 tube luminometer (Sirius Berthold, Pforzheim, Germany). The quick measurement FB12/Sirius software, version 1.4 (Berthold Detection Systems, Pforzheim, Germany), was used to calculate light emission as relative light units per second (RLU s^{-1}). Light emission was measured for three 5-s intervals with a 5-s delay before each interval, and the average of three readings was registered as a single measurement. The background light emission (Ibkg) from the reagent HS and the tube was monitored and allowed to decrease to a value below 50 RLU s^{-1} prior to registering a measurement. ATP was extracted from 100-µL aliquots of sample, by mixing for 5 s with 100 µL of B/S extraction solution from the ATP kit in a separate 4.0-mL polypropylene tube. Immediately after mixing, 100 µL of the obtained ATP extract mixture was added to the reagent HS tube in the FB12 tube luminometer, and the sample light emission (Ismp) was measured. Subsequently, 10 µL of an internal ATP standard was added to the reactant tube, and the standard light emission (Istd) was measured. The concentration of the ATP standard was to 10^{-7} M.

The ATP concentration of the analysed samples was calculated as follows:

$$\text{amol ATP mL}^{-1} = (\text{Ismp} - \text{Ibkg}) / ((\text{Ismp} + \text{std} - \text{Ibkg}) - (\text{Ismp} - \text{Ibkg})) \times 10^6 / \text{sample volume}$$

where I, represents the light intensity measured as RLU s^{-1}, smp represents sample, bkg represents the background value of the reagent HS, and std represents the standard (referring to a 10^{-7} M ATP standard).

2.8 Analysis of organic content with GC-MS

The extraction of organic content from the crushed rock was performed using a Soxhlet extractor (Jensen 2007) and ethyl acetate as solvent. 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 °C and the chromatograph oven was programmed in the range of 35–340 °C using helium as a carrier gas. Acquisition in the ion-trap was in the range 38–400 atomic mass units (amu). For evaluation, the chromatogram from a blank sample 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.

For the extraction of organic content from the circulating water in the FCFCS's liquid/liquid extraction was used. Extraction was performed in a separatory funnel. 200 ml of circulating water was extracted twice with 20 ml of hexane, the solvent aliquots were pooled and evaporated to a final volume of 1 ml using a stream of inert nitrogen gas. The results were background corrected versus a baseline chromatogram from a sample of 40 ml of hexane evaporated to 1 ml using the same procedure as for the water samples. The obtained extracts were analysed on the same GC-MS system as described above using the same conditions.

2.9 Eh analysis

Eh was analysed on the circulating water using a HACH HQ40d portable multi-parameter meter (HACH Lange AB, Sköndal, Sweden) equipped with an MTC101-05 probe (HACH Lange AB) installed in a flow cell connected to each analysed FCFCS.

2.10 Nutrients, major components and metals

Water samples from the groundwater of borehole KFM03A:4, section 633.5–650 m were taken in conjunction with the retrieval of the FCFCS's from the field and sent to ALS Scandinavia AB, Maskinv. 2, 183 71 Täby for analysis of nutrients, major components and metals according to the GV-3 Plus analysis package. Another sample from both FCFCS's was also taken at the end of the experiment and analysed as above.

2.11 H$_2$ analysis

Hydrogen gas from the circulating water from both FCFCS's was analysed after H$_2$ addition and at the end of the experiment on a DANI Master gas chromatograph using MXT-Molsieve 5A Plot 30 m × 0.53 mm × 50 μm and OPT 270M-MICRO thermal conductivity detector system, with argon as carrier gas.

2.12 Data processing, graphics and statistics

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

3   Results

3.1   Sulfide

Part 1: After the addition of 10 mM lactate to both FCFCS's the sulfide concentrations were measured repeatedly. As shown in Figure 3-1, a clear difference in sulfide concentration could be observed between the FCFCS's already at the second measuring point at day 6. The sulfide concentration then continued to rapidly increase in the FCFCS with biofilm while the FCFCS without biofilm remained at levels close to zero. At day 25 a small increase in sulfide concentration could be observed also for the FCFCS without biofilm. At the end of the measurements at day 40, approximately ten times more sulfide had been produced in the FCFCS with biofilm in comparison to the FCFCS without biofilm. This indicated that the bacterial sulfide producing population was far more active in the FCFCS that stimulated biofilm formation.

Part 2: After the addition of acetate and H_2 to both FCFCS's the sulfide concentrations were measured repeatedly. As shown in Figure 3-2, a clear difference in sulfide concentration could be observed between the FCFCS's already at the third measuring point at day 11. The sulfide concentration then continued to rapidly increase in both FCFCS's, however not as drastically and fast for the system without biofilm compared to the system with biofilm. At the end of the measurements at day 42, approximately 0.2 mM more sulfide had been produced in the FCFCS with biofilm in comparison to the FCFCS without biofilm. This indicated that the bacterial sulfide producing population was more active in the FCFCS that stimulated biofilm formation.
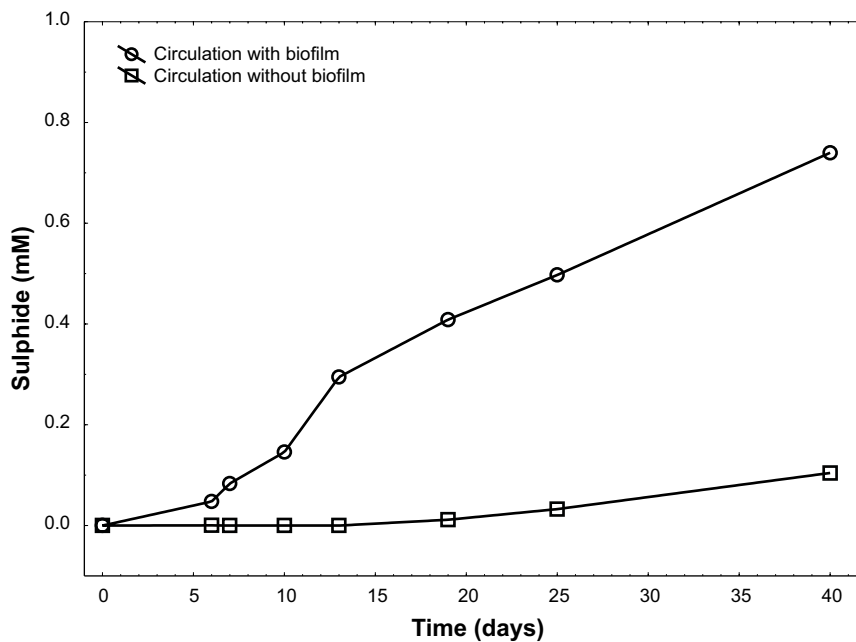
Figure 3-1. *Concentration of sulphide in the FCFCS's according to legend. Addition of lactate at day 0.*
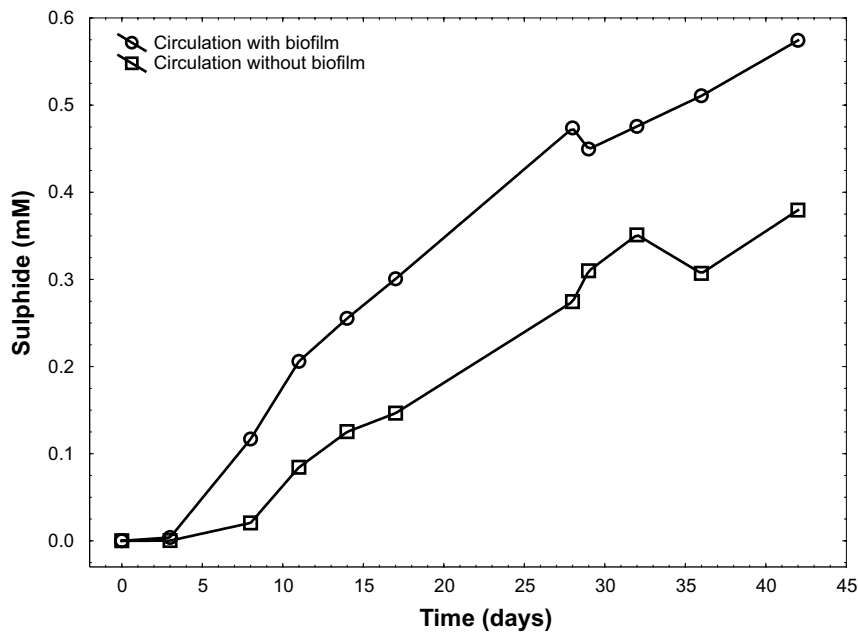
Figure 3-2. *Concentration of sulphide in the FCFCS's according to legend. Addition of acetate and H$_2$ at day 0.*

3.2 Sulfate

Part 1: The concentration of sulfate in both FCFCS's was analysed at the end of the experiment. The concentration of sulfate was measured to 49 mg L^{-1} and 160 mg L^{-1} in the FCFCS's with and without biofilm, respectively. At the start of the FCFCS installation in Forsmark the sulfate concentration in the groundwater from KFM03:A4 was measured to 185.3 mg L^{-1} (SKB Analysis, see appendix part 1). When sulfate and sulfide values are converted to mM (Table 3-1) the remaining sulfate and the amount of reduced sulfate agrees reasonably well with the sulfate concentration at the start of the experiment. However, some sulfide was not accounted for at the end of the experiment which could be explained by loss of sulfide as gas in connection with sampling. It could also be the case that sulfide was bound to surfaces as FeS in the FCFCS and therefore not analytically available in fluid samples. Finally, sulfide may have been oxidized to elemental sulfur (confer Tables 3-3 to 3-5).

Table 3-1. Measured concentrations of sulfate and sulfide in both FCFCS's at start and end of experiment part 1.

	Sulfate start (mM)	Sulfate end (mM)	Sulfide end (mM)	Sulfate end + Sulfide end (mM)
Circulation with biofilm	1.93	0.51	0.74	1.25
Circulation without biofilm	1.93	1.67	0.10	1.77

3.3 Lactate

Part 1: The concentration of lactate in the circulating water in the FCFCS's was measured to 5.82 mM and 7.01 mM in the FCFCS with biofilm and without biofilm at the end of the experiment, respectively. Of the 10 mM lactate that was added 4.18 mM was consumed by SRB or other bacteria in the system with biofilm, and 2.99 mM in the system without biofilm.

3.4 ATP

Part 1: The amount of ATP in the circulating water was approximately the same for both the FCFCS's (Table 3-2). That is, the numbers of active planktonic bacteria did not increase or decrease significantly if the system contained biofilm or not. However, in the biofilm approximately 70 percent more ATP was found compared to the circulating water. This argues that the bacteria in the biofilm were more active than planktonic bacteria in the circulating water.

Table 3-2. Analysed amounts of ATP in circulating water and biofilm. Since one millilitre of water approximately weighs one gram, the ATP values for the circulating water can be compared to the values for ATP in biofilm.

	ATP in circulating water (amol mL^{-1})	ATP in biofilm (amol g^{-1})
FCFCS with biofilm	184 000	308 000
FCFCS without biofilm	180 000	

Part 2: There was a clear difference in the amount of ATP where almost twice as much was found in the circulating water of the FCFCS with biofilm compared to the FCFCS without biofilm (Table 3-3). A substantial amount of ATP could also be observed in the attached biomass. This argues that the bacteria in the biofilm were more active than planktonic bacteria in the circulating water.

Table 3-3. Analysed amounts of ATP in circulating water and biofilm.

	ATP in circulating water (amol mL^{-1})	ATP in biofilm (amol g^{-1})
FCFCS with biofilm	102 000	185 000
FCFCS without biofilm	52 000	

3.5 Acetate

Part 2: The concentration of acetate in the circulating water in the FCFCS's was measured to 2.40 mM and 2.35 mM in the FCFCS with biofilm and without biofilm after one week of circulation, respectively. Roughly 3.4 mM of acetate was added to the systems at day 0. At the end of the experiment 2.17 mM and 2.11 mM were left in systems, respectively. This means that the amount of acetate consumption by SRB and other bacteria in both systems were approximately the same; ~ 1.3 mM.

3.6 Hydrogen gas (H$_2$)

Part 2: In the FCFCS with biofilm more than three times as much H$_2$ had been consumed compared to the FCFCS without biofilm at the end of the experiment. This again argues for a higher microbial activity in that system. At the start of the experiment equal amounts of H$_2$ was added to each FCFCS and as can been seen in Table 3-4, almost identical amounts had dissolved in the circulating water in both systems.

Table 3-4. Hydrogen gas analysed at the start and at the end of experiment and the consumed amount of H$_2$ for each FCFCS.

	START (vol%)	END (vol%)	Consumption (vol%)
FCFCS with biofilm	34.4	20.7	13.7
FCFCS without biofilm	34.6	30.3	4.3

3.7 Organic content – circulating water, part 1

The extractable organic content from the circulating water in the two FCFCS's did not differ significantly in substance composition. Most substances in the FCFCS without biofilm were identical to the system with biofilm. The concentration of the substances differed somewhat between the two FCFCS's. For those substances a + or a − sign indicates increase or decrease in concentration compared with FCFCS without biofilm in Table 3-6. Substances only detected in the FCFCS with biofilm are identified in bold.

Table 3-5. Extractable organic content from circulating water in FCFCS without biofilm, part 1. Substances identified according to NIST 14 library.

FCFCS without biofilm:

Substance	CAS #	Comment
alpha- Pinene	80-56-8	From natural sources
Oxalic acid, isobutyl nonyl ester	?	Possible product of petroleum degradation
Benzene, 1,2,3-trimethyl-	526-73-8	Possible petroleum residual
Decane	124-18-5	Possible petroleum residual
Cyclohexane, isocyanato-	3173-53-3	Probably contamination from plastic materials
beta- Ocimene	13877-91-3	From natural sources
Benzene, 1-ethyl-3-methyl	620-14-4	Possible petroleum residual
1-Hexanol, 2-ethyl-	104-76-7	Possible product of petroleum degradation
Pentadecane	629-62-9	Possible petroleum residual
trans-2-Undecen-1-ol	75039-84-8	Possible product of petroleum degradation
Thiourea, tetramethyl-	2782-91-4	
Benzothiazole	95-16-9	From natural sources
Carbonimidodithioic acid ester	18805-25-9	
Glycerol 1,2-diacetate	102-62-5	
Benzoic acid, trimethylphenyl ester	1504-38-7	From natural sources
2,4,8-Trimethyl- 1,2,3,4-tetrahydroquinoline	?	
4-Aminoquinaldine	6628-04-02	
4-Ethoxy-2-hydroxyoctanophenone	22198-47-6	
Butylated hydroxytoluene	128-37-0	Probably from natural sources
Diethyl phthalate	84-66-2	Probably contamination from plastic materials
2-(Methylmercapto)benzothiazole	615-22-5	
1,4-Naphthoquinone, 6-acetyl-2,5-dihydroxy-	13378-90-0	
Diphenylamine	122-39-4	
Benzophenone	119-61-9	
Benzenesulfonamide, N-butyl-	3622-84-2	Probably contamination from plastic materials
Elemental Sulfur	10544-50-0	Large amounts
Dibutylphthalate	84-74-2	Probably contamination from plastic materials
Hexadecanoic acid, methyl ester	112-39-0	Possible product of petroleum degradation
Diethylhexyl phthalate	117-81-7	Probably contamination from plastic materials
Diisononyl phthalate (DINP)	28553-12-0	Probably contamination from plastic materials
Inden-5-amine, 3-(4-aminophenyl)-2,3-dihydro-1,1,3-trimethyl-	?	

Table 3-6. Extractable organic content from circulating water in FCFCS with biofilm, part 1. +/– -sign indicates increase/decrease in concentration compared to FCFCS without biofilm. Unique substances compared to Table 3-5 are identified in bold. Substances identified according to NIST 14 library.

FCFCS with biofilm:

Substance	CAS #	+/–	Comment
alpha- Pinene	80-56-8		From natural sources
Oxalic acid, isobutyl nonyl ester	?		Possible product of petroleum degradation
Benzene, 1,2,3-trimethyl-	526-73-8		Possible petroleum residual
Decane	124-18-5		Possible petroleum residual
Cyclohexane, isocyanato-	3173-53-3	"+"	Probably contamination from plastic materials
beta- Ocimene	13877-91-3		From natural sources
Benzene, 1-ethyl-3-methyl	620-14-4		Possible petroleum residual
1-Hexanol, 2-ethyl-	104-76-7		Possible product of petroleum degradation
Pentadecane	629-62-9		Possible petroleum residual
trans-2-Undecen-1-ol	75039-84-8		Possible product of petroleum degradation
Thiourea, tetramethyl-	2782-91-4		
Benzothiazole	95-16-9	"+"	From natural sources
Propionic acid, 3-mercapto-, isooctyl ester	77916-53-1		
Carbonimidodithioic acid ester	18805-25-9		
Glycerol 1,2-diacetate	102-62-5		
Benzoic acid, trimethylphenyl ester	1504-38-7		From natural sources
2,4,8-Trimethyl- 1,2,3,4-tetrahydroquinoline	?		
4-Aminoquinaldine	6628-04-02	"–"	
2,4-Di-tert-butylphenol	96-76-4		Present in "Biofilm support mineral"
4-Ethoxy-2-hydroxyoctanophenone	22198-47-6		
Butylated hydroxytoluene	128-37-0		Probably from natural sources
Diethyl phthalate	84-66-2	"+"	Probably contamination from plastic materials
2-(Methylmercapto)benzothiazole	615-22-5		
1,4-Naphthoquinone, 6-acetyl-2,5-dihydroxy-	13378-90-0		
Diphenylamine	122-39-4		
Benzophenone	119-61-9		
Benzenesulfonamide, N-butyl-	3622-84-2	"+"	Probably contamination from plastic materials
Elemental Sulfur	10544-50-0		Large amounts
Dibutylphthalate	84-74-2		Probably contamination from plastic materials
Hexadecanoic acid, methyl ester	112-39-0		Possible product of petroleum degradation
Diethylhexyl phthalate	117-81-7		Probably contamination from plastic materials
Diisononyl phthalate (DINP)	28553-12-0		Probably contamination from plastic materials
Inden-5-amine, 3-(4-aminophenyl)-2,3-dihydro-1,1,3-trimethyl-	?		

3.8   Organic content – biofilm support (crushed rock), part 1

While the circulating water from the two FCFCS's had similar compositions of organic substances, the extracted organic content from the biofilm support mineral had a completely different profile (Table 3-7).

Table 3-7. Extractable organic content from biofilm support mineral. Substances identified according to NIST 14 library.

Biofilm support mineral:		
Substance	CAS #	Comment
Limonene	138-86-3	
Cyclopentanol	96-41-3	Possible product of petroleum degradation
Valeric acid ester	?	Possible product of petroleum degradation
Oxalic acid ester	?	Possible product of petroleum degradation
Glycerol 1,2-diacetate	102-62-5	
Oxalic acid, isobutyl nonyl ester	?	Possible product of petroleum degradation
2,4-Di-tert-butylphenol	96-76-4	
Elemental Sulfur	10544-50-0	Large amounts
Nonadecane	629-92-5	
Isopropyl myristate	110-27-0	
Phenol, 2-(2-pentyn-4-yl)-4-methyl-	?	
Dibutylphthalate	84-74-2	Probably contamination from plastic materials
Hexadecanoic acid, methyl ester	112-39-0	
Diethylhexyl phthalate	117-81-7	Probably contamination from plastic materials
Diisononyl phthalate (DINP)	28553-12-0	Probably contamination from plastic materials, large amounts

3.9   Organic content – circulating water and biofilm, part 2

The extractable organic content (EOC) from the circulating water in the two FCFCS's did not differ significantly in substance composition. Most substances in the FCFCS without biofilm were identical to the system with biofilm. The concentration of the EOC differed somewhat between the two FCFCS's (Table 3-8). The circulating water from the two FCFCS's had similar compositions of organic substances, however the EOC from the biofilm support mineral had a completely different profile, see following legend for explanation of the colour coding:

= Only in FCFCS with biofilm support
= All systems
= Both waters
= Only in biofilm

Table 3-8. Extractable organic content from circulating water in FCFCS and biofilm. Substances identified according to NIST 14 library. Substances in bold had large peaks indicating considerable amounts.

BIOFILM SUPPORT (crushed rock)		WATER, FCFCS with biofilm support		WATER, FCFCS without biofilm support	
Substance	CAS	Substance	CAS	Substance	CAS
Ethyl 3-acetoxybutyrate	27846-49-7	Benzothiazole	95-16-9	Benzothiazole	95-16-9
Decamethylcyclopentasiloxane	541-02-6	Cyclohexane, isothiocyanato-	1122-82-3	Cyclohexane, isothiocyanato-	1122-82-3
1,3,2-Dioxaborolane, 2-phenyl-	4406-72-8	Nonanoic acid	112-05-0	Nonanoic acid	112-05-0
1,3,2-Dioxaborolane, 4-methyl-2-phenyl-	4406-75-1	Carbonimidodithioic acid, methyl-, dimethyl ester -	18805-25-9	Carbonimidodithioic acid, methyl-, dimethyl ester -	18805-25-9
Diacetyl sulfide	3232-39-1	1,2-Dihydro-2,2,4-trimethylquinolin	147-47-7	1,2-Dihydro-2,2,4-trimethylquinolin	147-47-7
1,2-Benzenediczrboxylic acid	88-99-3	2,6-Di-tert-butyl-1,4-benzoquinone	719-22-2	2,6-Di-tert-butyl-1,4-benzoquinone	719-22-2
1,2,3-Trithiolane	6669-39-2	Cycluron	2163-69-1	Cycluron	2163-69-1
Hexathiane	13798-23-7	Hexathiane	13798-23-7	Hexathiane	13798-23-7
dl-Xylitol, cyclic 2,3-[phenylboronate]	–	Undecanoic acid	112-37-8	Undecanoic acid	112-37-8
[1,2,3,4]Tetrathiane	290-81-3	DEP	84-66-2	DEP	84-66-2
		2-Methylmercaptobenzothiazole	615-22-5	2-Methylmercaptobenzothiazole	615-22-5
		Benzophenone	119-61-9	Benzophenone	119-61-9
		2-Benzothiazolamine, N-ethyl-	28291-69-2	2-Benzothiazolamine, N-ethyl-	28291-69-2
		3,5-Di-tert-butyl-4-hydroxybenzaldehyde	1620-98-0	3,5-Di-tert-butyl-4-hydroxybenzaldehyde	1620-98-0
		Benzenesulfonamide, N-butyl-	3622-84-2	Benzenesulfonamide, N-butyl-	3622-84-2
		4-[4-Aminophenyl] pyrimidin-2-amine	–		
		3-Phenylthiane, S,S-dioxide	–	3-Phenylthiane, S,S-dioxide	–
		Tetramethylthiuram monosulfide	97-74-5	Tetramethylthiuram monosulfide	97-74-5
phthalate		phthalate		phthalate	
		Benzenesulfonanilide	1678-25-7	Benzenesulfonanilide	1678-25-7
		2-Aminodiphenylsulfone	4273-98-7	2-Aminodiphenylsulfone	4273-98-7
		1,3-Dicyclohexylurea	2387-23-7	1,3-Dicyclohexylurea	2387-23-7
S8	10544-50-0	S8	10544-50-0	S8	10544-50-0
Octadecanoic acid	57-11-4	unknown, perhaps mix			
Mono(2-ethylhexyl) adipate	4337-65-9	Dehydroabietic acid	1740-19-8		
BEHP	137-89-3	unknown			
		3-Methyl-1-penten-3-ol	918-85-4	3-Methyl-1-penten-3-ol	918-85-4
		unknown			
Acetic anhydride	108-24-7	3-methyl-1-penten-3-ol, isomer	86361-10-6		
4-Aminoisoxazolidin-2-one	4834-58-6	perhaps hexaethyleneglycol monoethyl ester			

3.10 Eh

Eh was measured at the end of the experiments part 1 and 2, on the circulating water in the FCFCS's. All FCFCS's, and specially the FCFCS's with biofilm, had Eh values indicating a strong reduction potential due to the hydrogen gas but also sulfide (Table 3-9). The measured Eh values correlated well with the microbial sulfate-reduction activity that took place in the systems. There was a big difference in Eh between the different experiments (part 1 and 2) but not between the systems in each experiment. The very low Eh in systems with H_2 and acetate is explained by the presence of H_2.

Table 3-9. Analysed Eh in the circulating water in all FCFCS with, and without biofilm.

| Experiment part	FCFCS	Eh in circulating water (mV)
1	Lactate +biofilm	−262.1
1	Lactate −biofilm	−206.5
2	Acetate, H_2 +biofilm	−585.6
2	Acetate, H_2 −biofilm	−565.7

4 Discussion

Since the future repository for spent nuclear fuel in Sweden is planned to be built deep underground in granitic bedrock, a vast surface area of water conducting fractures will be available for microbial biofilm formation. The effects of biofilm formation on groundwater chemistry and microbial activity should, therefore, be considered in the safety case. This report summarizes and compares the results from two separate experiments conducted with water from borehole KFM03A:4, section 633.5–650 m, in Forsmark.

4.1 Sulfide

In the systems with biofilm support material from part 1 and part 2 there was a small difference in sulfide production; same rate but slightly higher final amount with lactate compared to H_2 and acetate. The systems without biofilm support, however, showed a larger difference in sulfide production; much higher rate and final amount with hydrogen and acetate compared to lactate. This was probably due to that hydrogen is more easily metabolically accessible than lactate and this makes the advantage for the bacteria of residing in a biofilm smaller.

The ratio of solid material (biofilm support) and circulating water in the FCFCS's is not the same as in natural systems where surfaces on fractures stands for a much larger part of the habitable available space for microbes compared to the groundwater volume of the same system. Meaning that if all the surfaces on fractures contained biofilm in a natural system, the numbers of bacteria would be much higher than what could be found in the groundwater of the same system. In our laboratory setup the ratio between solid surfaces and water is smaller than it would have been in a natural system.

At any given time of the experiment the amount of sulfide produced by the biofilm versus the circulating water could be calculated by using the values in Figure 4-1, the volume of water (5 L) and the amount of biofilm support in one whole FCFCS (430 g). This shows how large the actual contribution of the biofilm was on the sulfide production which not correctly can be compared in Figure 4-1 since the volume of water is much larger in the FCFCS's compared to the volume of the biofilm support material. In Figure 4-2 normalized sulfide production values are shown.
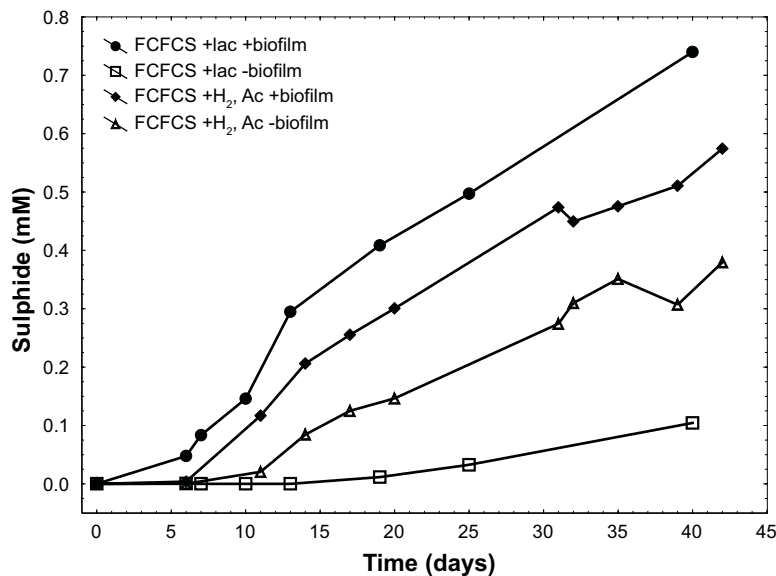
Figure 4-1. Concentration of sulphide in the FCFCS's according to legend. Addition of lactate or acetate and H_2 at day 0. Filled symbols represent FCFCS's with biofilm support.
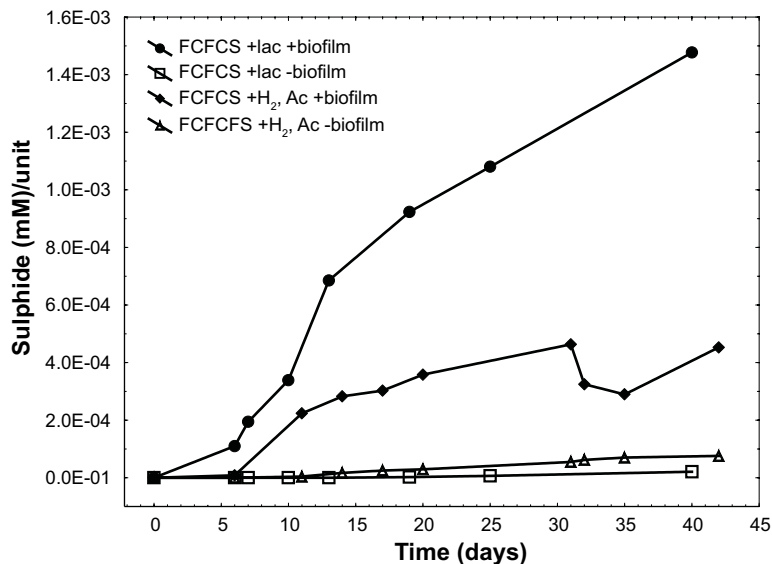
Figure 4-2. *Concentration of sulphide produced by biofilm and circulating water per gram of biofilm support or mL of circulating water respectively. Filled symbol represent FCFCS's with biofilm support.*

The following calculations were made on the data in Figure 4-1 to generate values in Figure 4-2:

1. (Measured sulfide amount in system with biofilm (mM) – Measured sulfide amount in system without biofilm (mM)) / Weight of biofilm support in FCFCS = Produced sulfide by biofilm per gram.

2. Measured sulfide amount in system without biofilm (mM) / Volume of water in FCFCS = Produced sulfide by water phase per mL

4.2 Lactate, acetate and hydrogen

As shown in Figure 4-3, the largest difference in consumption of added acetate and lactate between the FCFCS's with and without biofilm support material was in part 1 with lactate. Comparing that to the sulfide production (Figure 4-2) it was also in that experiment where the largest difference in sulfide production was observed. In part 2 with acetate, the differences were much smaller between FCFCS's with and without biofilm support. A large difference in hydrogen consumption was however observed. Even though the difference in sulfide production between the FCFCS's also was smaller in part 2, there was still a clear case that the FCFCS with biofilm support had both a higher final amount also a faster rate. This means that the sulfide-producing microbial population in both FCFCS's consumed similar amounts of acetate but the FCFCS containing biofilm produced more sulfide and consumed more hydrogen.
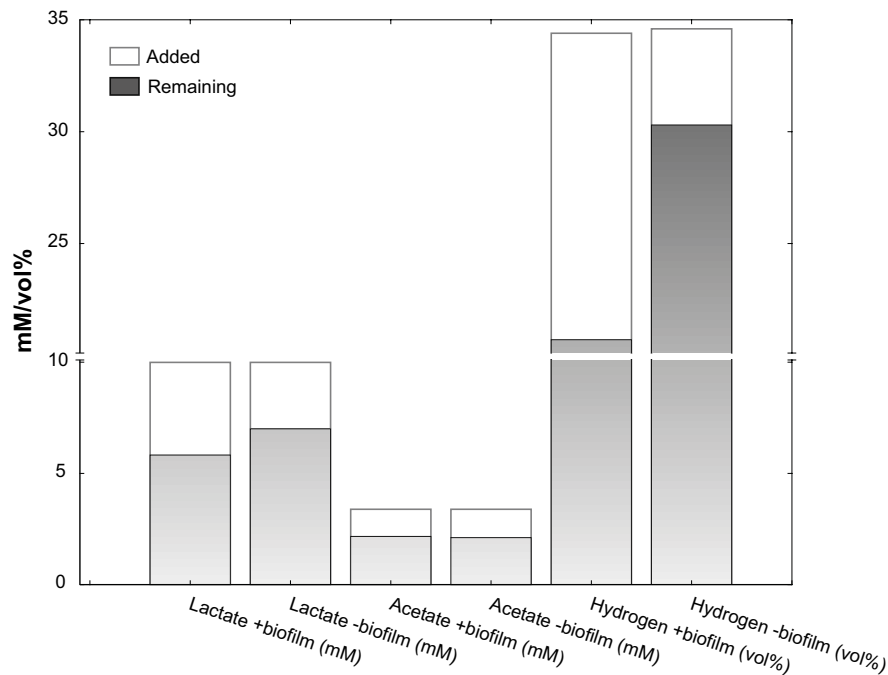
Figure 4-3. Added at start and remaining amounts at the end of the experiments (part 1 and 2) of lactate, acetate and hydrogen in FCFCS's with and without biofilm support material (± biofilm).

4.3   ATP

More ATP was produced in the water and biofilm in part 1 with lactate. However, approximately one month went by before the addition of lactate to the systems took place. This time difference could have played a role in ATP content, even if no analyzable amounts of sulfide before the lactate was added could be observed, and the time from the additions to analysis in both part 1 and 2 was approximately the same. The difference however, between the waters in the two systems was non-existing in part 1, but in part 2 the water in the system without biofilm had about half the ATP content compared to the system with biofilm (Table 4-1). The microbial population seems overall to have been more active when growing on lactate compared to acetate and hydrogen, especially bacteria in biofilm.

Table 4-1. Analysed amounts of ATP in circulating water and biofilm.

Experiment part	FCFCS	ATP in circulating water (amol mL^{-1})	ATP in biofilm (amol g^{-1})
1	Lactate +biofilm	184 000	308 000
1	Lactate −biofilm	180 000	–
2	Acetate, H$_2$ +biofilm	102 000	185 000
2	Acetate, H$_2$ −biofilm	52 000	–

In Table 4-1 there seemed to be more ATP per unit in the biofilm compared to the circulating water in both experiments. However, amol mL^{-1} and amol g^{-1} is not directly comparable. Therefore, the ATP content was re-calculated to ATP per cm^2 using the following input and assumptions:

- ATP content of biofilm per gram in biofilm and per milliliter in circulating water (Table 4-1).
- One crushed rock garnet grain used in the experiments as biofilm support material had an average weigh of 0.00351 g.
- The surface area of one average garnet grain was 0.02 cm^2.
- The dry weight (dw) in grams of the analysed biofilm support material.
- A natural water conducting fracture has a width of 0.1 cm.

The amount of ATP on each side of the hypothetical fracture (amol per 20 cm^2) was calculated using the following equation:

$$20 \times \left(amol\ mL^{-1} or\ g^{-1} / \left(\left(\frac{dw}{weight_{garnet}} \right) \times mantle\ surface_{garnet} \right) \right) \qquad (4\text{-}1)$$

Using equation 4-1, the amount of ATP on the hypothetical fracture walls was 1 080 000 and 630 437 amol for experiment part 1 and 2, respectively. If the fracture is 0.1 cm wide, 0.1 cm^3 or 0.1 mL of water will be able to fit between the two fracture walls per each cubic centimeter. 10 cm^2 of fracture wall will therefore come in contact with 1 mL of water on each side. Since the fracture has two sides, 20 cm^2 will in total come in contact with 1 mL of water. Knowing the amount of ATP in the circulating water per mL for the FCFCS's without biofilm support in experiment part 1 and 2 an ATP ratio between solid surfaces and water can be calculated by simply dividing one with the other. The results were that 6 and 12 times more water are needed compared to solid surfaces to represent the same amount of ATP for experiment part 1 and 2, respectively.

The amount of produced sulfide per unit at the end of the experiments for the biofilm and circulating water can also be divided by the total amount of ATP analysed from each sample type. This gives a value on how active the bacteria were in the biofilm and in the water. In the ratio column in Table 4-2 there are values illustrating how many times more active the sulfide-producing bacteria (SPB) were in the biofilms compared to the circulating water. In part 1 the SPB were 41 times more active and in part 2 they were 2 times more active.

Table 4-2. Produced sulfide per unit (mL or g) and degree of activity for sulfide-producing bacteria in biofilm and circulating water.

Produced sulfide by biofilm (mM)/g)	Produced sulfide by water (mM)/mL)	Analysed ATP biofilm (amol/g)	Analysed ATP water (amol/ml)	Produced sulfide by biofilm (mM/amol)	Produced sulfide by water (mM/amol)	Ratio
Part 1						
1.47×10^{-3}	2.09×10^{-5}	308 000	180 000	4.80×10^{-9}	1.16×10^{-10}	41
Part 2						
4.68×10^{-4}	7.49×10^{-5}	185 000	52 000	2.45×10^{-9}	1.46×10^{-9}	2

4.4 Organic content

The results from the organic content analysis from both experiment part 1 and 2 showed that the biofilm had its own unique substance profile with much fewer substances compared to the circulating water. The profiles for the circulating waters were very similar for each experiment but not between experiment part 1 and 2.

Many substances, possibly from petroleum degradation, were found in the systems. This is an indicator for microbial activity. There were larger numbers of compounds in the FCFCS's with hydrogen/acetate, which suggests this combination to be a better stimulator of microbial activity.

The extracted substances from biofilm contained a lot of non-water-soluble esters which can be considered as residues from microbial degradation. When the organic content from the biofilm support material was extracted these esters came along with the solid material. These types of compounds are often seen in borders between a solid and liquid phase where the hydrophilic acid end of the esters is in the water phase and the other hydrophobic end of the long carbon chain is attached to the solid phase.

4.5   Summary

In part 1, there was a clear case that the FCFCS with biofilm consumed significantly more lactate and produced more sulfide, especially when the results were normalized to milligram produced sulfide per unit (Figure 4-2). In part 1 the SPB in biofilm were truly favoured by the fact that they were able to form biofilm on the support material in the flow cells. In part 2, the case was not as clear. As stated in the earlier, the sulfide-producing microbial population in both FCFCS's consumed similar amounts of acetate but the FCFCS containing biofilm produced more sulfide. The SPB population clearly also in part 2 had an advantage of being in a biofilm since those bacteria produced more sulfide. Adapting the logic from part 1 the amounts of produced sulfide should also be similar if the consumption of food was similar. A possible explanation could be a tougher competition for the nutrients due the easy accessibility of hydrogen took place in part 2 were other types of bacteria got to the nutrients first to a greater extent than in part 1. This would have been true for both planktonic and attached bacteria which correlates well with the results for the acetate and hydrogen consumption. In addition, lactate is a large molecule to digest and the attached bacteria in part 1 seemed to be able to assimilate the lactate faster and to a greater extent than the planktonic bacteria. This advantage would also be smaller with a smaller molecule as acetate, which also agrees well with the results. In summary, when growing on acetate and hydrogen the advantage of being in a biofilm for the SPB population was smaller, however still significant.

Previous studies with lactate and acetate/hydrogen as carbon and energy source for attached underground bacteria has shown that lactate is a potent energy source. Acetate on its own was shown to be a moderately good but together with hydrogen it becomes an energy source that is very readily available compared to lactate (Ekendahl and Pedersen 1994, Pedersen and Ekendahl 1992a). The results from this project indicate similar findings. Lactate as nutrient resulted in the sulfide concentration and they SPB were 41 times more active in the biofilm compared to the circulating water (Figure 4-2 and Table 4-2). In the more readily accessible combination of acetate/hydrogen the difference between the biofilm and circulating water was smaller. Here the SPB in biofilm were only 2 times more active compared to the circulating water. In addition, the FCFCS without biofilm consumed approximately the same amounts of hydrogen and acetate as the FCFCS with biofilm.

Molecular biology methods have not been used in this project. DNA has however been extracted from the biofilm support material from FC's in part 1 with good yields. Biofilm material has also been saved from part 2 but DNA has not yet been extracted. Hence, there is a possibility to perform DNA analysis where the DNA profiles from the different subprojects can be compared to see if there is a pattern or other information about the microbial sulfide producing population. For example, could comparison of the DNA-profile with respect to the aps-gene be done to see differences in the genetic profile when growing on lactate and hydrogen/acetate respectively.

Conclusion

In part 1 there was a big difference in sulfide production between bacteria in biofilm and planktonic bacteria. The bacteria in the biofilm produced 71 times more sulfide per unit than the planktonic bacteria and were 41 times more active. In part two the difference was much smaller; the attached bacteria only produced 6 times more sulfide per unit and were 2 times more active than the free-living planktonic bacteria. The sulfide and ATP-data from these two experiments tells us three things:

- Approximately the same amount of sulfide is produced by free-living planktonic bacteria regardless if they grow on lactate or hydrogen/acetate.

- The bacteria produce more sulfide when they are attached to a surface in a biofilm. However, the advantage of being in a biofilm seems less favourable for the bacteria when growing on hydrogen/acetate. Probably due the fact that hydrogen is a more readily accessible energy source compared to lactate.

- If a water-conducting fracture is 0.1 cm wide it would take 6 and 12 times more water than solid surfaces to get the same amount of ATP for part 1 and 2, respectively. These results indicate that bacteria are much more active in a biofilm than as free-living planktonic cells. In addition, one factor regulating the degree of activity and the level of advantage of residing in a biofilm or not is the carbon and energy source available.

References

SKB's (Svensk Kärnbränslehantering AB) publications can be found at www.skb.com/publications.

Ekendahl S, Pedersen K, 1994. Carbon transformations by attached bacterial populations in granitic groundwater from deep crystalline bed-rock of the Stripa research mine. Microbiology 140, 1565–1573.

Eydal H S C, Pedersen K, 2007. Use of an ATP assay to determine viable microbial biomass in Fennoscandian Shield groundwater from depths of 3–1000 m. Journal of Microbiological Methods 70, 363–373.

Hallbeck L, Pedersen K, 2012. Culture-dependent comparison of microbial diversity in deep granitic groundwater from two sites considered for a Swedish final repository of spent nuclear fuel. FEMS Microbiology Ecology 81, 66–77.

Jensen W B, 2007. The origin of the Soxhlet extractor. Journal of Chemical Education 84, 1913–1914.

Jägevall S, Rabe L, Pedersen K, 2011. Abundance and diversity of biofilms in natural and artificial aquifers of the Äspö Hard Rock Laboratory, Sweden. Microbial Ecology 61, 410–422.

Pedersen K, 1982. Method for studying microbial biofilms in flowing-water systems. Applied and Environmental Microbiology 43, 6–13.

Pedersen K, 2001. Diversity and activity of microorganisms in deep igneous rock aquifers of the Baltic Shield. In Fredrickson J K, Fletcher M (eds). Subsurface microbiology and biogeochemistry. Chichester: Wiley, 97–139.

Pedersen K, 2005. Äspö Hard Rock Laboratory. MICROBE. Analysis of microorganisms and gases in MICROBE groundwater over time during MINICAN drainage of the MICROBE water conducting zone. SKB IPR-05-29, Svensk Kärnbränslehantering AB.

Pedersen K, 2012. Subterranean microbial populations metabolize hydrogen and acetate under in situ conditions in granitic groundwater at 450 m depth in the Äspö Hard Rock Laboratory, Sweden. FEMS Microbiology Ecology 81, 217–229.

Pedersen K, 2013. Metabolic activity of subterranean microbial communities in deep granitic groundwater supplemented with methane and H2. The ISME Journal 7, 839–849.

Pedersen K, 2014. Microbial life in terrestrial hard rock environments. In Kallmeyer J, Wagner D (eds). Microbial life of the deep biosphere. Berlin: De Gruyter, 63–81.

Pedersen K, Ekendahl S, 1992a. Assimilation of CO_2 and introduced organic compounds by bacterial communities in groundwater from southeastern Sweden deep crystalline bedrock. Microbial Ecology 23, 1–14.

Pedersen K, Ekendahl S, 1992b. Incorporation of CO_2 and introduced organic compounds by bacterial populations in groundwater from the deep crystalline bedrock of the Stripa mine. Journal of General Microbiology 138, 369–376.

Pedersen K, Ekendahl S, Tullborg E-L, Furnes H, Thorseth I G, Tumyr O, 1997. Evidence of ancient life at 207 m depth in a granitic aquifer. Geology 25, 827–830.

Pedersen K, Arlinger J, Eriksson S, Hallbeck A, Hallbeck L, Johansson J, 2008. Numbers, biomass and cultivable diversity of microbial populations relate to depth and borehole-specific conditions in groundwater from depths of 4–450 m in Olkiluoto, Finland. The ISME Journal 2, 760–775.

SKB, 2010. Design and production of the KBS-3 repository. SKB TR-10-12, Svensk Kärnbränslehantering AB.

Nutrients, major components and metals

Table A-1. Nutrients, major components and metals analysed in the groundwater of KFM03A:4 at the time of the FCFCS installation and from the circulating water from FCFCS with and without biofilm at the end of experiment part 1. Red numbers indicate significant increase in relation to the other systems.

Element	Unit	KFM03A:4	FCFCS with biofilm	FCFCS without biofilm
Ca	mg/l	1640	1560	1550
Fe	mg/l	0.523	0.0376	1.45
K	mg/l	17.9	14.4	14.3
Mg	mg/l	58.2	53.6	53.4
Na	mg/l	2020	2120	2110
Si	mg/l	6.82	6.22	5.96
Al	µg/l	2.38	**19.2**	9.6
As	µg/l	n.a	0.155	< 0.1
Ba	µg/l	180	**284**	193
Cd	µg/l	< 0.05	0.0125	0.00663
Co	µg/l	< 0.05	0.0128	**0.186**
Cr	µg/l	< 0.1	57.6	**325**
Cu	µg/l	< 0.5	< 0.1	0.207
Hg	µg/l	< 0.002	0.0306	0.0332
Mn	µg/l	355	334	**514**
Mo	µg/l	**39.2**	0.643	4.41
Ni	µg/l	0.642	0.892	**10.9**
P	µg/l	< 40	2.96	2.44
Pb	µg/l	< 0.3	0.0964	0.129
Sr	µg/l	17100	17600	17700
Zn	µg/l	< 2	0.437	0.43
V	µg/l	< 0.05	**11.8**	1.54
S	mg/l	66.7	n.a	n.a
Br	µg/l	37900	n.a	n.a
I	µg/l	125	n.a	n.a
Li	µg/l	37.9	n.a	n.a
Total hardness	°dH	n.a	231	229
Turbidity	FNU	n.a	**310**	16
Conductivity	mS/m	1614	1590	1600
pH		7.7	7.4	7
Alkalinity	mg HCO3/l	19.3	**210**	50
CODMn	mg/l	n.a	293	394
Ammonium	mg/l	n.a	< 0.050	< 0.050
Ammonium nitrogen	mg/l	n.a	< 0.040	< 0.040
Phosphate	mg/l	n.a	< 0.040	< 0.040
Phosphor	mg/l	n.a	< 0.010	< 0.010
Nitrate	mg/l	n.a	0.73	0.55
Ion	**Unit**	**KFM03A:4**	**FCFCS with biofilm**	**FCFCS without biofilm**
Nitrate	mg/l	n.a	0.16	0.12
Fluoride	mg/l	1.53	**240**	**301**
Chloride	mg/l	5649	**6040**	5520
Sulfate	mg/l	185.3	49	160
Nitrite	mg/l	n.a	< 1.50	< 0.400
Nitrite nitrogen	mg/l	n.a	< 0.375	< 0.100

Table A-2. Nutrients, major components and metals analysed in the groundwater of KFM03A:4 at the time of circulation start and from the circulating water from FCFCS with and without biofilm at the end of the experiment, part 2. Red numbers indicate significant difference in relation to the other systems.

Element	Unit	KFM03A:4	FCFCS with biofilm	FCFCS without biofilm
Ca	mg/l	1580	1570	1560
Fe	mg/l	0.345	0.0194	0.00413
K	mg/l	14.5	13	12.9
Mg	mg/l	49.3	50.3	51
Na	mg/l	2050	2040	2050
Si	mg/l	5.66	5.7	5.73
Al	µg/l	3.37	4.5	1.43
As	µg/l	<0.1	0.313	0.145
Ba	µg/l	149	144	159
Cd	µg/l	0.0368	0.0402	0.0245
Co	µg/l	0.0155	0.0079	0.0145
Cr	µg/l	0.656	0.19	1.31
Cu	µg/l	<0.2	<0.1	0.15
Hg	µg/l	<0.002	0.00684	0.0129
Mn	µg/l	264	**67.2**	239
Mo	µg/l	**16.4**	43.7	37
Ni	µg/l	0.512	0.825	1.02
P	µg/l	<2	4.93	1.28
Pb	µg/l	0.0679	<0.01	<0.01
Sr	µg/l	18900	18300	18300
Zn	µg/l	<0.4	0.96	<0.2
V	µg/l	0.0267	0.0342	0.0358
total hardness	°dH	233	231	230
turbidity	FNU	1.8	0.46	<0.2
conductivity	mS/m	1600	1590	1590
pH		7.3	7.9	8.3
alkalinity	mg HCO3/l	14	26	23
Nitrite	mg/l	<0.0328	<0.0050	<0.0050
Nitrite nitrogen	mg/l	<0.0100	<0.0020	<0.0020
CODMn	mg/l	11.7	16	14.5
Ammonium	mg/l	<0.320	0.109	0.102
Ammonium nitrogen	mg/l	<0.248	0.084	0.079
Phosphate	mg/l	<0.040	<0.040	<0.040
Phosphate phosphor	mg/l	<0.010	<0.010	<0.010
Nitrate	mg/l	<2.00	<1.00	<2.00
Nitrate nitrogen	mg/l	<0.40	<0.10	<0.10
Fluoride	mg/l	1.39	1.93	1.7
Chloride	mg/l	5820	5760	5540
Sulfate	mg/l	203	**178**	216

SKB is responsible for managing spent nuclear fuel and radioactive waste produced by the Swedish nuclear power plants such that man and the environment are protected in the near and distant future.

skb.se