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Development and evaluation of methods for sampling and analysis of bacterial 16S rDNA diversity in groundwater and biofilms of hard rock aquifers

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Abstract

Active, growing, and diverse populations of attached microorganisms on solid-water interfaces, so called biofilms, in deep groundwater was discovered more than two decades ago. It was suggested that biofilms could influence geochemical rock-water interactions such as dissolution and precipitation processes. When such biofilms develop in natural aquifers, they could influence the mobility of radionuclides escaping from future spent nuclear fuel repositories. This report presents the third part in a series of four parts of a method development programme to sample, analyse and characterize microbial biofilms on solid surfaces in deep aquifers. During drilling of a 2500 m deep hole close to the mountain Åreskutan in Jämtland, fracture surfaces from rock-groundwater and rock-rock interfaces were sampled for quantification of adenosine-5'-triphosphate (ATP) and DNA. Before the drilling was started, laboratory trials were conducted to find the best way to sample with different types of sampling swabs and sample storage techniques. New types of swab materials were successfully tested and utilized for sampling of biofilm materials from newly drilled fracture surfaces. Sampling was easy to perform and the swab samples could be stored at room temperature. Further, this report describes how a pressure filtration method was developed to collect enough quantities of DNA for diversity analysis of sequence libraries. This filtering method can be used for *in situ* sampling of sparsely populated groundwater. By high pressure filtration of a large volume of groundwater (> 100 L) the amount of cells for DNA extraction was significantly increased compared to smaller volumes (0.5–2 L) collected and filtered in the laboratory. In addition to obtain more DNA, filtering a large volume of groundwater will reduce possible variations in cell numbers and diversity over volume compared to smaller volumes of groundwater. Finally, a part of a gene in the extracted DNA from pressure filtrated groundwater from four boreholes in the Äspö hard rock laboratory tunnel and DNA from biofilms in flow cells (FCs) attached to these boreholes were sequenced. The FCs were loaded with glass beads and garnet grains as solid supports for biofilm development. With this method, the diversity of the microbial communities could be thoroughly characterized using 454 FLX Titanium 16S rDNA v6v4 pyrosequencing. Differences in community composition between samples were detected and, due to the high number of sequences obtained, rare microorganisms present at only low levels, i.e., below 0.1 % of the community, was found. The amounts of biomasses and the DNA library diversities in groundwater and on the solid materials were compared and evaluated.

Sammanfattning

För över 20 år sedan upptäcktes att det finns aktiva, växande och komplext sammansatta populationer av mikroorganismer på sprickytor i djupa grundvatten. Dessa fastsittande mikroorganismer benämns ofta för biofilmer och man fann att biofilmer kan inverka på utbytesreaktioner mellan grundvatten och berget genom utfällnings- och upplösningsreaktioner. När sådana biofilmer växer i naturliga spricksystem med grundvatten har man funnit att de kan påverkar hur radionuklider rör sig från ett slutförvar för radioaktivt avfall om det uppstått en läcka i en kapsel. Den här rapporten beskriver den tredje i en serie av fyra delar av ett forskningsprogram som syftar till att utveckla metoder för att provta, analysera och karaktärisera mikrobiella biofilmer på fasta ytor i akvifärer på stora djup. Under 2014 borrades ett 2500 m djupt hål nära Åreskutan i Åre, Jämtland. Under borrning kunde forskare provta sprickytor som varit exponerade för grundvatten och sprickytor som historiskt varit i kontakt med grundvatten men som vid provtagning läkts ihop och därmed var utan vatten. Dessa sprickytor provtogs för analys av adenosin-5'-trifosfat (ATP) och DNA. Innan provtagningarna i Åre startades genomfördes en metodutveckling i laboratoriemiljö för att testa olika typer av provtagningssvabbar och ta fram den bästa metoden för att lagra proverna fram till dess analys blev möjlig. En ny typ av svabb på marknaden visade sig vara mycket lämplig när den testades på biofilmerna i borrkärnor från borrhålet i Åre. Provtagningen var enkel att utföra och proverna för DNA analys kunde förvaras i rumstemperatur. Prover för ATP förvarades i frysbox. En filtreringsmetod för insamling av förhållandevis stora mängder DNA från grundvatten har också utvecklats inom detta forskningsprogram. Metoden är lämplig för grundvatten med låga antal mikroorganismer. Genom att under högt tryck filtrera en större volym (> 100 L) grundvatten direkt från borrhål i underjordsanläggningar fick man ett betydligt bättre utbyte av DNA vid extraktionen jämfört med filtrering av enstaka litrar i laboratoriet. Förutom ett bättre utbyte av DNA innebär filtrering av en stor volym att eventuella variationer i antal och sammansättning av mikrobpopulationerna i grundvattnet utjämnas. En del ave en gen i det DNA som insamlats genom filtrering, och det DNA som insamlats från mikroorganismer från fasta ytor i flödesceller har sekvensbestämts. Flödescellerna laddades med glaskulor och korn av granatmineral på vilka mikroorganismerna kunde fästa och växa. Med hjälp av denna flödescellmetod kunde mångfalden i biofilmer analyseras med 454 sekvenseringsteknik där en del av DNA:t i en förutbestämd gen, här 16S rDNA, sekvensbestämdes. Likheter och olikheter mellan biofilmer från olika grundvatten och på de två olika materialen kunde analyseras i stor detalj eftersom metoden går på djupet i data och även fångar upp sällsynt förekommande mikroorganismer, långt under en förekomst på 0,1 % av totala antalet förekommande arter och släkten. Med denna metod klarlades tydliga skillnader så väl som likheter i populationerna som växte på de olika analyserade typerna av material och grundvatten.

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1 Introduction

Active, growing, and diverse populations of attached microorganisms on solid-water interfaces, so called biofilms, in deep groundwater were discovered more than two decades ago (Ekendahl et al. 1994, Ekendahl and Pedersen 1994, Pedersen and Ekendahl 1992). This discovery indicated that biofilms could influence geochemical rock-water interactions such as dissolution and precipitation processes. Accordingly biofilms have been investigated and found to influence the sorption of trace elements and radionuclides onto glass and rock surfaces (Anderson et al. 2006). When biofilms develop in natural aquifers, they, consequently, may influence the mobility of radionuclides escaping from future spent nuclear fuel (SNF) repositories. Hence, it has been deemed crucial to investigate the presence, diversity and activity of biofilm microorganisms under *in situ* conditions relevant for aquifers leading to and from future SNF repositories. This report presents the third part in a series of four parts of a method development programme to sample, detect and identify microbial diversity of biofilms in the deep aquifers.

The first of two published reports from the method development programme describes how biofilms could be investigated using microscopic analysis of total number of cells (TNC), the analysis of adenosine-5'-triphosphate (ATP), DNA extraction and cultivation methods for aerobic and anaerobic microorganisms (Bengtsson et al. 2013). Different methods for sampling of microorganisms attached to rock surfaces were developed and tested. Initial sampling tests showed that tested cotton and rayon swab techniques released fibres and thereby interfered with the TNC and ATP analyses. It was at that time not possible to find an alternative swab material that would hold together sufficiently during sampling not to interfere with downstream processing. This was especially a problem for the TNC analysis where fibres released from the swab limited the sight field in the fluorescence microscope, masking and hiding cells behind the fibres. The procedure to collect samples by scraping with a scalpel proved to be a superior method over swabs with better yields and no sight limiting fibres in the microscope. However, the scraping released minerals from the stone which interfered by fluorescence in the microscope. There also appeared to be interference from rock mineral with the ATP analysis and the DNA extraction. Further tests with new swab materials was deemed important. In conclusion, during the first part of the programme it was demonstrated possible to detect microorganisms on natural fracture surfaces in a drill core retrieved during drilling at 400 m depth in the Äspö hard rock laboratory tunnel. Cultivation of microorganisms from fracture surfaces was successful but cultivation suffers from biases, far from all microorganisms in deep groundwater can be cultivated. Therefore, extraction and analysis of DNA was tested. It was deemed important to mitigate interferences during extraction and amplification of the nucleic acids because the amount of DNA available on most natural fractures is minute. It was concluded very important that all sampling should commence directly after drill core retrieval; sending cores may be very detrimental to the viability of biofilm microorganisms and the extractability of DNA and ATP.

The second report described the adaptation of a flow cell (FC) method for *in situ* development, sampling and analysis of microbial biofilms on solid materials introduced in groundwater flowing from deep aquifers (Eriksson et al. 2014). This biofilm collection was considered as an alternative to collect microorganisms from the flowing water with filtration of limited volumes groundwater (0.5–2 L), a sampling technique that often results in amounts of DNA too small for a quality assured nucleic acid analysis (Salter et al. 2014). The method employs FCs with solid surface materials that were connected to tubes feeding the FCs with groundwater under *in situ* pressure from packed off sections in boreholes at various groundwater pressures. Planktonic microorganisms then adhered, grew, multiplied and formed biofilms on the solids. Good quality DNA could be extracted and quantified from the solids.

This report describes how new swab materials were successfully utilized for sampling of biofilm materials from newly drilled fracture surfaces. The swabs were developed specifically for ATP and DNA collection and analysis. In addition to efficient sampling, these swabs were expected to swiftly release the sample material into the sample tubes but without interfering fibres from the swabs. The multidisciplinary Swedish Deep Drilling Program project 'Collisional Orogeny in the Scandinavian Caledonides' (COSC, (http://www.ssdp.se/projects/cosc/) drilled a 2500 m deep hole close to the mountain Åreskutan in Jämtland, Sweden. During drilling fresh fracture surfaces from

rock-groundwater and rock-rock interfaces were sampled for ATP and DNA. Before the drilling was started, laboratory trials were conducted to find the best way to sample with different new types of sampling swabs and storage techniques. Further, this report describes how a pressure filtration method was developed to collect large quantities of DNA for diversity analysis of sequence libraries. Finally, the DNA from pressure filtrated groundwater from four boreholes in the Äspö hard rock laboratory tunnel and DNA from FCs attached to these boreholes were sequenced. The FCs were loaded with glass bead and garnet grains as solid supports for biofilm development. The amounts of biomasses and the DNA library diversities from these two materials were compared and evaluated.

2 Materials and methods

2.1 Sampling methodology

2.1.1 Pressure filtration of groundwater for DNA analysis

Groundwater was pressure filtrated using high pressure, stainless steel 47 mm filter holders (X4504700, Millipore AB, Solna, Sweden) prepared with 0.22 µm pore size water filters from the MO BIO Power Water kit filter units. The filter holders were equipped with pressure relief valves (Swagelok SS-RL3S6MM, SWAFAB, Sollentuna, Sweden) and a pressure gauge that enabled adjustment of a pressure drop over the filter between 200 and 400 kPa relative to the ambient aquifer pressure (Figure 2-1). Groundwater was filtered during 15–16.5 hours at a flow rate of 0.2 L per minute from each of the boreholes KA2198A, KA3110A, KA3385A and KF0069A01 in the Äspö Hard Rock Laboratory (HRL) tunnel (Table 2-1). Sample dates were 2012-02-02 for KA2198A and 2012-07-20 for KA3110A, KA3385A and KF0069A01.

Table 2-1. Sampling data for boreholes in Äspö Hard Rock Laboratory tunnel investigated with pressure filtration for DNA analysis of planktonic bacterial diversity.

Sampled borehole sites in the Äspö tunnel	Depth (masl)	Volume filtered groundwater (L)	Time (hours)	Flowrate through pressure filter L/min)
KA2198A	−300	192	16	0.20
KA3110A	−400	180	15	0.20
KA3385A	−420	186	15.5	0.20
KF0069A01	−450	198	16.5	0.20

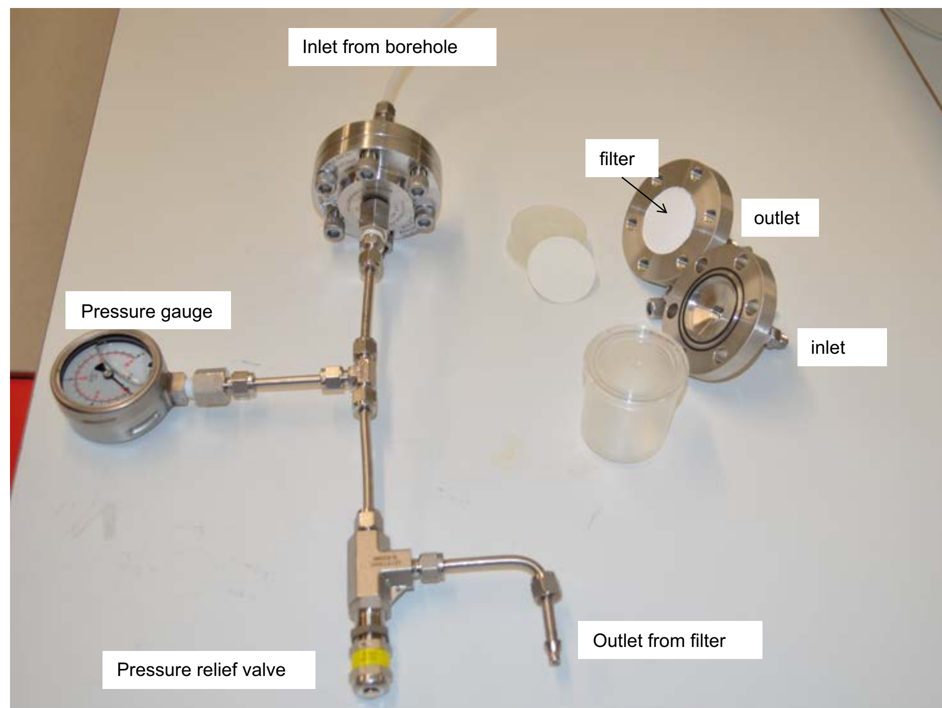

Figure 2-1. *Pressure filtration unit for sampling of bacteria in groundwater. Fully mounted unit is shown to the left; an opened filter holder with filter is shown to the right.*

2.1.2 Flow cells for collection of bacterial DNA in biofilms

This sampling method utilised FCs with surfaces of glass beads and garnet grains to grow biofilms *in situ* in flowing groundwater at the investigated borehole sites KA2198A, KA3110A, KA3385A2 and KF0069A01 in the Äspö HRL tunnel. In previous experiments regarding the enrichment of biofilm we used grains of bedrock that came from the same geographical origin as the borehole where the microbes and groundwater came from (Pedersen 2012a, b, Pedersen et al. 2014). These rock surfaces had a tendency to be porous and unattached minerals and particles often disrupted the subsequent analysis of the biofilm. We therefore decided to use commercially available enrichment surfaces consisting of garnet grains and glass beads. FCs used in this project had a stainless steel shells (length 300 mm, diameter 65 mm) and were lined with polyvinyldifluoride (PVDF) plastic (Figure 2-2). Each FC had a 120-mm-long PVDF insert with a 22 × 32 mm opening that supported ~ 100 g of garnet grains (0.7 mm in diameter) and glass beads (1 mm in diameter), per FC for microbial adhesion and biofilm growth. The garnet grains were delivered from MoBio Laboratories (USA) and are of molecular grade meaning they were sterile, DNA-free and RNAse/DNAse-free. Glass beads were sterilized by heating to 450 °C for 5 hours in a muffle furnace. Glass beads were of the brand Assistant and were purchased from WVR International product number 201-0276.

One FC was connected to each of the boreholes KA2198A, KA3110A, KA3385A2 and KF0069A01 in the Äspö Hard Rock Laboratory (HRL) tunnel (Figure 2-2). Groundwater was let through the FCs for attachment of planktonic microorganisms on the glass and garnet surfaces to develop biofilms. The FCs were equipped with a pressure gauge and a pressure relief valve that enabled adjustment of a pressure drop over the FCs between 200 and 400 kPa relative to the ambient aquifer pressure. The groundwater was let through the FCs at varying flow rates (Table 2-2). The FCs were connected to groundwater from to 2013-05-23 to 2013-06-24. Immediately after disconnection from groundwater, the FCs were transported in coolers to the laboratory for analysis of the biofilms.

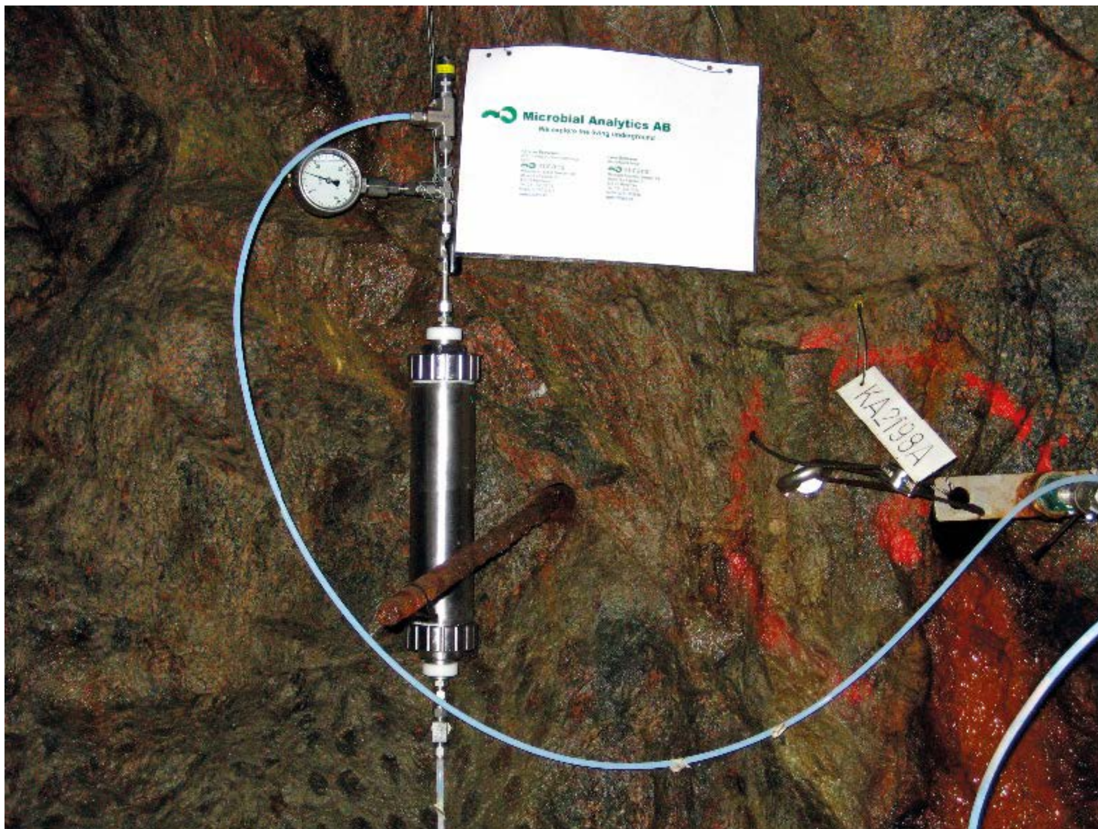

Figure 2-2. *A flow cell connected to the borehole 2198A in the Äspö Hard Rock Laboratory tunnel.*

Table 2-2. Sampling data for boreholes in Äspö Hard Rock Laboratory tunnel investigated with flow cells for DNA analysis of biofilms (masl: meter above sea level).

Sampled borehole sites in the Äspö tunnel	Depth (masl)	Biofilm growth time (days)	Total volume water through the FCs (l)	Flowrate through FCs (ml/min)
KA2198A_1	−300	33	13 900	293
KF0069A01_2	−400	33	77 700	1635
KA3110A_2	−420	33	15 200	320
KA3385A2_2	−450	33	13 500	285

2.1.3 Swabs for DNA and ATP collection

The COSC programme provided a unique opportunity to further develop and test new swab types for collection of DNA and ATP. The drilling program targets the far-travelled (> 400 km) allochthons of the Scandinavian Caledonides and their emplacement across the Baltoscandian foreland basin onto the platform of continent Baltica (Lorenz et al. 2015). COSC-1 was drilled in the summer of 2014, and targeted the high-grade metamorphic complex of the Seve Nappes and its contact to underlying allochthons. An international science team, including expertise on Himalaya-Tibet and other young orogens, was running the science program. The drilling was carried out utilizing the new Swedish scientific drilling infrastructure, located at Lund University, an Atlas Copco CT20 diamond core-drilling rig, with versatile drilling equipment, providing the ideal platform for core-drilling to 2500 m depths. COSC-1 drilling operations and the directly related on-site investigations were financed by International Continental Drilling Program and the Swedish Research Council. Researchers from Microbial Analytics Sweden got the opportunity to collect biofilms on fresh bedrock fracture surfaces during the drilling.

The drill cores were investigated for fresh natural fractures with wet surfaces, likely to have been exposed to groundwater. Fracture surfaces within these criteria were sampled for biomass investigation with the swab technique. Sampling for ATP-analysis was performed by adding extracting reagent from the ATP Biomass kit HS directly on the fracture surface. This was to lyse cells in the biofilms to release ATP (See Lundin 2000 for details). After cell lysis the fracture surface was gently scrubbed with a Copan ATP-free polyamide swab (Copan art.no. 710-0462, VWR) to absorb the released intracellular ATP (Figure 2-3). Then the top of the swab was separated from the applicator and stored in 1.5 mL Eppendorf sample tubes and frozen until analysis. DNA sampling was performed in a similar way using Copan FloqSwab™ (art.no: 4479438; Thermo Fisher Scientific). The swabs were rubbed gently over the moist fracture surface directly after retrieving the drill core to collect cells for subsequent DNA extraction. Until the time for analysis the swabs were stored in the provided sample tube in room temperature, according to the manufacturer's instructions. The sample positions along the COSC-1 borehole are given in Table 2-3.

2.2 Analytical procedures

2.2.1 ATP analysis on garnet and glass surfaces or swabs

The ATP Biomass Kit HS (no. 266-311; BioThema, Handen, Stockholm, Sweden) was used to determine total ATP for estimating the biomass in the samples. The sample preparations for ATP analysis was similar for both the FC and fracture surface samples; the beads and grains from the FCs or the frozen swabs, thawed in room temperature, were added 1 mL of extractant reagent BS, vortexed vigorously for 30 seconds and placed dark in room temperature for 30 minutes. Thereafter the ATP was analysed as previously described, tested in detail, and evaluated for use with Fennoscandian Shield groundwater (Eydal and Pedersen 2007).

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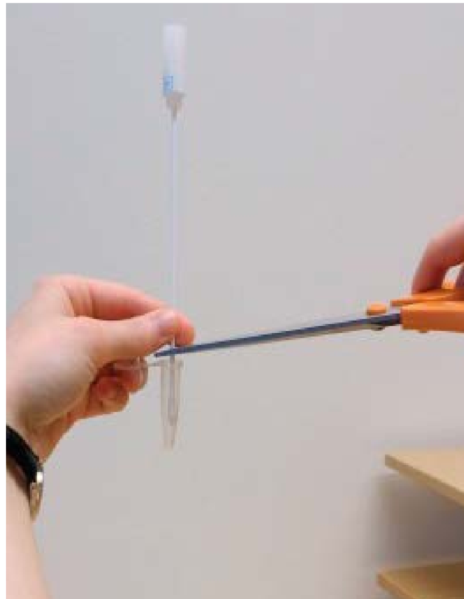

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***Figure 2-3.** Sampling with swab on fracture surface to detect ATP, indicating the number of living cells. 1. Water-conductive fracture surface detected in a drillcore. 2. ATP extracting reagent is added to the surface. 3. Released intracellular ATP is collected with swab. 4. The top of the swab is put into an Eppendorf tube and stored in freeze until analysis.*

2.2.2 Nucleic acids analysis

Genomic DNA from each sample was extracted using PowerWater® DNA Isolation Kit (cat. no. 14900) from MO BIO Laboratories, Carlsbad, CA, USA. (no. 14900-100; Immuno Diagnostics Oy, Finland) according to the manufacturer's protocol. The DNA extractions were thereafter stored at −20 °C until further processing commenced.

The filters from the high pressure filtration equipment were removed from the filter holder with sterile equipment and placed in provided sample tubes from the MO BIO Power Water kit. Tubes with filters were stored frozen at −20 °C until DNA extraction.

Table 2-3. Overview sample positions with swabs in the COSC_1 drillhole Åre, Sweden.

Sample name	Sample date	Borehole depth (m)	Comments from sampling
Test KaP	2014-05-27	114.84	Drillcore that was stored on surface for 24 h before sampling
309-Z	2014-06-04	974.35	Porous rock in otherwise compact gneiss
312-Z	2014-06-04	985	Cavity in calcite
338-Z	2014-06-06	1058.37	Cavity in ”rotten” mafic* layer
395-Z	2014-06-17	1225.75	Fractures along Karst**
401-Z	2014-06-18	1242.62	Fractures along Karst, opened w/hammer, corresponding mud sample at 10:22 am
427-Z	2014-06-21	1320.7	Porous mafic band w/ fractures, corresponding mud sample at 13:55 am
457-Z	2014-06-26	1406.3	Small fracture in mica-rich bands of gneiss, corresponding mud sample at 05:12 am
494-Z	2014-07-02	1406.3	Fracture in mafic band in gneiss. Possibly not pre-existent before drilling
499-Z	2014-07-02	1514.05	Biotite dominated fracture
507-Z (60–70cm)	2014-07-04	1530.7	Talk*-filled fracture, re-opened by pressure unloading, swabbed and scraped
507-Z (15–25cm)	2014-07-04	1550.5	Talk-filled fracture, reopened by drilling
509-Z	2014-07-04	1552.5	Fracture opened w/ hammer
515-Z	2014-07-04	1575.2	Filled fracture, re-opened
561-Z	2014-07-18	1706.5	Natural fracture opened w/ hammer
565-Z	2014-07-19	1725.8	Sampled natural fracture
647-Z	2014-08-17	2199.6	Low angle fracture

* Mafic is an adjective describing a silicate mineral or rock that is rich in magnesium and iron; the term is a portmanteau of the words “magnesium” and “ferric”.

** Karst topography is a landscape formed from the dissolution of soluble rocks such as limestone, dolomite, and gypsum. It is characterised by underground drainage systems with sinkholes and caves.

Approximately 6 g garnet grains were collected from each FC using sterile equipment and placed directly into DNA extraction vessels provided by the manufacturer. The weight of 6 g corresponded to a surface area of ~34.2 cm^2 assuming average diameter of grains to be 0.7 mm. Likewise approximately 6 g glass beads g were collected from each FC using sterile equipment and placed directly into DNA extraction vessels provided by the manufacturer. The weight of 6 g corresponding to a surface area of ~81.2 cm^2 assuming average diameter of grains to be 1.0 mm. The surfaces of glass beads and garnet grains were kept separated. This was to be able to compare for any differences in the amounts of biofilm microorganisms and the diversity of bacteria growing in biofilms on the glass surfaces compared to the garnet surface. The method has been shown reproducible elsewhere (Pedersen et al. 2014).

2.2.3 Quantification of extracted dsDNA

Total extracted nucleotide concentrations were measured using the Nanodrop ND-1000 UV-vis spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA) and double stranded (ds)DNA concentrations were measured fluorometrically using the Stratagene MX3005p fluorometer with MXPro software (Agilent Technologies Inc., Santa Clara, CA, USA) and the Quant-it™ Picogreen reagent kit from Molecular Probes (cat. no. P7589; Invitrogen, San Diego, CA, USA), according to the manufacturer’s specifications. The extracted DNA was stored at −20 °C and subsequently used for sequencing.

2.2.4 454 FLX Titanium 16S rDNA v6v4 pyrosequencing

A Bacterial 16S rDNA v6v4 amplicon library for sequencing was generated by using one forward and four reverse primers. Forward primer (565F) was TGGGCGTAAAG and reverse primers (1064R) were 1064R-1 CGACAGCCATGCANCACCT, 1064R-2 CGACAACCATGCANCACCT, 1064R-3 CGACGGCCATGCANCACCT and 1064R-4 CGACGACCATGCANCACCT. Conditions

for the PCR reaction was; 1X Platinum HiFi Taq polymerase buffer, 1.6 units Platinum HiFi polymerase, 3.7 mM $MgSO_4$, 200 uM dNTPs (PurePeak polymerization mix, ThermoFisher), and 400 nM primers. 5–25 ng of sample DNA was added to a master mix to a final volume of 100 µl and this was divided into three replicate 33 µl reactions. A no-template negative control for each molecular identifier (MID) was included. Cycling conditions included an initial denaturation at 94 °C for 3 minutes; 30 cycles of 94 °C for 30 seconds, 57–60 °C for 45 seconds, and 72 °C for 1 minute; and a final extension at 72 °C for 2 minutes using an Bio-Rad mycycler. The quality and concentration of the amplicon library was evaluated by using the Agilent Tapestation 2000 instrument from Agilent according to manufacturer's protocol. The reactions were cleaned and products under 300 bp were removed using Ampure beads at 0.75 × volume (Beckman Coulter, Brea CA). The final products were resuspended in 100 µl of 10mM Tris-EDTA +0.05 % Tween-20, quantified using PicoGreen Quant-IT assay (Life Technologies), and assayed once again on the Tapestation 2000 instrument. Amplicons were further titrated in equimolar concentration before emulsion-PCR based on their dsDNA concentrations. A GS-FLX Titanium Sequencer was used to generate pyrotag sequence reads with the Roche Titanium reagents.

After sequencing, data was run through a quality control process. Each read was trimmed for primer bases from the beginning and the end of each read, and sequences likely to be of low-quality based on assessment of pyrosequencing error rates was removed (Huber et al. 2007). A Bioinformatic Trimming anchor site (565F-a) TGGGCGTAAAG was used to trim sequences. The 454 pyrotagsequence processing to assign a taxonomic classification was done by the tag mapping methodology Global Alignment for Sequence Taxonomy (GAST) (Huse et al. 2008) were the reference database of 16S rRNA genes, RefSSU, were based on the SILVA database (Pruesse et al. 2007). If two-thirds or more of the full-length sequences share the same assigned OTU, the tag is assigned to that OTU. Tags that did not match any reference tag by the Basic Local Alignment Search Tool (BLAST) were not given a taxonomic assignment. Normalized sample count in percent was used for alpha diversity analysis and visualization of the datasets. After clustering of data the representativeness of sequences was tested by rarefaction analysis, Abundance-based coverage estimator (ACE) and unbiased richness estimate (named Chao) indices were used to estimate richness (confer Sogin et al. 2006). To statistically estimate abundance and evenness for each sample Shannon and Simpson indices were calculated. Distance calculations for sequence similarities were done by using the Morisita-Horn algorithms. Beta diversity compares different communities based on their composition relative to sample metadata such as type of material for biofilm development, depths or sample date. Beta diversity was studied through various scripts in QIIME (Quantitative Insights Into Microbial Ecology) comparing the samples in the dataset. An even sampling depth is crucial for meaningful beta diversity analysis in this study we chose to evenly sample as many sequences as possible.

2.2.5 Statistical analyses and data visualization

The sequencing data was analysed and evaluated using the Visualization and Analysis of Microbial Population Structure (VAMPS) web site (vamps.mbl.edu) and the QIIME software version 1.8.0. Data graphics design and statistical analyses were performed in Statistica 10 (Statsoft Inc., Tulsa, OK, USA).

3 Results

3.1 ATP analysis of flow cell samples

For each sample the analysed ATP (amol/cm^2) was recalculated to estimate the number of cells, by using the average value of amount of intracellular amol ATP per cell for bacteria in deep groundwater, 0.43 amol/bacterium (Eydal and Pedersen 2007). After analysis, the garnets and beads from each sample were washed, dried and weighed for calculation of the total garnet and bead surface areas. The number of cells was then divided by the total area of the respective total garnet of bead surface area to obtain the approximate number of cells per cm^2. The largest amount of ATP (amol/cm^2) was found for the KA2198A FC, for both the glass and garnet surfaces. When comparing the glass with garnet surfaces, the largest amount of ATP was found on garnet surfaces for all samples except for KA2198A, where the largest amount of ATP was found on the glass beads (Table 3-1).

Table 3-1. Analysed amounts of extracted ATP on glass beads and garnet grains with estimated numbers of cells per cm^2 for each surface material.

Sample	Garnet ATP (amol/cm^2)	Glass ATP (amol/cm^2)	Ratio of ATP (Garnet/Glass)	Garnet estimated (cells/cm^2)	Glass estimated (cells/cm^2)
KA2198A	1.97×10^6	8.47×10^7	0.023	45.8×10^5	1.97×10^8
KA3110A	2.75×10^5	6.17×10^4	4.5	6.40×10^5	1.43×10^5
KA3385A2	9.00×10^3	1.00×10^3	9.0	0.209×10^5	2.33×10^3
KF0069A01	7.95×10^3	1.86×10^3	4.3	0.185×10^5	4.32×10^3

3.2 Nucleic acids analysis of flow cell samples

DNA was extracted from the garnet and bead surfaces and the largest amounts of nucleic acids was obtained from the KA2198A samples, both for the garnet as well as for glass biofilms (Table 3-2). The detected amounts of biomass using the ATP assay correlated with the amounts of nucleic acids. The amounts of nucleic acids were calculated to cell numbers using the average amount of DNA in the typical groundwater bacterium, i.e. *Desulfovibrio aespoeensis*, which is 649 Daltons/base pair $\times$ 3 629 109 bases (Locus CP002431) = 2.36×10^9 Daltons cell^{-1} = 2.36×10^9 Daltons cell^{-1} $\times$ $1.6605402 \times 10^{-24}$ g/Dalton = 3.9×10^{-15} g DNA/cell. After the DNA extraction from the bead and the grain surfaces they were washed, dried and weighed and calculated for total area in cm^2. The mass of extracted DNA in ng were divided by the total surface area to obtain the mass of DNA (ng/cm^2). Using the average mass of nucleic acids per cell for *D. aespoeensis*, the number of extracted cells per cm^2 of surface material could be calculated. The extracted mass of DNA per surface area (ng cm^2) was divided by 3.9×10^{-15} g (DNA/cell) to give these numbers. It should be noted that DNA results were reported previously (Eriksson et al. 2014). However, the quantification of DNA was repeated after the publication of that report with a new, more sensitive kit, at a detection limit of 10 ng DNA. The revised values are shown in Table 3-2.

Table 3-2. Analysed amounts of extracted DNA on glass beads and garnet grains with estimated numbers of cells per cm^2 for each surface material.

Sample	Garnet estimated DNA (ng/cm^2)	Glass estimated DNA (ng/cm^2)	Ratio of DNA (Garnet/Glass)	Garnet estimated (cells/cm^2)	Glass estimated (cells/cm^2)
KA2198A	4.26×10^{-9}	1.71×10^{-8}	0.25	10.9×10^5	43.8×10^5
KA3110A	5.37×10^{-10}	3.19×10^{-10}	1.8	1.38×10^5	0.818×10^5
KA3385A2	5.85×10^{-10}	3.69×10^{-10}	1.6	1.50×10^5	0.946×10^5
KF0069A01	1.77×10^{-9}	9.61×10^{-10}	1.8	4.54×10^5	2.46×10^5

3.2.1 Analysis of bacterial 16S rDNA diversity in groundwater and flow cell samples

Sampling intensity was in the range of 13 000 to 23 000 reads (Table 3-3). All rarefaction curves display a good sampling depth by having similar shapes approaching a plateau at the highest sample sizes indicating that only a few more taxa would have been detected with a higher sampling intensity (Figure 3-1). Rank abundance curves showed a typical pattern for environmental samples were a few species are abundant but most are rare (not shown). This ecological pattern is accounted for when interpreting diversity by using species diversity indices which not only interpret diversity by richness but also evenness. Alpha diversity calculations for each sample is shown in Table 3-3. In general were the groundwater samples more diverse compared to biofilm samples. Groundwater sample KA2198A_1 had a total of 229 taxa, also having the largest estimated ACE and Chao diversities of 264.4 and 373.9, respectively. The Shannon and inverse Simpson diversity indices indicate that the samples KA3110A_2 and KA3385A_2 were highly diverse and had even distributions of taxa.

For the biofilm samples KA2198A_glass was the most diverse sample having 174 different taxa. Further, that sample had the highest ACE and Chao estimator values when compared to other biofilm samples. When diversity indices for both KA2198A_glass and KA2198A_garnet were calculated, almost the same numbers were obtained indicating that these two samples were equally diverse.

When comparing glass and garnet samples both the KF0069A01 samples and the KA2198A samples were closely related as indicated by the dendrogram generated by a Morisita–Horn distance calculation using an un-weighted pair group method with arithmetic mean (UPGMA) for the tree construction with taxonomic depth at the genus level (Figure 3-2). A representation regarding which taxa and to which abundance that taxa occurred in respective sample type is shown in Table 3-4.

There was no clear agreement in diversity between the groundwater and the biofilm samples. This may be to a large part explained by the one year length in time between sampling occasions. Microbial diversity might have changed over time as a result of other activities in the tunnel that influence groundwater flow and composition.

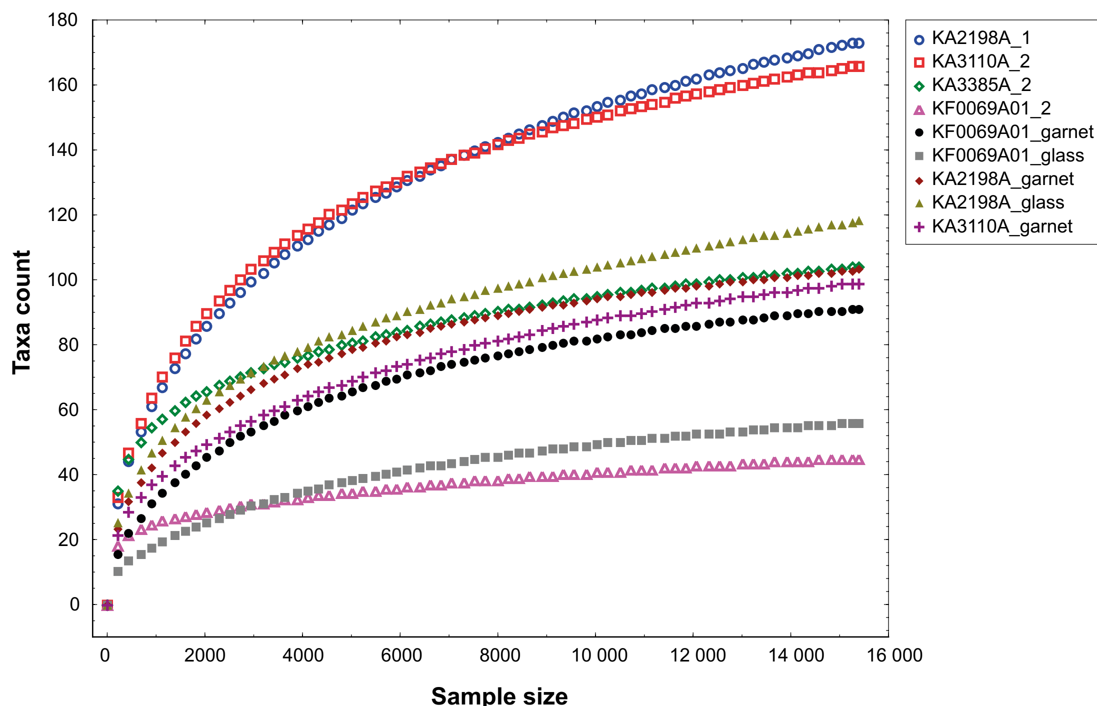

Figure 3-1. *Rarefaction curves for bacterial 16S rDNA v4v6 dataset. Each curve represents a single sample and sampling occasion.*

Table 3-3. Samples from groundwater with the corresponding flow cell samples sorted decreasing diversity by number of taxa at > 0 % abundance. The table shows decreasing diversity by depth below ground surface. Amounts of extracted double-stranded bacterial DNA analysed fluorometrically using the Stratagene MX3005p fluorometer with MXPro software and the Quant-it Picogreen reagent kit from Molecular Probes; observed and estimated diversity at genus level (> 0 % abundance) in groundwater bacterial 16S rDNA v4v6 sequence libraries. The > 0.1 % and > 1 % abundance taxa number was generated at genus level or the highest annotated rank.

Sample	Depth (masl)	Sample type	Sampling depth, i.e., number of sequences	Number of taxa at > 0 % abundance	Number of taxa at ≥ 0.1 % abundance	Number of taxa at ≥ 1 % abundance	ACE[1]	Chao[2]	Shannon-Weaver diversity Index	Inverse Simpson diversity Index
KA2198A_1	−300	groundwater	15 380	229	55	13	264.4	373.86	2.14	3.52
KA2198A_glass	−300	biofilm	22 823	174	45	12	201.28	264.25	1.9	3.23
KA2198A_garnet	−300	biofilm	16 388	131	43	8	142.28	185.45	1.85	3.23
KA3110A_2	−400	groundwater	16 227	219	60	16	242.52	347.13	2.27	3.65
KA3110A_garnet	−400	biofilm	11 890	123	32	12	139.47	163.26	1.76	3.09
KA3385A_2	−420	groundwater	22 830	129	53	22	141.83	169.91	2.4	3.81
KF0069A01_garnet	−450	biofilm	13 551	114	29	6	128.51	198.5	1.51	2.92
KF0069A01_glass	−450	biofilm	14 630	89	14	7	105.5	125.3	1.39	2.89
KF0069A01_2	−450	groundwater	13 241	53	22	13	62.53	95.67	1.8	3.38

[1] Abundance-based coverage estimator.

[2] Unbiased richness estimate.

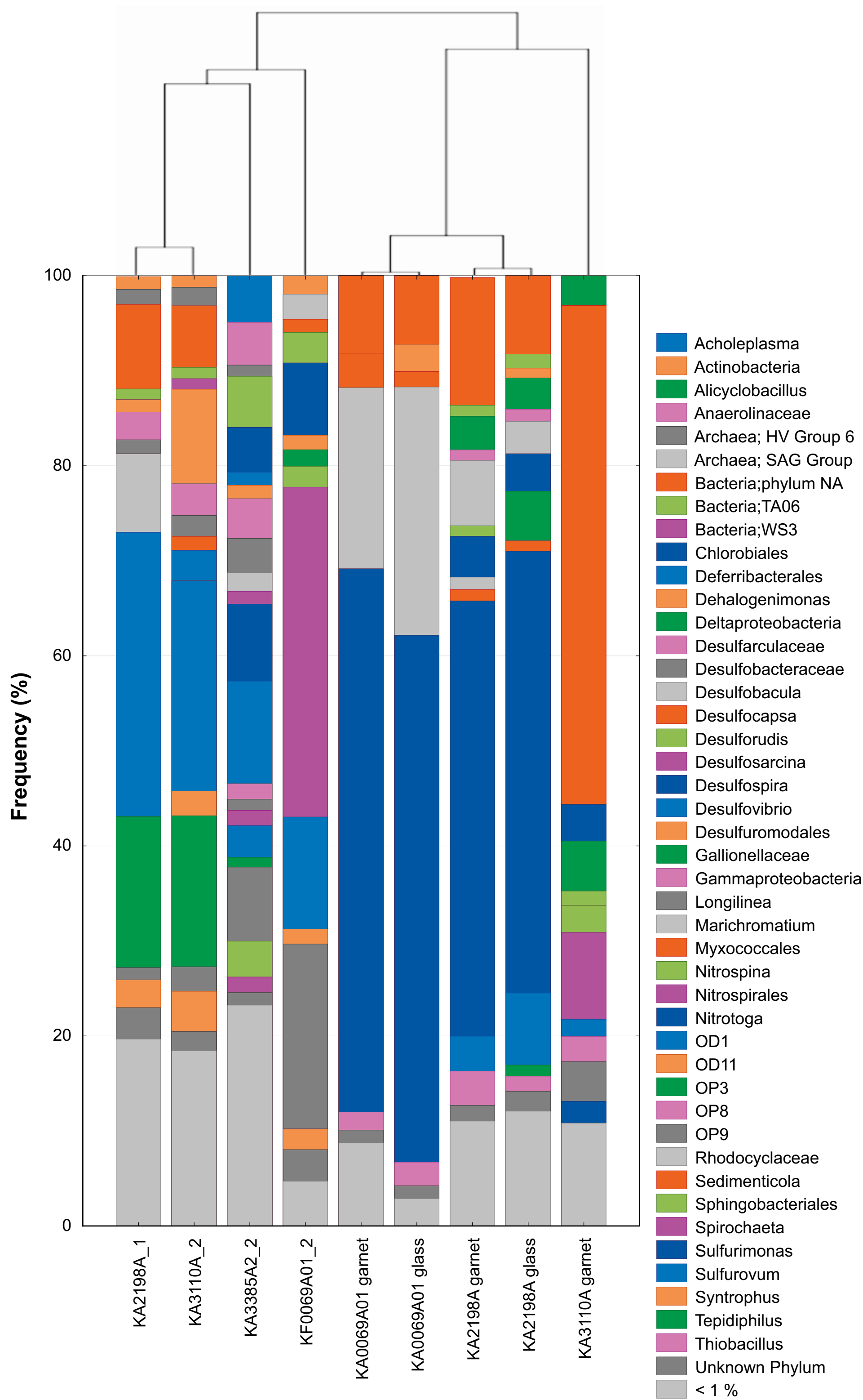

Figure 3-2. *Frequency of taxonomic assignment for 16S Bacteria rDNA v4v6 pyrotagsequence libraries for samples from the Äspö tunnel. Sequences with ≥1% abundance frequency are shown. The clustering graph on top of the bar graph shows a Morisita–Horn distance measure illustrated using an unweighed pair group method with arithmetic mean (UPGMA) for the tree construction with taxonomic depth at genus level.*

Table 3-4. Percent occurrence of Taxa in bacterial 16S rDNA v4v6 sequence libraries from groundwater and biofilms at ≥1 % abundance as illustrated in table below.

Taxa	KA2198A_1	KA2198A garnet	KA2198A glass	KA3110A_2	KA3110A garnet	KF0069A_201	KF0069A01 garnet	KF0069A01 glass	KA3385A2_2
Acholeplasma									4.91
Actinobacteria	1.33			1.22		1.95			
Alicyclobacillus					3.13				
Anaerolinaceae									4.49
Archaea; Deep Sea Hydrothermal Vent Group6	1.62			1.94					1.19
Archaea; SAG Group						2.63			
Bacteria; phylum NA	8.86	13.45	8.24	6.51	52.5	1.39			
Bacteria;TA06	1.14	1.15	1.48	1.16		3.21			5.37
Bacteria;WS3				1.12					
Chlorobiales					3.86	7.64			4.72
Deferribacterales									1.38
Dehalogenimonas	1.29		1.03	9.94		1.49			1.4
Deltaproteobacteria		3.54			5.26	1.77			
Desulfarculaceae	2.92			3.34					4.18
Desulfobacteraceae	1.48			2.24					3.61
Desulfobacula									1.99
Desulfocapsa				1.43			8.15	7.22	
Desulforudis					1.51				
Desulfosarcina									1.31
Desulfospira									8.11
Desulfovibrio				3.22					
Desulfurivibrio									10.8
Desulfuromodales								2.86	
Gallionellaceae			3.31						
Gammaproteobacteria		1.12	1.28						1.63
Longilinea									1.17
Marichromatium	8.27	6.85	3.4						
Myxococcales							3.64	1.64	
Nitrospina		1.11			2.85	2.16			
Nitrospirales					9.14	34.72			1.61
Nitrotoga		4.3	3.94						
OD1	29.9			22.1	1.81	11.79			3.34
OD11				2.61		1.58			
OP3	15.9		5.22	15.89					1.02
OP8					2.64				
OP9	1.29			2.58	4.17	19.46			7.79
Rhodocyclaceae		1.31					19.05	26.12	
Sedimenticola		1.2	1.08						
Sphingobacteriales									3.77
Spirochaeta									1.66
Sulfurimonas		45.8	46.5		2.31		57.17	55.45	
Sulfurovum		3.67	7.59						
Syntrophus	2.93			4.23		2.18			
Tepidiphilus			1.17						
Unknown Phylum	3.30	1.65	2.10	2.02		3.32	1.34	1.36	1.31
< 1 %	19.77	11.24	12.1	18.45	10.82	4.71	8.75	2.88	23.24

3.3 Analysis of swab samples from COSC-1

All swab samples were analysed for amounts of ATP and extracted for amounts of DNA to detect possible presence of microorganisms on the sampled bedrock fracture surfaces during the COSC-1 drilling project in Åre, Sweden.

3.3.1 ATP analysis of swab samples

The results for ATP in the 17 swab samples are presented in Table 3-5 and displays amounts of ATP in the swabs between 8.92×10^3 up to 1.46×10^5 ATP (amol). Since the swabbed surface areas were not exactly registered, the results show the total amount of sample in the swabs, which was extracted in 1 ml Extraction BS solution from the ATP Biomass Kit HS.

Table 3-5. Detected amounts of ATP and DNA in the swabs of fracture surfaces. (b.d.: measured concentration was below detection limit of 10 ng per swab).

Sample name	Sampling date	Depth below ground surface (m)	ATP in surface swab (amol)	DNA in surface swab (ng)
Test KaP	2014-05-27	114.81–115.10	8.92×10^3	26.9
309-Z	2014-06-04	−974.35	2.23×10^4	14.2
312-Z	2014-06-04	−985	4.38×10^4	16
338-Z	2014-06-06	−1058.37	1.10×10^5	17.7
395-Z	2014-06-17	−1225.75	1.46×10^5	23.0
401-Z	2014-06-18	−1242.62	9.54×10^4	14
427-Z	2014-06-21	−1320.7	4.71×10^4	48.4
457-Z	2014-06-26	−1406.3	8.97×10^4	12.3
494-Z	2014-07-02	−1514.05	3.99×10^4	27.4
499-Z	2014-07-02	−1530.7	1.37×10^4	b.d.
507-Z (60–70 cm)	2014-07-04	−1550.5	1.29×10^5	b.d.
507-Z (15–25 cm)	2014-07-04	−1552.5	1.27×10^5	b.d.
509-Z	2014-07-04	−1556.5	2.75×10^4	b.d.
515-Z	2014-07-04	−1575.2	1.74×10^4	b.d.
561-Z	2014-07-18	−1706.5	1.45×10^4	36.7
565-Z	2014-07-19	−1725.8	2.80×10^4	b.d.
647-Z	2014-08-17	−2199.6	4.82×10^3	b.d.

3.3.2 Nucleic acid analysis of swab samples

The DNA extraction methodology detected amounts of DNA above 10 ng per swab in 10 of the17 swab samples (Table 3-5). A selection of two samples, 401-Z and 427-Z, is presently in progress for 454 pyrosequencing and the results will be included in the last report for this method development programme, during 2016. The amount of DNA is re-calculated according to Section 3.2 to number of cells for the 5 samples with the largest masses of DNA (Table 3-6).

3.3.3 Drillmud samples from COSC-1 compared to swabs

At some depths the drilled bedrock was porous and when drilling the fracture surfaces were brittle and difficult to swab for sampling. In these cases, the swab samples were supplemented with a drillmud sample. The drillmud sample was not expected to correlate with swab sample, because the drillmud was diluted with water from a nearby creek on ground. There were detectable amounts of nucleic acids in the drillmud samples (Table 3-7). Two drillmud samples, 401-Z and 427-Z, were extracted for DNA and will be sequenced as a contamination control. The work with sequencing, typing and identifying species of bacteria in the two drillmud samples is presently in progress for 454 pyrosequencing and the results will be included in the last report for this method development programme, during 2016.

Table 3-6. Results for swab samples with the five largest amounts of ATP and DNA.

Sample	ATP in surface swab (amol)	ATP estimated number of cells	DNA in surface swab (ng)	DNA estimated number of cells
Test KaP	8.92×10^3	2.07×10^4	26.9	7.66×10^6
395-Z	1.46×10^5	3.39×10^5	23.0	5.90×10^6
427-Z	4.71×10^4	1.09×10^5	48.4	12.4×10^6
494-Z	3.99×10^4	9.28×10^4	27.4	7.03×10^6
561-Z	1.45×10^4	3.37×10^4	36.7	9.40×10^6

Table 3-7. DNA results for analysed drillmud compared with the corresponding swab sample.

Sample	Swab ATP (amol)	Drillmud ATP (amol)	Swab DNA (ng)	Drillmud DNA (ng)
401-Z	9.54×10^4	b.d.	b.d.	161
427-Z	4.71×10^4	b.d.	48.4	1229

4 Discussion

4.1 Evaluation of sampling methods

4.1.1 Pressure filtration of groundwater for DNA analysis

When investigating the microbial diversity in deep groundwater using DNA analysis, the often encountered challenge has been to sample sufficient water and microorganisms ($> 5 \times 10^9$ cells) to obtain enough amounts of extracted bacterial DNA from living biomass to avoid reagent contamination biases (Salter et al. 2014). This is because there are usually relatively low numbers of microorganisms in deep groundwater ($< 10^5$/mL). The filtering method can be used for *in situ* sampling of sparsely populated groundwater. By filtering a large volume of groundwater (> 100 L) the amount of cells for extraction can be significantly increased compared to smaller volumes collected and filtered in the laboratory. In addition to obtain more DNA, filtering a large volume of groundwater will reduce possible variations in cell numbers and diversity over volume compared to smaller volumes of groundwater. Finally, filtering of collected groundwater in the laboratory generally experience problems with oxygen induced and/or pressure release related precipitates that clog filters. This effect is circumvented in the pressure filtration method.

4.1.2 Flow cells for collection of bacterial DNA in biofilms

The use of flow cells was introduced in this project during 2013 (Bengtsson et al. 2013) and further discussed in 2014 (Eriksson et al. 2014). The strategy behind the FC method was to induce attachment and growth of microorganisms in the groundwater passing through the FC and form biofilms on the glass and garnet surfaces. With this technique it is possible to investigate microbial diversity in groundwater where pressure filtration is impossible, for instance on the ground surface with pumped boreholes. Pumps used in boreholes do not give enough pressure for a successful pressure filtration. The FC method can be used to sample *in situ*, is straightforward and technically simple to perform. By using different solid surface material it is possible to investigate preferences by planktonic cells for different material such as in this case garnet or glass. The method requires at least 4–5 weeks of microbial attachment and biofilm development time to obtain enough biomass for DNA extraction. During the attachment and growth time the FCs may need intermittent supervision to ensure that the wanted pressure and flow rate of the groundwater is retained. The flow cell method has been tested, used and evaluated previously (Pedersen 2012a, b, 2013, Pedersen et al. 2014) In brief, colonisation of deep rock aquifers occurs when new fractures are opened by seismic events. Colonisation and biofilm development is driven by microorganisms in the groundwater that fills new fractures. Consequently, the FCs mimic such a process. Because the rare biosphere is large (confer table 3.3) full diversity from biofilms will be present in groundwater. It will be the conditions in the flow cell that determine which of these microorganisms that will prosper and dominate on the surfaces in the FCs (Jägevall et al. 2011).

4.1.3 Swabs for DNA and ATP collection

Investigation of biofilms spot on fresh bedrock fracture surfaces obtained during drilling is challenging, again due to expected low numbers of microorganisms available for sampling. Previously, rayon and cotton swabs were tested and released fibres were found to interfere with the analytical procedures (Bengtsson et al. 2013). Here, we used a new type of swabs that was developed to strongly absorb all biomass from the fracture surfaces. The swabs released cells for ATP and DNA readily in the extraction for analysis of the samples. There was no interference from swab material with the analytical procedures. The swab technique returns real time data of the microbial biomass and diversity on bedrock fracture surfaces. Sampling was easy to perform and the swab samples could be stored either in room temperature or freezer to be analysed later. This is a large advantage when working in the field without access to a freezer. The swabs are expensive to purchase in relation to rayon and cotton swabs, but still not very expensive. The cost is in the range of 1–2 € per swab. The DNA swabs are intended to be used in crime scene investigations where often only traces of DNA material can be expected. The DNA swabs detected DNA in 10 of 17 COSC-1 samples which probably is realistic when the sample description in Table 2-3 is reviewed. The samples

without detectable DNA were fractures opened during drilling, i.e. not exposed to deep groundwater. The absence of DNA in these samples then indicate that contamination from the drillmud, and by the sampling crew, was limited, i.e. below the limit of detection for the DNA quantification analysis.

4.2 Evaluation of analysis methods

The ATP and DNA extraction methods were evaluated in detail in the previous report (Eriksson et al. 2014). The new results presented here concern microbial diversity in groundwater and in biofilms using 454-pyrosequencing.

High-throughput sequencing by means of 454 pyrosequencing is based on emulsion PCR and does not require the preparation of clone libraries before sequencing. DNA extracted from the biomass can be directly used for the analysis of microbial communities based on the 16S rDNA gene. Using sequencing platforms, such as GS FLX Titanium sequencing on the 454 sequencing platform (Roche, Basel, Switzerland), it is possible to obtain thousands of sequences both cost and labour efficiently compared with previously used sequencing techniques. The method produces a huge number of sequences covering most microorganisms in the sampled populations, providing conclusive information about genus/species diversity. The massive number of sequence library data then have to be processed using bioinformatics tools. Recently, there has been a change from use of the 454 pyrosequencing platform to use of the Illumina sequencing platform. This is because the 454 pyrosequencing platform was recently bought by Hoffman LaRoche who soon after that announced the discontinuation of the 454 sequencing platform in 2013 in favour for their own Illumina platform. From 2015 and onwards sequencing will, therefore, be performed using the Illumina platform. With the high-throughput sequencing methods the composition of the microbial communities can be thoroughly characterized. The differences in community composition between samples can be accurately detected and, due to the high number of sequences obtained, rare micro-organisms present at only low levels, i.e., below 0.1 % of the community, can be detected (Bowen et al. 2012).

The usefulness of high-throughput sequencing is illustrated by the information in Figure 4-1. The proportion of important genera, such as the sulphate-reducing bacteria (SRB) can be revealed. The data is limited to four observations but do still show clear differences in the representation of SRB between the sampled groundwater locations, possibly related to differences in groundwater origin and composition.

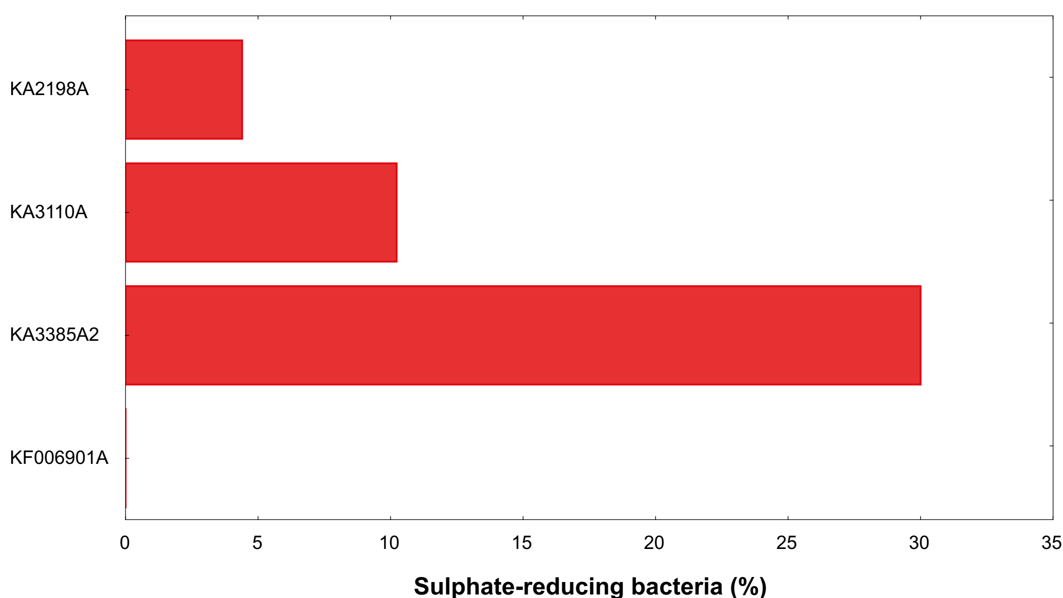

Figure 4-1. *The proportion of 454pyrosequences belonging to sulphate-reducing genera. Data are extracted from Table 3-4.*

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