P-14-18

Further methods development for sampling and analysis of microbial abundance and distribution on fracture surfaces in granitic bedrock

Lena Eriksson, Andreas Bengtsson, Johanna Edlund,
Björn Hallbeck, Linda Johansson, Lisa Rabe,
Frida Ekström, Karsten Pedersen

Microbial Analytics Sweden AB

December 2014

Svensk Kärnbränslehantering AB
Swedish Nuclear Fuel
and Waste Management Co

Box 250, SE-101 24 Stockholm
Phone +46 8 459 84 00

ISSN 1651-4416
SKB P-14-18
ID 1431173

Further methods development for sampling and analysis of microbial abundance and distribution on fracture surfaces in granitic bedrock

Lena Eriksson, Andreas Bengtsson, Johanna Edlund,
Björn Hallbeck, Linda Johansson, Lisa Rabe,
Frida Ekström, Karsten Pedersen

Microbial Analytics Sweden AB

December 2014

This report concerns a study which was conducted for SKB. The conclusions and viewpoints presented in the report are those of the authors. SKB may draw modified conclusions, based on additional literature sources and/or expert opinions.

Data in SKB's database can be changed for different reasons. Minor changes in SKB's database will not necessarily result in a revised report. Data revisions may also be presented as supplements, available at www.skb.se.

A pdf version of this document can be downloaded from www.skb.se.

Abstract

This report describes the progress of project number two, in order of four, to develop methods for sampling, detection and analysis of microbial biofilms on fracture surfaces in the bedrock. As the majority of microorganisms in the subsurface environment are assumed to adhere on surfaces, modelling of biogeochemical processes based solely on analyses of groundwater thus becomes uncertain with large margins of error. The projects is performed as commissioned by SKB, Swedish Nuclear Fuel and Waste Management Company, to develop methods to be used during investigations of fracture surfaces on site for investigations for long term storage of nuclear waste in Forsmark, Sweden. The project has so far proved to be useful to detect microorganisms on natural fracture surfaces on a drill core, retrieved from drilling at 400 m depth in the Äspö research site. In this part project, the main focus was to develop a method to enrich microbial biofilms on surfaces, *in situ,* on the sites for investigation. The enrichment was considered necessary because biofilms in natural environment contain very small amounts of DNA, often below detection level for nucleic acid analysis. The method used flowcells, containing enrichment surface materials, connected to tubes leading groundwater out of the bedrock. The intention was to lead the groundwater through the flowcell at ambient pressure, where some of the planktonic microorganisms would adhere and form biofilms on the enrichment surfaces. To allow the microorganisms enough time to form biofilms, the flowcells were connected to flowing groundwater at the sites from three weeks up to three months. When the enrichment surfaces were analysed at the laboratory, the results showed that it was possible to enrich detectable amounts biofilms using either microbiological or nucleic analysis.

Sammanfattning

Denna rapport beskriver status i ett utvecklingsprojekt, delförsök nummer två i ordningen av fyra, med avseende att ta fram metoder för provtagning samt detektion av biofilmer på sprickytor i berggrund. Då majoriteten av alla mikroorganismer i underjorden antas sitta fast på ytor, blir modellering av biogeokemiska processer baserad enbart på analyser av grundvatten därmed osäker med stora felmarginaler. Syftet var att ta fram metoder som kan användas för provtagning samt detektion av mikoorganismer på sprickytor från borrningar i Äspötunneln samt vid platsundersökningar i Forsmark för långtidsförvaring av kärnbränsleavfall. I projektet har hittills metodik för provtagning och detektion testats och utvecklats som visat att det var möjligt att upptäcka mikroorganismer på naturliga sprickytor i en borrkärna som hämtades från borrning vid 400 m djup i Äspölaboratoriets tunnel. I detta delförsök har en metod för anrikning av mikrobiella biofilmer på ytor vidareutvecklats, då det ansågs nödvändigt eftersom biofilmer i naturlig miljö innehåller mycket liten mängd DNA, ofta under detektionsnivå för molekylära analysmetoder. Metoden utformades för anrikning av biofilmer *in situ,* i s.k. flowcells, cylinderformade behållare innehållande olika typer av ytmaterial, vilka kopplades till grundvatten som pumpades upp ur borrhål med olika djup i berggrunden. Grundvattnet flödades genom inkopplad flowcell under veckor till månader, för att låta mikroorganismer fästa till och därmed kunna bilda biofilmer anrikningsytorna. Resultaten visade att det var möjligt att anrika detekterbara mängder biofilmer för både mikrobiologiska och molekylärbiologiska analysmetoder.

Contents

1 Introduction 7

2 Materials and methods 9
2.1 Phase 1: Enrichment surface materials 9
2.1.1 Selection of surface material for enrichment of biofilms 9
2.1.2 Enrichment surfaces of rock 9
2.1.3 Enrichment surfaces of glass 9
2.2 Enrichment, sampling and analysis 9
2.2.1 Enrichment of microbial biofilms 9
2.2.2 Sampling 9
2.2.3 ATP assay 10
2.2.4 DNA extraction assay 10
2.2.5 Estimates of the amount of biofilms in the test material 10
2.3 Phase 2: Biofilms enrichment *in situ* 11
2.3.1 Enrichment *in situ* 11
2.3.2 Sampling methods 11
2.3.3 ATP-analysis 11
2.3.4 DNA extraction 12

3 Results 13
3.1 Rock enrichment surface 13
3.2 Glass enrichment surface 13
3.3 Comparison between the detection methods 13
3.4 Other observations at the *in situ* trials 13

4 Discussion 17
4.1 Comparison between the enrichment surfaces 17
4.2 ATP-analysis versus DNA-extraction 17
4.3 Additional factors affecting the biofilms enrichment 17
4.4 Future experiments and method developments 17

References 19

1 Introduction

This report presents the work of developing methods for the sampling and detection of biofilms on fracture surfaces in bedrock. Previous projects (Jägevall et al. 2011) showed that it was difficult to obtain sufficient amounts of DNA to be detected with the methods available in molecular biology. A method development ordered by SKB was therefore performed in 2011, where the previous tests were supplemented with additional methods for sampling and measuring the quantity of biofilms. These methods had a higher sensitivity than the molecular methods that were used earlier (Bengtsson et al. 2013).

A major methodological development for SKB divided into a four part experiment was then instigated in 2012. This was to further develop methods for the study of microbial abundance and distribution of fractures in natural granitic rock aquifers, including this interim report that is number two in the series. In the first part of the experiment (Bengtsson et al. 2013), a methodology for enriching biofilms in the laboratory was adopted and these methods were developed to eventually be used *in situ*. After enrichment, microbial biofilms was detected with both microbiological and molecular methods, both of which showed promising results.

Part two of the method development was divided into two phases. The emphasis in the first phase was on the selection of enrichment surfaces, sampling of the biofilms and the optimization of our analytical methods. For detection and estimation of the amount of biofilms after enrichment, we optimized our methods to extract ATP and DNA. The results of the measured amounts of extracted ATP or DNA were then used to calculate the estimated number of cells per cm^2 of the enrichment surfaces. Phase number two continued the efforts *in situ*, by trials to enrich biofilms on the surfaces of the natural microbial environment. Groundwater from boreholes in the bedrock was connected to flowcells containing enrichment surfaces of both rock and glass. By allowing groundwater to flow through the flowcell, our hypothesis was that planktonic microbes found in the groundwater would attach and form biofilms. The enrichment time for the each borehole ranged between 3 to 8 weeks and trials were carried out both in the Äspö tunnel outside Oskarshamn, Sweden and at Olkiluoto, Finland. In Olkiluoto we had access to boreholes at the research facility for repository of high and intermediate level nuclear waste. Our hope was also to get sufficient samples of material from these trials to be able to perform a genomic sequencing of DNA from these areas. With the genomic data collected from the samples we would be able to identify and map the species of microbes that could be found at the different bedrock research sites. Documented material, methods and data from these studies will form the basis for future sampling and assist in the process of enriching biofilms on the fracture surfaces in the bedrock.

2 Materials and methods

2.1 Phase 1: Enrichment surface materials

2.1.1 Selection of surface material for enrichment of biofilms

One of the main criteria when selecting materials for the enrichment attempts was to select the ones that had the highest ability for microbes to attach to the surfaces, thus increase the amount of the sample material. It was also important to use surfaces that resemble natural materials, i.e. to mimic fracture surfaces in the bedrock.

2.1.2 Enrichment surfaces of rock

In previous experiments regarding the enrichment of biofilms we used grains of bedrock that came from the same geographical origin as the borehole where the microbes and groundwater came from. These rock surfaces had a tendency to be porous and the unattached minerals and particles often disrupted the subsequent analysis of the biofilms. We therefore decided to use a commercially available enrichment surface consisting of crushed garnet minerals (hereafter the garnet will be referred to as "rock"). They have an average size of 0.70 millimetres per grain and were supplied sterile. They are inert to the surrounding particles and reagents in order to not affect the downstream analysis after DNA extraction.

2.1.3 Enrichment surfaces of glass

As an alternative material to rock, we had previously used slides of glass as an enrichment surface. In earlier experiments we had observed that microbes attached to and formed biofilms on these slides and they were also relatively easy to detect under a microscope (Bengtsson et al. 2013). The slides, however, required more space in the flow cell and generated a smaller overall surface area for microbes to attach to. We therefore performed a test using glass beads as the enrichment surface. Thanks to the spherical shape of the beads, the glass required less space in the flow cell, but had a larger surface area for enrichment compared with the glass sheets used in previous attempts. The glass beads used in the experiments were 1 millimetre in diameter and were sterilized in a muffle furnace at 450°C for 4.5 hours.

2.2 Enrichment, sampling and analysis

2.2.1 Enrichment of microbial biofilms

The first phase was performed by using a bacterial culture infused in a saline medium that were circulated in an anaerobic pumping system, as seen in the picture below. The circulating bacteria in saline solution were led through two flow cells with holders containing the glass beads and the crushed rock for the biofilms enrichment. The bacterial culture used for the enrichment was of the species *Shewanella putrefaciens*, containing cell numbers of approximately $2.5 \cdot 10^5$ cells mL^{-1}, which formed well detectable amounts of biofilms on surfaces. The enrichment in the circulation system generated plenty of sample material to be used for optimization of sampling and detection methods. Enriching biofilms from natural groundwater is more of a challenge, as this contains less number of cells, usually in the range of 10^4–10^5 cells mL^{-1}. It was therefore very important to optimize the enrichment conditions, such as flow rate, pressure in the flow cell and type of surface material, to gain sufficient sample material of biofilms to be detected.

2.2.2 Sampling

The flow cell was opened and portions of the surface materials were collected for detection and analysis for presence of biofilms. Each surface material of glass and rock was handled separately and rinsed with sterile DNA and RNase free phosphate buffered saline (PBS) to remove planktonic microbes, since we only wanted to detect the adhered microbes. The sample materials were distributed into tubes and any excess fluid was removed before the sample went on to downstream analysis.

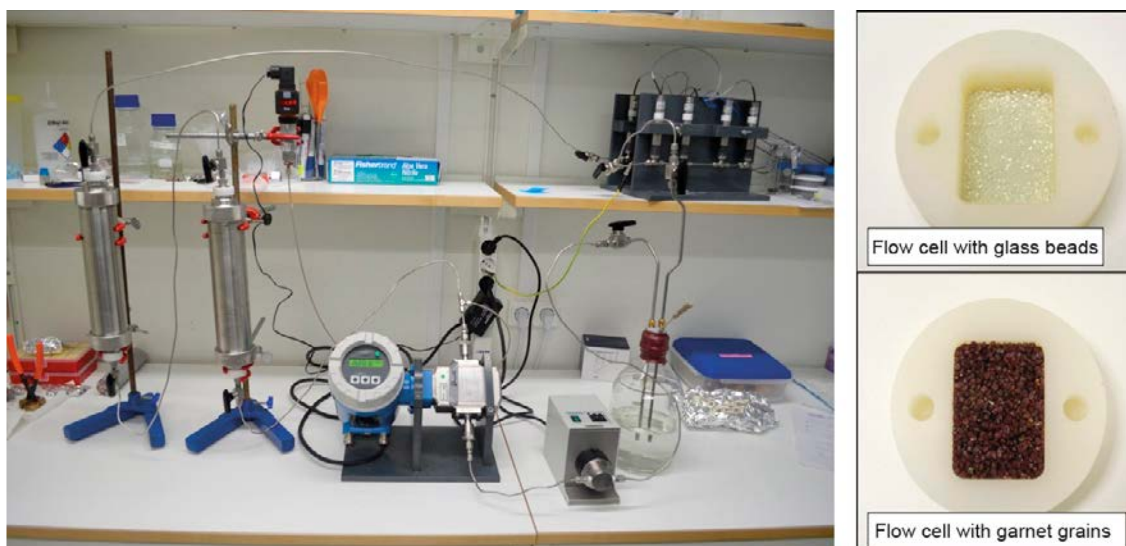

Figure 2-1. Circulating system in the laboratory for enrichment of biofilms on glass and garnet beads in the flowcells. At the top right, picture from above of a flowcell filled with glass beads and below with chrushed garnet grains.

2.2.3 ATP assay

Because biofilms are difficult to remove from surfaces, which are necessary in order to dissolve the cell walls and release the intracellular ATP, we extended the extraction time before the analysis. Except for that, the ATP assay was performed according to the method described by Eydal and Pedersen (2007), directly on the enrichment surfaces of rock or glass.

2.2.4 DNA extraction assay

Optimization tests for extraction of DNA from the biofilms were made in the laboratory experiments with two different kits; Power Water DNA Isolation Kit and Power Biofilms DNA Isolation Kit, both from MoBio. The principal of both these kits is to lyse the microorganisms by a combination of heat, detergent, and mechanical force against specialized beads. The cells are lysed both chemically and mechanically in around 10 minutes by shaking on a vortex. The released DNA is then bound to a silica spin filter, washed and the DNA is recovered in Tris-buffer. The kits only differ in content by one lysing reagent, which comes with the Power Biofilm kit, that are supposed to have higher efficiency to lyse glycolipids in biofilms. The kits were evaluated if any of these gave a higher amount of DNA after the extraction of the biofilms from the samples. Extraction was performed according to the supplier's manual, directly on the surfaces. We had to adjust the amount of reagent for the Power Biofilm Kit, since it is only intended to use on very small amounts of sample material. Quantification of the extracted DNA was performed by using Quant-It Pico green High Sensitive Kit 0–20 ng, which was supplied by Invitrogen.

2.2.5 Estimates of the amount of biofilms in the test material

After ATP analysing and DNA extraction, the enrichment material was washed thoroughly in tap water and dried over night at 105°C. The clean and dry enrichment material was then weight and by dividing the total weight by the weight of each bead or grain, the numbers of beads/grains in the material were calculated. The total area of enriched material was then calculated by multiplying the numbers of grains/beads by the calculated area of each bead/grain in cm^2.

Each rock grain had an approximate diameter of 0.7 millimetres and a weight of about 3.51 milligrams per grain. The corresponding parameter per glass bead was 1 millimetre in diameter and it weighed approximately 2.22 milligrams per bead.

The following formulas were then used to calculate the average area of enrichment in cm^2:

1 glass bead (1 mm in diameter) weighed 2.22 milligrams. The surface area per 1 mm glass bead was calculated by the formula $4\,\pi \cdot r^2$. k. The average area of each glass bead was $3.14 \cdot 10^{-3}\,cm^2$. The total enrichment area of each sample was then calculated by dividing total weight of the enriched material by the weight for each bead, to get the number of beads. To get the total enrichment area, the numbers of beads were multiplied with the average area of each bead in cm^2.

1 grain of the rock (diameter = 0.7 mm) weighed in average 3.51 milligrams. The surface area of $1.54 \cdot 10^{-3}\,cm^2$ per grain was calculated using the formula $4\,\pi \cdot r^2$. The grains are not exactly smooth and spherical, but they are quite round grains, with a size of 0.7 mm in diameter, and thus we chose to use the same formula as for the glass beads. The total enrichment area of each sample was weighed and the then calculated by dividing the weight of each grain to get the number of grains in the enrichment material. The number of grains was multiplied with the total area of each grain in cm^2.

The results of detected and quantified amount of extracted ATP and DNA in each sample were recalculated on terms of the equivalent number of cells on enrichment area as follows:

- By assuming that each bacterial cell contains 0.4 amol ATP (Eydal and Pedersen 2007), the amounts of measured ATP per sample were divided with 0.4 for the total number of cells. The results were then divided by the calculated enrichment area in cm^2 for each sample.
- We assumed that the amount of DNA content in each cell is equivalent to that of *Desulfovibrio aespoeensis*, a common microbe in deep biosphere groundwater. The DNA has an average weight of $3.9 \cdot 10^{-15}$ g $cell^{-1}$ (Pedersen et al. 2014). The amount of extracted DNA was divided by the assumed weight of DNA per cell to calculate the total number of cells in each sample. The number of cells was divided by the total enrichment area to estimate the number of cells per cm^2 in each sample.

2.3 Phase 2: Biofilms enrichment *in situ*

2.3.1 Enrichment *in situ*

For the *in situ* experiments the flow cells were prepared with sterile enrichment surfaces. Each flow cell was connected to flowing groundwater from boreholes in the bedrock, either in the Äspö tunnel in Sweden or in Olkiluoto, Finland. The groundwater was flowed through each connected flow cell, for between three to nine weeks, allowing microbes to form biofilms on the surface materials. Enrichment time also depended on when we had access to the sampling site in the tunnels. During the enrichment period the flow rate of groundwater through each flow cell was noted in regular intervals, in order to identify any factors that could affect the microbe's ability to attach and form biofilms. We hypothesized that a higher water flow would increase the number of microbes that passed the enrichment surfaces and thereby extend the time to get into the flow cell and form biofilms. But a higher water flow could also mean increased turbulence, with the result that it becomes more difficult for microbes to attach, as they are flushed by too fast. In Finland we could not influence the flow rate from surface boreholes during the enrichment time, since the boreholes during this period were occupied for additional sampling for other parameters. Therefore we could only record data and observations from these experiments. During the collection of samples in the Äspö and ONKALO tunnels, we were able to adjust the influence of flow rate and pressure in each flow cell.

2.3.2 Sampling methods

After enriching, the flowcell was detached from the borehole and transported back to the laboratory for further tests and analysis. The excess groundwater was removed from the flowcell, leaving only the surface material. The materials were divided into rock and glass and washed with PBS to remove any planktonic microbes before proceeding with further analysis.

2.3.3 ATP-analysis

An ATP analysis was performed according to the optimization of the earlier methods on glass and rock surfaces. After analysing the materials, they were washed, dried and weighed in order to calculate their total surface area in cm^2.

Figure 2-2. *A flowcell connected to borehole ONK-PVA8, for biofilm enrichment in the ONKALO tunnel, Finland.*

2.3.4 DNA extraction

During all of the earlier experimental DNA extractions, the best results were obtained while using the Power Water DNA isolation kit. Therefore we decided to continue using this when analysing the materials from the *in situ* trials. We suspected that even with the enrichment time given, there would not be much biofilms to detect and because of this as much as possible were extracted from each enrichment material. As with the ATP-analysis, the materials were kept separated, washed, dried and weighed in order to calculate their total surface area in cm^2.

Figure 2-3. *A flowcell connected to borehole KA0069 for biofilm enrichment in the Äspö tunnel, Sweden.*

3 Results

3.1 Rock enrichment surface

A total of 10 samples were collected *in situ* from different boreholes, and we could detect and quantify biofilms in all of them using the ATP-analysis. The concentrations of nucleic acids were measured after completing a DNA-extraction, and were noticeable in 7 of the samples. (See Table 3-1.) The 3 enrichment surfaces where the levels of extracted nucleic acids were below detection level were stated as < 0.02 ng/µL and no further calculations were made. These 3 samples also had the lowest measured ATP-levels. The average number of cells on the rock samples was between 10^5 and 10^6 per cm^2, measured and calculated in accordance to the extracted DNA. The number of cells calculated using the ATP-analysis was between 10^4 and 10^7 per cm^2. In all tests but one, we detected more biomass on the rock than we did on the glass beads.

3.2 Glass enrichment surface

The number of cells per cm^2 on the glass surface was calculated using the results from the ATP-analysis and the extracted DNA, the same as in Section 2.2.5. In all the tests but one, the microbes seemed to prefer attaching and forming biofilms on the rock surface rather than on the glass. (see Figure 3-2.) The same pattern could be observed after both the ATP-analysis and the DNA extraction. After DNA-extraction, it was calculated that the average number of cells were between 10^4 and 10^6 per cm^2, compared to between 10^3 and 10^8 per cm^2 after using the ATP-analysis.

3.3 Comparison between the detection methods

The ratio between the two methods of analysis concluded that we could detect more biofilms using the ATP-analysis compared to the DNA quantification. Since there are very small amounts of the sample materials to begin with, it is important to take the loss of material into consideration when making a comparison between methods. Also, we do not know the exact amount of amol ATP or DNA per cell, as the samples include a number of different microbes. Since it is not clear what kind of different microbes that are in the samples we can only do a preliminary comparison between the two analysis methods. However, we can be certain that the results we got are at least the minimum quantities that were in the samples.

3.4 Other observations at the *in situ* trials

The highest levels of biofilms were detected using ATP-analysis when the flow rate of water was between 150 to 350 mL min^{-1}. After enrichment from the borehole using a lower flow rate than 150 mL min^{-1}, only small amounts of biofilms were detected even though the enrichment time were longer in that trial.

Table 3-1. Detailed chart for the *in situ* enrichment of biofilms in the Äspö tunnel and Olkiluoto, Finland.

Borehole	Depth (m)	Enrichment time (days)	Flow rate mL min^{-1}	Ground water (L)	ATP in water (amol mL^{-1})	ATP biofilms (rock) amol ATP/cm^2	ATP biofilms (glass) amol ATP/cm^2	DNA biofilms (rock) g/cm^2	DNA biofilms (glass) g/cm^2	Ratio (rock) ATP/DNA cells/cm^2
Äspö										
KA3110A	400	33	320	1.52E+04	–	2.75E+05	6.17E+04	5.40E–10	3.20E–10	4.97
KA3385A:1	420	33	285	1.35E+04	–	9.00E+03	1.00E+03	< 0.02	< 0.02	–
KA0069	450	33	1,635	7.77E+04	–	7.95E+03	1.86E+03	2.20E–09	6.40E–10	0.04
KA2198	300	33	293	1.39E+04	–	1.97E+06	8.47E+07	4.30E–09	1.70E–08	4.47
Olkiluoto										
OL-KR55	286.9–288.4	29	240	1.00E+06	2.23E+04	8.29E+05	7.88E+03	3.50E–09	1.90E–09	2.31
OL-KR57	57–61	35	225	1.13E+07	3.90E+03	9.68E+04	5.68E+03	< 0.02	< 0.02	–
OL-KR56	1,154–1,158	33	160	1.61E+04	4.89E+04	1.81E+05	1.42E+04	3.80E–09	5.80E–09	0.46
ONK-PVA8	293	67	1	9.65E+02	1.70E+05	1.67E+05	1.29E+04	4.80E–09	1.20E–09	0.34
OL-KR55	863–866	27	150	9.70E+01	7.20E+03	6.35E+06	7.48E+05	1.70E–08	1.04E–09	3.64
OL-KR54	364.5–368	30	160	6.91E+03	3.80E+03	3.31E+04	3.32E+03	< 0.02	< 0.02	–

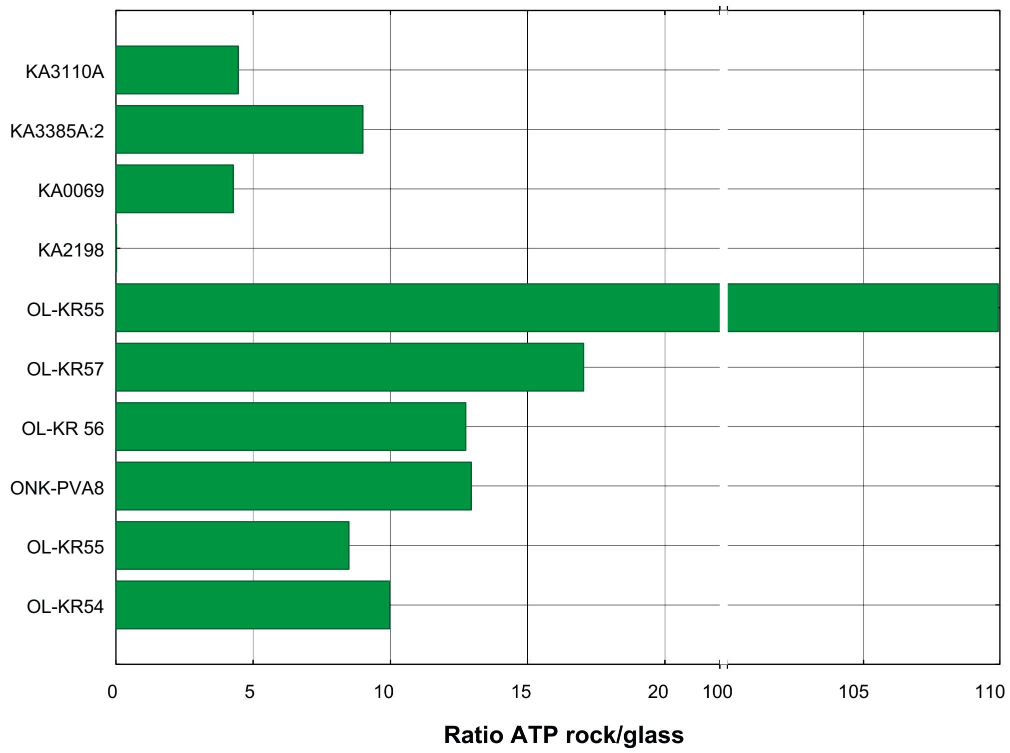

Figure 3-1. *Ratio of detected biomass using ATP on rock compared to glass.*

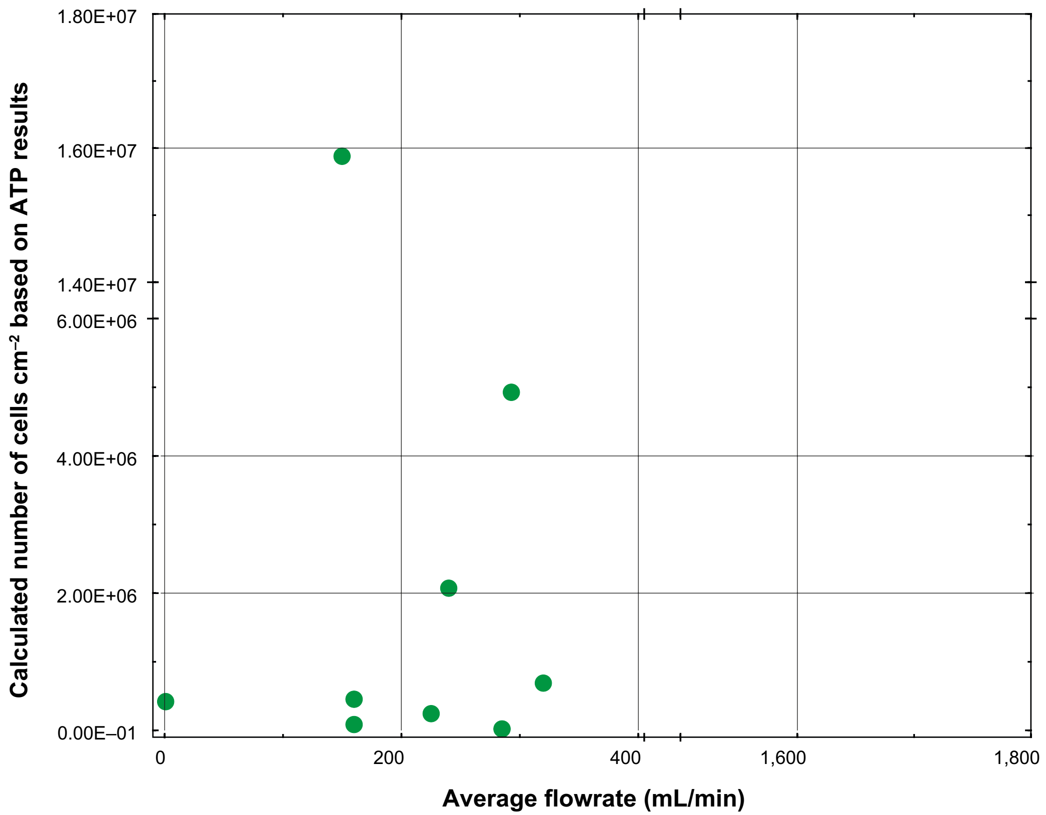

Figure 3-2. *Detected amount of biomass compared to an increasing flow rate.*

4 Discussion

4.1 Comparison between the enrichment surfaces

In all of the trials we used glass beads as an alternative enrichment surface to the rock, in order to see if any of the surface materials attracted more microbes than the other. It would also be interesting to examine whether some species of microbes prefer a certain surface rather than the other. This comparison will be executed 2014 by studying the sequence libraries from glass and rock biofilms. In all the trials except for one, the results pointed towards rock being the preferred surface, as more biofilms were detected there than on the glass.

4.2 ATP-analysis versus DNA-extraction

A comparison between the two measuring methods proved that we consistently extracted less DNA than ATP, when calculated into cells per cm^2. We saw the same results for both the rock and the glass surfaces. Since ATP can only be measured in living cells while DNA can be detected in both living and dead cells, the results should be the opposite. However, we do know that the results from DNA extractions, even with optimized methods, typically turns out to be lower than 100% of what was originally in the sample. Usually this does not affect the final result more than one factor of two. Our hypothesis is therefore that the results show the least amount of microbes that we can expect to find per cm^2 on either rock or glass using our measuring methods.

4.3 Additional factors affecting the biofilms enrichment

There are some factors that can influence the microbes ability to adhere on surfaces and form biofilms, such as the flow rate, the depth, salinity or the natural occurrence of microbes in the water. These factors varied a lot between the boreholes during the *in situ* trials. As we did not have the ability to affect these factors, more than adjusting the actual enrichment time, we cannot come to any certain conclusions, but only note what we can read from the data in Table 3-1 and Figure 2-3. Here we can see that the highest levels of biofilms were detected using ATP-analysis and when the water flow was set to between 150 and 350 mL min^{-1}. This could indicate that using a higher flow rate will have a negative effect on the microbes ability to adhere to surfaces and form biofilms as they are flushed through the flowcell too fast. To prevent this from happening in future attempts when using a high flow rate, the enrichment time should be prolonged accordingly. Also, if the flow rate in the borehole is lower than 150 mL min^{-1}, that could have a negative effect on the microbes ability to form biofilms, since the low flow rate would allow less water to pass through the flowcell, and thereby giving less microbes the chance to adhere to the enrichment surfaces. It is essential to prolong the enrichment time in both cases, but also in the attempts where we actually detected biofilms. That would give us more sample materials to work with during the analysis.

4.4 Future experiments and method developments

All the extracted DNA samples from the enrichment surfaces were kept for sequencing in order to examine what species of organisms were detected from each borehole. This data will be processed using bioinformatics in phase 3 of the experiment during 2014. We will keep developing our methods regarding the enrichment of biofilms in groundwater from boreholes, both sampling methods and analysis. In phase 3 the main focus will be on sampling and detection of biofilms on recently fractured surfaces where there is no access to groundwater or enrichment. We are continuing the efforts to further improve our methods for sampling, cultivation and analysis in order to identify and map the microbes that live in the bedrock.

References

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

Bengtsson A, Eriksson L, Pedersen K, 2013. Methods development for analysis of microbial abundance and distribution of fractures in natural granitic rock aquifers. SKB P-13-52, Svensk Kärnbränslehantering AB.

Eydal H S C, Pedersen K, 2007. Use of an ATP assay to determine viable microbial biomass in Fennoscandian Shield groundwater from depths of 3–1,000 m. Journal of Microbiological Methods 70, 363–373.

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, Bengtsson A F, Edlund J S, Eriksson L C, 2014. Sulphate-controlled diversity of subterranean microbial communities over depth in deep groundwater with opposing gradients of sulphate and methane. Geomicrobiology Journal 31. doi:10.1080/01490451.2013.879508.