Report
R-16-09
August 2016

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SVENSK KÄRNBRÄNSLEHANTERING

ISSN 1402-3091
SKB R-16-09
ID 1541355
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Keywords: 454 sequencing, ATP, Biomass, Flow cells, Nucleic acids, Sulphate-reducing bacteria biofilms.

Abstract

Investigations of potential sites for a spent nuclear fuel (SNF) repository in Sweden have revealed diverse cultivable populations in all analysed groundwater samples from depths of a few meters down to approximately 1000 m. The presence of active microbial populations in repository environments must consequently be addressed to facilitate safe implementation of geological disposal of SNF. It has repeatedly been show that subterranean microorganisms rapidly attach to mineral surfaces and that attached microorganisms are at least as metabolically active as are planktonic microorganisms. While there were well developed methods for the analysis of numbers and diversity of planktonic microorganisms in deep groundwater there were no methods for assaying numbers and diversity of attached microorganisms on aquifer fracture surfaces during the site investigations. New methods for sampling attached microorganisms had to be developed for determination of amount of biomass per surface area. The methodologies should also be applicable for determination of microbial diversity. The first part of this project addressed 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. Different methods for sampling of microorganisms attached to rock surfaces were developed and tested. Extraction and analysis of DNA was tested as well. The second part of the project was on 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. The project also investigated new swab materials utilized for sampling of biofilm materials from newly drilled fracture surfaces. Finally, this project developed a pressure filtration method to collect large quantities of DNA from planktonic microorganisms for diversity analysis of sequence libraries. The DNA from pressure filtrated groundwater from boreholes and DNA from FCs attached to these boreholes were sequenced with a 454-pyrosequencing methodology. The FCs were loaded with glass beads and garnet grains as solid supports for biofilm development. The amounts of biomasses and the DNA sequence library diversities from these two materials were compared and evaluated.

Three methods were developed. The first method utilises swabs for detachment of microorganisms from fracture surfaces in conjunction with drilling operations. The second method collects planktonic microorganisms with a filtration equipment. The third method involves flow cells with garnet grains that are overflown with groundwater for a period of time and thereafter sampled and analysed for ATP and DNA. All three developed methods can be used in a series. Swabbing and analysis of ATP and DNA during drilling will produce a data archive with information on original diversity on aquifer surfaces. The analysis can await data from flow logging and found water conducting aquifer samples can be further processed. If such aquifers are packed off and pumped, the filtration method will provide information on planktonic diversity while the use of FCs will provide data on microbial attachment, growth diversity.

Sammanfattning

Vid undersökningar av potentiella platser för förvar av använt kärnbränsle i Sverige har det i samtliga analyserade grundvattenprover, från djupet av några meter ner till cirka 1000 m, påvisats olika populationer av mikroorganismer. Förekomsten av aktiva mikrobiella populationer i de tänkta förvarens miljöer bör kartläggas för att kunna bedöma den långsiktiga säkerheten av geologisk slutförvaring av SNF. Upprepade gånger har undersökningar påvisat att mikroorganismer i underjordiska miljöer, så som i djup berggrund, snabbt fäster till sprickytor och att dessa är minst lika metaboliskt aktiva som de mikroorganismer som cirkulerar fritt i grundvatten. Även om det vid platsundersökningarna funnits väl utvecklade metoder för bestämning av antal och diversitet av fritt cirkulerande mikroorganismer i djupa grundvatten, saknades metoder för att samla in provmaterial och analys av mikroorganismer som vidhäftar på sprickytor i berggrunden. Nya metoder för provtagning av mikroorganismer i biofilm på sprickytor behövde därför utvecklas, både för bestämning av mängd biomassa per ytarea samt för mikrobiell diversitet. Den första delen av detta projekt inriktades mot undersökning av mikrobiella biofilmer med hjälp av mikroskopi för att bestämma totala antalet celler (TNC), analys av adenosin-5'-trifosfat (ATP), DNA-extraktion samt odlingsmetoder för aeroba och anaeroba mikroorganismer. Olika metoder för provtagning av mikroorganismer som lever i biofilm fäst på sprickytor i berggrunden testades. I den andra delen av projektet utvecklades en ny typ av flödescell (FC) för *in situ* provtagning av mikrobiella biofilmer på vattenförande sprickor i djup berggrund. I projektet testades också nya typer av provtagningssvabbar för provtagning av biofilm från färska vattenförande sprickytor i berggrund. Slutligen har det inom detta projekt utvecklats en tryckfiltreringsmetod där stora mängder DNA från mikroorganismer i grundvatten samlats in för att utföra diversitetsanalys mot ett sekvensbibliotek. Från tryckfilter som filtrerat grundvatten från ett borrhål har mikrobiellt DNA extraherats och sekvenserats med en 454-pyrosekvenseringsmetodik. Mängd biomassa och diversitet av DNA från de testade provtagningsmetoderna jämfördes och utvärderades mot DNA i sekvensbiblioteket.

Sammanfattningsvis utvecklades tre metoder. I den första metoden används speciella svabbar för provtagning av mikroorganismer från sprickytor i samband med borrning i berggrund. Den andra metoden samlar fritt cirkulerande mikroorganismer i grundvatten med hjälp av en filtreringsutrustning. Den tredje metoden använder flödesceller fyllda med granatkorn som fungerar likt anrikningsytor som flödas igenom med grundvatten från ett borrhål under en bestämd period. Mikrobiell biofilm som anrikats på granatkornens ytor i FC extraheras och analyseras för att mäta mängd ATP samt DNA per ytenhet. De tre utvecklade metoderna kan användas i en serie. Svabbprov tas på färska vattenförande sprickor under borrning som därefter analyseras för att kvantifiera mängd ATP och DNA, vilket kommer att kunna generera data med information om naturligt förekommande mikrobiell diversitet på de undersökta sprickytorna. Denna metod kan kombineras med data från flödesloggning vid borrning och pumpning samt filtrering av grundvattenprover för att kunna bearbetas ytterligare och ge mer kompletterande information. Genom att tryckfiltrera grundvatten som pumpas upp vid borrning i berggrund kan man få prover som ger information om antal och diversitet av fritt cirkulerande mikroorganismer i grundvattnet. I kombination med FC-metodiken kommer man också kunna erhålla prover som ger information om de mikroorganismer som istället fäster på ytor i det omgivande borrhålet.

Contents

1 Introduction

Plans to dispose of spent nuclear fuel (SNF) wastes contained in copper shielded 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 1000 m in Sweden (Hallbeck and Pedersen 2012) and Finland (Pedersen et al. 2008). Oxygen-, nitrate-, iron-, manganese-, and sulphate-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 geochemical data pertaining, for example, to Eh, Mn^{2+}, Fe^{2+}, S^{2-}, and $\delta^{34}S$, 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).

The presence of active microbial populations in repository environments must be addressed to facilitate safe implementation of geological disposal of SNF. There are three main effects of microorganisms in the context of a SNF repository situated approximately 500 m underground in bedrock of the Fennoscandian Shield, as follows:

1. Sulphide production by sulphate-reducing bacteria (SRB) under anaerobic and reduced conditions – sulphide is corrosive to the copper canisters (Pedersen 2010).
2. Bio-mobilization and bio-immobilization of radionuclides, and the effects of microbial metabolism on radionuclide mobility (Anderson et al. 2011, Johnsson et al. 2006, Pedersen 2005c).
3. O_2 reduction by microorganisms using organic carbon and methane as electron donors and the maintenance of anoxic and reduced conditions (Kotelnikova and Pedersen 2000, Pedersen 2012a). O_2, in addition to sulphide, is corrosive to the copper canisters that will be used to dispose of SNF, and radionuclides are generally more mobile under oxidizing than under reducing conditions.

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 2012b, 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 were no information on numbers and diversity of attached microorganisms.

Previously, it was assumed that attached and planktonic microbial diversity is similar but there was little experimental evidence for this assumption. More data has to be collected to test this assumption. New methods for sampling attached microorganisms must be developed for determination of amount of biomass per surface area. The methodologies should also be applicable for determination of microbial diversity. The development of procedures for sampling is a methodological challenge. One option is to collect drill cores directly after retrieval to the ground surface and identify open, water conducting aquifers that can be sampled. This procedure was tested in the Äspö HRL tunnel and the risk for drill water contamination was evaluated (Jägevall et al. 2011). Scalpels were used to collect biofilm material. The obtained results for biofilms agreed with the groundwater composition, and the risk for drill water contamination was mitigated by the use of triple tube drilling. It was concluded that drill core analysis is possible, at least in tunnel environments, but more tests are needed before a quality assured method can be defined. Colonisation of deep rock aquifers occurs when new fractures are opened by seismic events. Biofilm development on such new fracture surfaces would

start with planktonic microorganisms in the groundwater that fills new fractures. Consequently, flowing groundwater over solid surfaces, e.g. crushed rock, would mimic such a process. After drilling of a borehole, packers can be installed and water from selected aquifers can be obtained. A second option for the analysis of biofilms is to install flow cells (FCs) with solid surfaces for the attachment of planktonic microorganisms and growth of biofilms. The use of FCs for analysis of diversity and activity has been successful in tunnel environments on two boreholes in the Äspö HRL (Jägevall et al. 2011, Pedersen 2012a, b) and two boreholes in the Onkalo HRL in Olkiluoto, Finland (Pedersen 2013, Pedersen et al. 2014a). Further development of this methodology may result in a quality assured process for analysis of microbial diversity in deep aquifers using groundwater from surface and tunnel boreholes.

The first part of this project addressed 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. Different methods for sampling of microorganisms attached to rock surfaces were developed and tested. Extraction and analysis of DNA was tested as well. Another part of the project was on the adaptation of a FC method for *in situ* development, sampling and analysis of microbial biofilms on solid materials introduced in groundwater flowing from deep aquifers. The method employs FCs with solid surface materials that were connected to tubes feeding the FCs with groundwater from packed off sections in boreholes at various groundwater pressures. Planktonic microorganisms then adhered, grew, multiplied and formed biofilms on the solids. DNA and ATP was extracted and quantified from the solids. The project also investigated new swab materials 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 the different new types of sampling swabs and storage techniques. Finally, this project developed a pressure filtration method to collect large quantities of DNA from planktonic microorganisms for diversity analysis of sequence libraries. The DNA from pressure filtrated groundwater from boreholes in the Äspö HRL tunnel, Sweden and DNA from FCs attached to these tunnel boreholes and surface boreholes in Olkiluoto, Finland, were sequenced. The FCs were loaded with glass beads 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 and enumeration of cell numbers and biomass

2.1.1 Total number of cells

The total number of cells (TNC) was determined using the acridine orange direct count method as devised by Hobbie et al. (1977) and modified by Pedersen and Ekendahl (1990). The acridine orange dye binds to nucleic acids and is fluorescent in blue light. The acridine orange method is described in detail in several papers (e.g. Pedersen et al. 2008). Briefly described, samples were suction filtered (−20 kPa) onto 0.22-µm-pore-size Sudan black-stained polycarbonate filters, 13 mm in diameter, mounted in stainless steel analytical filter holders. The filtered cells were stained for 5–7 min with 200 µL of a 10 mg L^{-1} acridine orange solution, dried, and mounted between microscope slides and cover slips using fluorescence-free immersion oil. The number of cells was counted under blue light (390–490 nm), using a band-pass filter for orange light (530 nm), in an epifluorescence microscope at 1000 × magnification. At least 600 cells and 15–30 microscopic fields (1 field = 0.01 mm^2) were counted on each filter. The expected distribution of cells on the filters should follow a normal distribution. Usually, three subsamples filtered on three filters were counted, and the average of these three results is reported together with the standard deviation of the mean. Finally, the personnel conducting microscope counting must be inter-calibrated; otherwise, there may be different interpretations of what should be counted.

2.1.2 Plate count of cultivable heterotrophic aerobic bacteria

Petri dishes with a diameter of 9 cm containing agar with nutrients were prepared as described elsewhere (Pedersen and Ekendahl 1990) for determining the numbers of cultivable heterotrophic aerobic bacteria (CHAB). Briefly, ten-times dilution series of water samples were made in sterile analytical grade water (AGW) containing 1.0 g L^{-1} of NaCl and 0.1 g L^{-1} K_2HPO_4; 0.1 mL portions of each dilution were spread with a sterile glass rod on the plates in triplicate. The plates were incubated for 8 days at 20 °C in the dark, after which the number of colony-forming units was counted; plates with 20–200 colonies were counted.

2.1.3 Cultivable fungi

For determination cultivable numbers of fungal cells, Sabouraud agar containing 40 g L^{-1} of dextrose, 10 g L^{-1} of peptone and 10 g L^{-1} agar (Prolabo, order no AX0222456, VWR, Stockholm Sweden) was prepared to Petri dishes. Briefly, ten-times dilution series of water samples were made in sterile AGW containing 1.0 g L^{-1} of NaCl and 0.1 g L^{-1} K_2HPO_4; 0.1 mL portions of each dilution were spread with a sterile plastic rod on the plates in triplicate. The plates were incubated for 14 days at 20 °C in the dark after which the number of colony-forming units was counted; plates with 20–200 colonies were counted.

2.1.4 Most probable number of sulphate reducing bacteria

An anaerobic medium for determining the most probable number (MPN) of SRB was prepared according to the procedures described by Hallbeck and Pedersen (2008). The MPN procedure results in a protocol with tubes that scored positive or negative for growth. The results of the tube analyses were rated positive or negative compared with control levels. Three dilutions of five parallel tubes were used to calculate the MPN of SRB, according to the calculations found in Greenberg et al. (1992). The lower and upper 95 % confidence intervals for the MPN method applied to five parallel tubes equalled approximately 1/3 and 3 times the obtained values, respectively. The detection limit for the methods using five parallel tubes with 1 mL of sample per first tube is of 0.2 cells mL^{-1}.

2.1.5 Adenosine-5'-triphosphate (ATP)

Adenosine-5'-triphosphate (ATP) transports chemical energy within cells for metabolism. ATP is a multifunctional nucleotide used in cells as a coenzyme. It is often called the "molecular unit of currency" of intracellular energy transfer. It is produced by cellular respiration, photosynthesis, or fermentation and used by enzymes and structural proteins in many cellular processes, including biosynthetic reactions, motility, and cell division. One molecule of ATP contains three phosphate groups, and it is produced by ATP synthase from inorganic phosphate and adenosine diphosphate (ADP) or adenosine monophosphate (AMP). Metabolic processes that use ATP as an energy source convert it back into its precursors, so ATP is continuously recycled in organisms. The average bacterium contains 1×10^{-18} moles (amol) of ATP, but this concentration varies significantly with cell size and metabolic status. Active cells have more ATP than do inactive, non-metabolizing cells. The analysis of ATP thus captures both biomass and activity.

The ATP Biomass Kit (HS no. 266–311; BioThema, Handen, Stockholm) was used to determine total ATP in the biofilms. The ATP biomass method used here has been described and tested in detail, and evaluated for use with Fennoscandian Shield groundwater (Eydal and Pedersen 2007). Typically, three subsamples were extracted and analysed and the average of these three results was reported together with the standard deviation of the mean. The method reports ATP, which correlates with the activity and size of the cells in a sample. A sample containing large, active cells will consequently contain more ATP than will a sample containing the same number of small, inactive cells.

2.1.6 Test bacteria

During the method development, several different pure cultures of bacteria were used as proxies for biofilm microorganisms.

Pseudomonas aeruginosa

Pseudomonas aeruginosa was ordered from Culture Collection, University of Göteborg (CCUG 56489) and cultured on solid nutrient agar. A large colony of *P. aeruginosa* grown on tryptic soy agar plates (Oxoid Art. No. PO0163) was reconstituted in a tube containing 10 mL of sterile isotonic Phosphate Buffer Solution (PBS) with pH 7.4 (order no. AM9625, Ambion, Invitrogen, Thermo Fisher, Sweden) to approximately 10^7 cells mL^{-1} and used for inoculation of biofilm experiments.

Pseudomonas fluorescens

Pseudomonas fluorescens, originally isolated from borehole KAS03 at the island of Äspö was ordered from Culture Collection, University of Göteborg (CCUG 32456 A) and cultured in 100 mL Erlenmeyer flasks containing 50 mL aerobic nutrient broth (Sharlau Art. No. 02-140) medium (13 g L^{-1}), sealed with a sterile cotton top. Flasks were kept shaking at 150 rotations per minute in a dark room with a temperature of 30 °C.

Shewanella putrefaciens

Shewanella putrefaciens, originally isolated from borehole KR0013 (70 meter below sea level (m.b.s.l.)) at Hålö next to island of Äspö, was ordered from Culture Collection, University of Göteborg (CCUG 32456 B) and cultured in anoxic NRB-medium. This medium has been described in detail elsewhere (Pedersen et al. 2008).

Pseudomonas stutzeri

Pseudomonas stutzeri, originally isolated from borehole KR0013 (70 m.b.s.l.) at Hålö next to island of Äspö, was ordered from Culture Collection, University of Göteborg (CCUG 36651) and cultured aerobically in NRB-medium.

Desulfovibrio aespoeensis

The bacterial type strain *Desulfovibrio aespoeensis* Aspo-2 (DSM 10631T) originally isolated from deep granitic groundwater (borehole KAS03 at a depth of 600 m) at the Äspö HRL (Motamedi and Pedersen 1998) was ordered from Deutsche Sammlung von Mikroorganismen and cultured in 300 mL anoxic flasks containing SRB-medium. The medium used for cultivation has been described elsewhere (Pedersen et al. 2008). 1 mL of a culture containing 10^8 cells mL^{-1} was transferred to a tube containing 9 mL of sterile PBS, generating approximately 10^7cells mL^{-1}.

2.2 Nucleic acid extraction, quantification and analysis

2.2.1 DNA extraction

Three different extraction kits were tested for extraction of total genomic DNA from sample materials using the manufacturer's respective protocol.

- PowerWater® DNA Isolation Kit, (order no. 14900-100-NF, MO BIO Laboratories, Immuno diagnostics, Hämeenlinna, Finland).
- PowerBiofilm® DNA Isolation Kit, (order. no. 12988-10), MO BIO Laboratories, Immuno diagnostics, Hämeenlinna, Finland).
- PowerSoil® DNA Isolation Kit, (order. no. 12888-100, MO BIO Laboratories, Immuno diagnostics, Hämeenlinna, Finland).

The extracted DNA was quantified and subsequently stored at −20 °C until 454 pyro-sequencing.

2.2.2 Quantification of extracted double stranded DNA

Extracted nucleotide eluates were first quantified using a ND-1000 UV-vis spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA), for quality control of the extraction efficiency and purity.

Double stranded (ds) DNA concentrations were quantified 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.

2.2.3 454 FLX Titanium 16S rDNA v6v4 pyrosequencing

A Bacterial 16S rDNA v4v6 amplicon library for sequencing was generated with degenerated forward (518F, CCAGCAGCYGCGGTAAN) (Marteinsson et al. 2013) and reverse primers (1064R, CGACRRCCATGCANCACCT) (Huber et al. 2007). Conditions for the PCR reaction was; 1X Platinum HiFi Taq polymerase buffer, 1.6 units Platinum HiFi polymerase, 3.7 mM $MgSO_4$, 200 µM dNTPs (PurePeak polymerization mix, ThermoFisher), and 400 nM primers. Between 5 and 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 sample series 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 a Bio-Rad mycycler. The quality and concentration of the amplicon library was evaluated with Agilent Tapestation 2000 instrument from Agilent according to manufacturer's protocol. The reactions were cleaned and products under 300 base pairs were removed using Ampure beads at 0.75 × volume (Beckman Coulter, Brea CA). The final products were re-suspended in 100 µL of 10mM Tris-EDTA + 0.05 % Tween-20, quantified with 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 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, barcode key was identified and removed and sequences likely to be of low-quality based on assessment of pyrosequencing error rates was removed

(Huse et al. 2007). A Bioinformatic Trimming anchor site (565F-a) TGGGCGTAAAG was used to trim sequences to the same biological length. Further sequences were screened for chimeras with the UCHIME algorithm (Edgar et al. 2011). The 454 pyrotag sequence clustering into operational taxonomic units (OTUs) was done by an open reference OTU picking methodology using the USEARCH algorithm which uses both a de novo and reference based approach (Edgar 2010). After OTU-picking a representative sequence from each OTU was selected and used for processing to assign a taxonomic classification based on the Greengenes 13_8 database (http://greengenes.lbl.gov/cgi-bin/nph-index.cgi).

After classification of data the representativeness of sequences was tested by rarefaction analysis and Chao as well as abundance-based coverage (ACE) indexes were used to estimate richness. Samples were not normalized or subsampled when alpha- or beta diversity was analysed (McMurdie and Holmes 2014). To statistically estimate abundance and evenness for each sample, Shannon and Simpson indices were calculated. Distance calculations for phylogenetic tree construction were done by Unifrac distance measure and Unweighted Pair Group Method with Arithmetic Mean (UPGMA). The generated tree was visualized in FigTree v 1.4.0 software. To compare groups and visualize relationships of samples based on their composition of OTUs a Principal Coordinate Analysis (PCoA) was done in the Emperor software allowing for three-dimensional visualization of clustering based on metadata for samples (Vázquez-Baeza et al. 2013).

2.2.4 Bioinformatic processing, statistical analyses and data visualization

The amplicon 16S rDNA sequencing data was analysed and evaluated with the Quantitative Insights into Microbial Ecology software (QIIME) version 1.9.1. (www. http://qiime.org). Data graphics design and statistical analyses were performed in Statistica 13 (Statsoft Inc., Tulsa, OK, USA).

2.2.5 Sequence Read Archive (SRA) submission

The sequence reads were submitted to the Sequence Read Archive (SRA) and was given the following accession numbers: SRP076858 for the Äspö tunnel and COSC_1 drillhole reads. SRP076834 for the Olkiluoto reads.

2.3 Development and testing of methods for sampling and analysis of attached microorganisms on fracture surfaces

2.3.1 Tools for removal of biofilms on fracture surfaces

Two sampling methods were evaluated for the ability to efficiently collect cells from core fracture surfaces, scraping with scalpel and swabbing.

Scalpels

The scraping method was adopted from Jägevall et al. (2011). First, 2 mL PBS were applied on the sampling surface followed by incubation for 5 minutes to enhance the removal of biofilm microorganisms from the fracture surface. Subsequently, a single use sterile scalpel (Swann-Morton, order no. 133-310030, ThermoFisher Scientific, Sweden) was used to scrape and detach bacteria from the surface. Detached surface sample material was mixed in the added PBS on the surface which was transferred by pipetting the mix to an Eppendorf tubes for downstream analysis.

Swabs

Several different swab materials were tried out during this work. They were DNA-free cotton swab, (LP ITALIANA SPA order no. LPIT111298, VWR, Sweden), sterile rayon swab (LP ITALIANA SPA, order no. LPIT111698, VWR, Sweden), ATP-free polyester swab (order no 710-0462), sterile alginate swab (order no 710-0429), sterile viscose swab (order no 710-0457) all from COPAN diagnostics via VWR, Sweden and COPAN4N6FLOQSwabs™, flocked swab with scalpel shaped tip for forensic use (order no 4479438, Life technologies, Thermo Fisher, Sweden). The swabbing method

involved swabbing of the fracture surface with a swab soaked in a swabbing solution. Fracture surfaces were swabbed to collect sample material and thereafter the tip of swab was detached from the handle and placed in an Eppendorf tube to be stored and analysed according to the instructions for each downstream analysis.

Several of the tested swabs in the array above released fibres in various amounts to the sample material which disturbed the downstream analysis. During the time frame of this project, a new type of COPAN swab was developed for forensic investigations of crime scenes where very low amounts of cells and DNA challenge the investigators. The flocked COPAN swab was easy to use for sampling fracture surfaces, samples can be stored at room temperature in the transfer tube and the swab did not release fibres to samples. The flocked COPAN swab was chosen as the preferred swab for DNA sampling for sampling when it came on the market in 2014. The manufacturer COPAN could not guarantee that the flocked swabs were free of ATP molecules. Consequently, we chose to proceed using the COPAN ATP-free rayon swabs for ATP sampling. At the end of the project, a new type of swab specifically developed for ATP came on the market in 2015, the Hy-Lite Swab (Merck ATP-free 50-pack, Order no1.30103.0001.1 VWR/ or Th. Geyer). This swab will be used in future sampling (Appendix A1.1).

2.3.2 ATP from fracture surfaces

Fracture surfaces were swabbed with ATP-free polyester swabs from COPAN. Each swab sample was placed in an Eppendorf tube containing 1 mL of B/S extractant from the ATP Biomass Kit, vortexed for 2 minutes and stored in the dark for a minimum of 30 minutes before analysis (2.1.5). Tests were performed to optimize sample storage procedures for laboratory and field conditions.

Test of ATP sample storage procedures

Approximately 50 mm pieces of sterile rock were placed in a 2 L beaker with aerobic NRB-medium. A pure culture of *P. stutzeri* was pre-cultured to 10^8 cells mL^{-1} and 0.5 mL was used as inoculum. The beaker with rock and bacteria was placed in a shaking water bath at 65 r.p.m. and 30 °C for 3 days to grow biofilms on the rock surfaces. Three 50 × 50 mm surfaces were swabbed with ATP-free polyester swabs and analysed immediately. This procedure was repeated in triplicate and the swabs were stored in room temperature, refrigerator and in a freezer for 48 h before analysis. Storage in room temperature showed higher ATP values than did refrigerator and freezer, probably because of microbial growth in the swab during warm storage. It was decided that freezer should be used for storage.

2.3.3 DNA from drillcore fracture surfaces

Sample for DNA extraction was sampled from fracture surface by scraping with scalpel or swabbing. The tip of the swab was placed in an Eppendorf tube and stored at −20 °C until extraction and quantification (2.2.1).

Test of DNA sample swabs and storage procedures

Approximately 50 mm pieces of sterile rock were placed in a 2 L beaker with aerobic NRB-medium. A pure culture of *P. stutzeri* was pre-cultured to 10^8 cells mL^{-1} and 0.5 mL was used as inoculum. The beaker with rock and bacteria were placed in a shaking water bath at 65 r.p.m. and 30 °C for 3 days to grow biofilms on the rock surfaces. Six 50 × 50 mm surfaces were swabbed with DNA-free viscose swabs and stored in refrigerator (3 samples) and in a freezer (3 samples) for 48 h before analysis. The best yield and quality was obtained with storage in freezer.

A second test was performed using the flocked COPAN swab with scalpel shaped tip. DNA/RNAase free microscope slides were added with increasing concentrations of *P. stutzeri* which was let dry for a couple of minutes and then swabbed. The swabs were extracted and the amount of obtained DNA was analysed on the ND-1000 UV-vis spectrophotometer (Nanodrop). The yield ratio and quality was not good. Therefore, a new set of experiments were started which tested the yield and quality with and without the swab in the lysis step. The best yield and quality was obtained without the swabs in the lysis step.

2.3.4 Sources of fracture surfaces in drill cores

The development of the swab method utilised drill cores from drilling operations at Äspö HRL and in Åre, Sweden. Drill cores were inspected for possible water conducting fractures. Selected such fracture surfaces were sampled as described next.

Äspö HRL

Drill cores in storage from drilling of KJ0052F01 (Pedersen 2000) were used to create sterile fresh fracture surfaces.

Drilling at Äspö HRL was done with triple tube drilling (50.2 mm core diameter) with 3 m length of the cores from the NASA3009A and NASA3067A boreholes. Drilling was launched in November 2011 and 18 m per day where drilled. The task was to give access to drill cores of water-bearing fracture surfaces immediately after removal. This type of sampling was previously tested and did prove to be successful (Jägevall et al. 2011). When one core with water-bearing fractures was obtained it was sampled by performing Äspö staff in the tunnel and documented with digital photography. Immediately after sampling one core was wrapped in a plastic film and preserved under vacuum in an aluminium bag and sent to the laboratory. The original plan suggested that several samples should be collected, but the personnel on site abandoned and ignored the approved activity plan, thereby minimizing the number of cores to only one.

Åre COSC-1

The COSC programme provided a unique opportunity to further develop and test new COPAN swab types for collection of DNA and ATP. The drilling program targeted 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). The COSC-1 drilling operations started on 1 May 2014 and were completed on 26 August 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 depth. COSC-1 drilling operations and the directly related on-site investigations were financed by International Continental Drilling Program and the Swedish Research Council (Figure 2-1). Researchers from Microbial Analytics Sweden got the opportunity to, in collaboration with the science team on site, collect biofilms on fresh bedrock fracture surfaces during the drilling. Drilling operations were conducted 24 h day^{-1} with initially two drillers per 12 h shift and three drillers below 545 m depth (after 2 weeks of drilling). The exact depth of COSC-1 is 2495.8 m.

2.3.5 Analysis of sterile fracture surfaces - Negative controls

Sterile fracture surfaces were prepared as follows. Dry drill cores from drilling of KJ0052F01 with a diameter of 76 mm were put in a sterile plastic container filled with sterile deionised water (analytical grade) for approximately one week to allow the cores to become water-saturated. At the time of analysis, the cores were mechanically split with a hammer, generating two sterile fracture surfaces. The two fracture surfaces of the core were mounted horizontally in a vice. A sterile plastic film was wrapped around the top of each core to prevent liquids from running off the surface.

Half of one fracture surface area was thoroughly swabbed with a rayon swab soaked with PBS. The swab was then put in a tube with 1 mL PBS and analysed for TNC. The other half surface area was swabbed in the same manner but with an ATP-free viscose swab and then transferred to a tube containing 1 mL extracting solution B/S (see ATP-analysis section). This tube was then analysed for ATP content.

The opposite fracture surface was treated with 2 mL PBS after which the whole surface was thoroughly scraped with a sterile scalpel. After scraping the solution was pipetted up and transferred to Eppendorf tubes. The solution was then analysed for ATP content and TNC.

Figure 2-1. *The COSC-1 drill site with the drill mud reservoirs in front, drill strings on top and the drill rig in the background. The blue "bugs-lab" is visible in in the left background.*

2.3.6 Analysis of fracture surfaces with known amounts of bacteria

Sterile fracture surfaces were prepared, sampled and analysed as in 2.3.5. However, before swabbing and scraping, the surfaces were treated with 1 mL of a *P. fluorescens* culture (10^7 cells mL^{-1}) of which the culture liquid was allowed to be almost completely absorbed into the core rock before sampling.

2.3.7 Sampling and analysis of KA3065A01 fracture surfaces

At the arrival to Micans the fractures were first inspected to confirm their authenticity (not created as effect of drilling). To prevent the fractures from drying they were moistened with sterile AGW containing 1.0 g L^{-1} of NaCl and 0.1 g L^{-1} K_2HPO_4 and wrapped with plastic film before sampling. The two fractures on each side of the core were labelled #1 and #2, were #1 corresponded to 24.17 m drillhole length and #2 to 23.97 m drillhole length. Each fracture was divided into four equally large sectors, one for each downstream analysis, according to Table 2-1.

All four sectors were thoroughly scraped with a sterile scalpel to release the biofilm from the surface (Figure 2-2). After scraping each sector was swabbed with a DNA/RNA-free viscose swab which was subsequently transferred to the appropriate sampling vessel, one swab for each sector. The samples were then analysed according to each method as stated above.

To verify the sampling method, each fracture #1 and #2, were treated with an equal mix of *D. aespoeensis* and *P. aeruginosa* cultures, with 10^7 cells mL^{-1}. Four mL of this mix were added to the fractures by pipette and the liquid was allowed to soak into the rock for 20 min. When the fractures were still moist but without free liquid the sampling procedure described above was repeated.

Table 2-1. Sectors of fracture with corresponding downstream analysis and sampling vessel.

No	Analysis	Treatment
1	Most probable number of sulphate-reducing bacteria	9 mL anaerobic SRB medium
2	Cultivable heterotrophic aerobic bacteria	1 mL PBS
3	ATP-analysis	Extraction solution B/S
4	DNA extraction	PowerSoil® DNA Isolation Kit

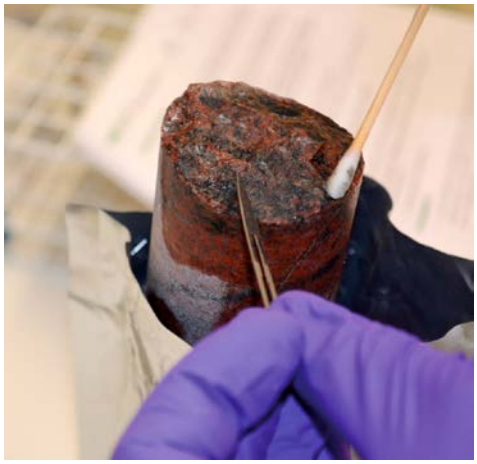

Figure 2-2. *Sampling of one of the KA3065A01 fracture surfaces showing scalpel and a viscose swab.*

2.3.8 Sampling and analysis of COSC-1 fracture surfaces

The sample positions and their characteristics along the COSC-1 borehole are given in Table 2-2. 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 (Figure 2-3). 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 an ATP-free rayon swab to absorb the released intracellular ATP. Then the top of the swab was separated from the applicator and stored in 1.5 mL Eppendorf sample tubes and frozen until analysis.

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

Sample name	Sample date	Borehole depth (m.b.s.l.)	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	985.00	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. Drill mud was also sampled
427-Z	2014-06-21	1320.7	Porous mafic band w/ fractures, corresponding mud sample at 13:55 am. Drill mud was also sampled
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

m.b.s.l = meter below sea level.

* Mafic is 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.

DNA sampling was performed in a similar way using the Copan FloqSwab™ (Figure 2-4). The swabs were rubbed gently over the moist fracture surface directly after retrieving the drill core to collect cells for subsequent DNA extraction with the PowerWater® DNA Isolation Kit. Until the time for analysis the swabs were stored in the provided sample tube in room temperature, according to the manufacturer's instructions. Drillmud and porous material were sampled to 50 mL polypropylene tubes (Figure 2-5).

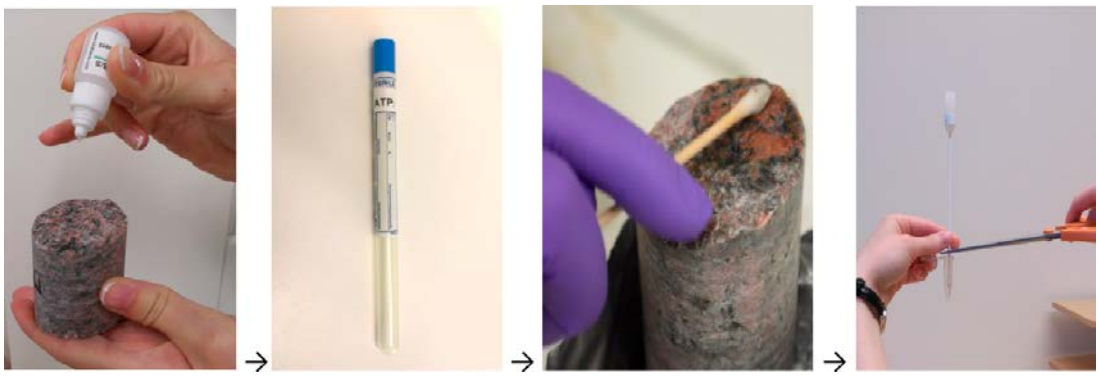

Figure 2-3. *Sampling procedure for ATP: Add 2–3 drops from the bottle containing Extractant B/S on the surface. Rub thoroughly all over the surface with the swab marked ATP. Place the swab in the provided 1,5 mL Eppendorf tube. Cut off the swab at 2 cm from the tip and close the cap on the tube. Store the ATP samples in freezer.*

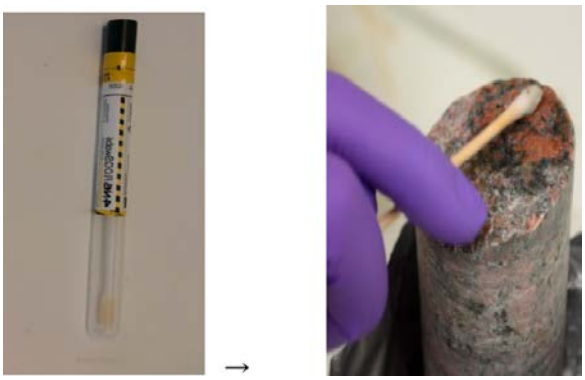

Figure 2-4. *Sampling procedure for DNA: Sample the opposite fracture surface with the black swab for DNA. Put the swab back into the provided tube. Note the sample number on the tube and on the sampling list. Store the DNA samples in room temperature.*

Figure 2-5. *Sampling of mud and sand material for DNA and ATP: When an aquatic fracture surface consists of porous materials of mud and soil minerals, as much as possible of the materials should be collected with a sterile scrape and into a 50 mL polypropylene tube. Sample both opposite surfaces separately. Scrape material from the fracture surface and place the material into a tube marked "ATP" and repeat on the corresponding surface and place the sample material in a tube marked "DNA". Note sample identification on the tubes and on the sample list. Store these samples in the freezer.*

2.4 Development and testing of methods for sampling and analysis of attached microorganisms on solids in flow cells

2.4.1 Flow cells and solids used for biofilm development

The development of this sampling method utilised flow cells (FCs) with surfaces of rock, glass or garnets to grow biofilms *in situ* in the laboratory and in flowing groundwater at borehole sites in the Äspö HRL tunnel and in Olkiluoto, Finland. FCs used in this project had a stainless steel shells (length 300 mm, diameter 65 mm) and were lined with polyvinyldifluoride (PVDF) plastic (Figure 2-6). Each FC had a 120-mm-long PVDF insert with a 22×32 mm opening that supported ~100 g of garnet grains (0.70 mm, Bulk 500 g, order no. 13123-05, Immuno Diagnostic Oy, Finland.) and glass beads (1 mm Hecht Assistant, art.no 201-0276 VWR, Sweden), per FC for microbial adhesion and biofilm growth. The garnet grains were of molecular grade meaning they were sterile, DNA-free and RNAse/DNAse-free. The glass beads were sterilized by heating to 450 °C for 5 hours in a muffle furnace. Crushed rock from Äspö HRL drill cores were heat sterilized at 160 °C for 5 h and tested as a material for attachment and growth.

With a different type of PVDF insert, glass slides and slides of rock could be used as support for biofilms during the development of sampling and analysis methods (Figure 2-7). All glass slides (dimensions $60 \times 24 \times 0.8$ mm) used in the flow cells were heated for 4 hours at 475 °C in a muffle furnace to ensure the glass surface was hydrophilic and free of organic material before insertion into the FCs. The granite for these experiments came from the drill-core taken from borehole KJ0052F03 in Äspö HRL (Pedersen 2000). This rock was cut in to slides with dimensions of $30 \times 24 \times 0.8$ mm and then polished on both sides to have a surface comparable to the glass slides for biofilm adhesion. The slides were cleaned and disinfected with 70 % ethanol and air dried before insertion into the FC. This set-up was used previously to investigate the influence of biofilms on sorption of radionuclides (Anderson et al. 2006).

2.4.2 Laboratory flow cell experiments with pure cultures and field tests

The laboratory FC experiments were performed by using bacterial cultures infused in an artificial groundwater without carbon sources that were circulated in the circulation system shown in Figure 2-8. Carbon sources was added later, during the experiments. The circulating medium with bacteria was led through two FCs with holders containing heat sterilised glass beads, crushed rock grains, and flat glass and rock surfaces, for biofilm enrichment. Garnet grains were sterile from the producer. The rock grains were heat sterilized at 160 °C for 5 hours.

Experiment 1

The circulation system was disinfected with a 22.5 mg L^{-1} ClO_2 solution for 1 hour and subsequently washed with sterile AGW 5 times before start of the experiment. A bacterial culture of *D. aespoeensis* was used for the enrichment with approximately 5×10^5 cells mL^{-1}. Flow cell number 1 was loaded with crushed rock and FC number 2 was loaded with flat glass and rock surfaces. The crushed rock and flat surfaces were sampled after 1, 4, 6, 13 and 20 days. Total number of cells were counted on flat glass surfaces and ATP and DNA were extracted from all surface materials.

Experiment 2

Experiment 1 was repeated with the following modifications. A bacterial culture of *S. putrefaciens* was used for the enrichment with approximately 2.5×10^5 cells mL^{-1}. Flow cell number 1 was loaded with garnet grains and FC number 2 was loaded with flat glass and rock surfaces. The crushed rock in experiment 1 interfered with the analyses, probably due to release of small mineral particles, and were, therefore, excluded in this experiment. The garnet and flat surfaces were sampled after 1, 3 and 8 days.

Experiment 3

Two different DNA extraction kits, PowerWater and PowerBiofilm, were tested and evaluated on glass beads and garnet grains exposed to flowing groundwater in Olkiluoto from drillholes OL-KR54 (365.5–369 mbls), OL-KR55 (863–866 mbls) and OL-KR56 (1154–1158 mbls).

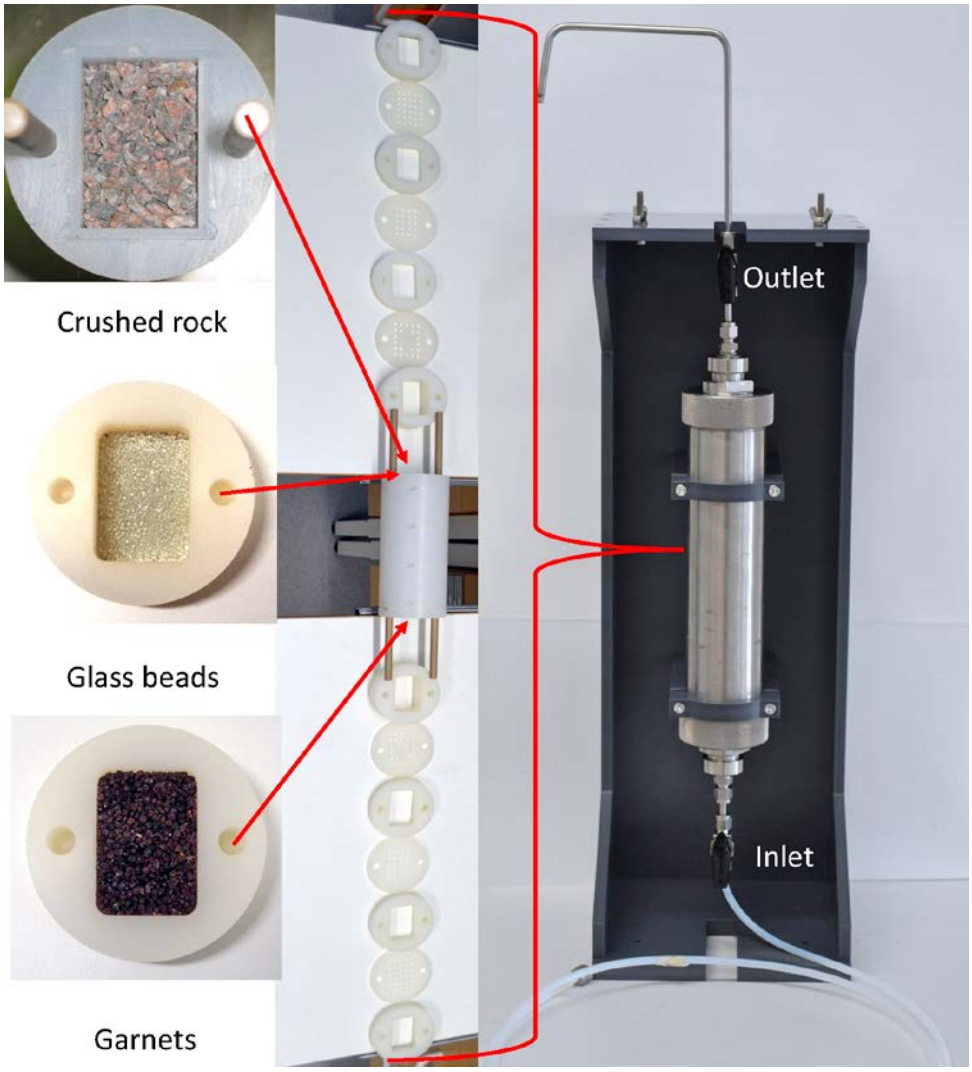

Figure 2-6. *Flow cell unit for sampling of attached microorganisms from groundwater. Fully mounted unit is shown to the right; the interior parts of an opened flow cell is shown to the left. Flow diffusor system that equalize the flow over the solid material in the middle and holder for glass beads and garnet grains to the left.*

Figure 2-7. *Mounted pressure-resistant flow cell with glass slides. Three flow diffusers distribute the flow evenly over all test surfaces in the test pile. The steel is not in contact with the groundwater.*

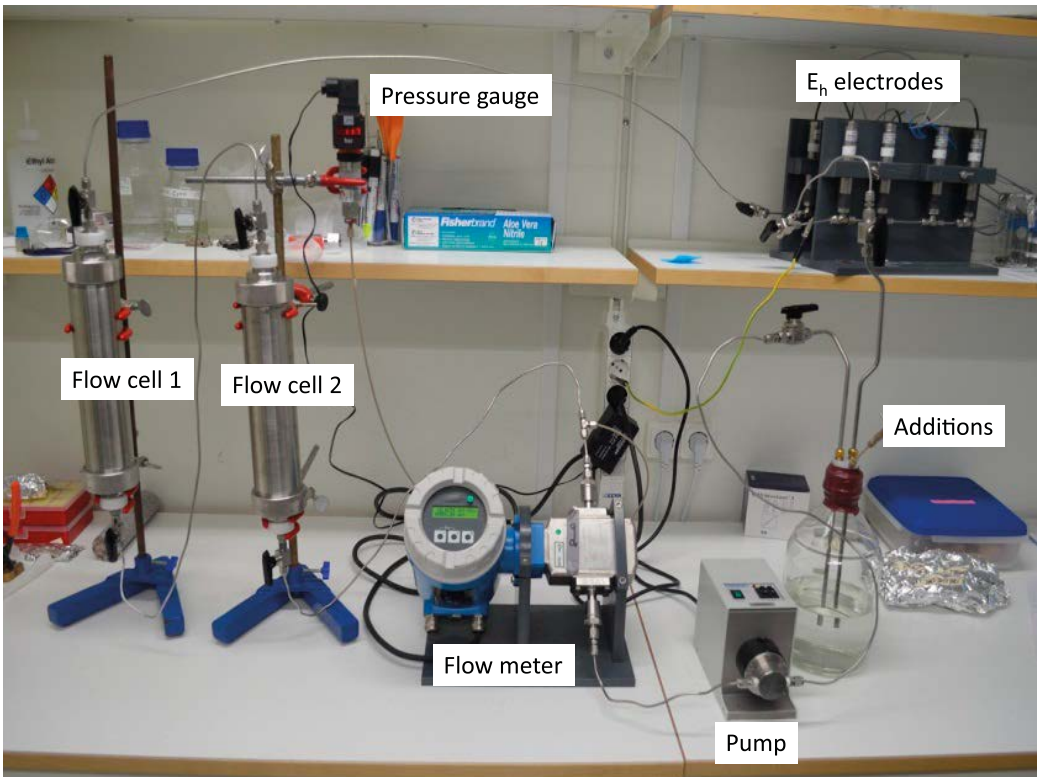

Figure 2-8. *The circulation system in the laboratory with two flow cells for enrichment and analysis of ATP and DNA in biofilms.*

2.4.3 Optimization of DNA extraction

Optimization tests for extraction of DNA from the biofilms were made in these laboratory experiments with the PowerWater DNA Isolation Kit and the PowerBiofilm DNA Isolation Kit. This was done to evaluate if any of the kits yielded a higher amount of DNA after the extraction of the biofilm 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 Picogreen High Sensitive Kit 0–20 ng, which was supplied by Invitrogen.

2.4.4 Estimating the amount of biofilm on the test material

After performing the ATP analysis and DNA extraction, each sample of enrichment material was washed, dried and then weighed. By using the weight and diameter of each garnet grain or glass bead in the sample materials the total area was calculated in cm^2. Each garnet grain had an approximate diameter of 0.7 mm and an average weight of 3.51 mg per grain. The corresponding parameters per glass bead were 1 mm in diameter and an average weight of 2.22 mg.

The following formulas were then used to calculate the average area available for attachment on the enrichment solids in cm^2:

One grain of the garnet (diameter = 0.7 mm) weighed in average 3.51 mg. The surface area of 0.7 mm per grain was calculated using the formula $4 \pi \times r^2$. The total enrichment area of each sample was then calculated by multiplying the number of grains with the total area of each grain in cm^2. One glass bead (1 mm in diameter) weighed 2.22 mg. The surface area per 1 mm glass bead was likewise calculated by the formula $4 \pi \times r^2$. The same calculations were used on the glass as with the rock.

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 (amol) were divided with 0.4 which to obtain the total number of cells. The results were then divided by the calculated enrichment area in cm^2 for each sample.

The amounts of nucleic acids were calculated to cell numbers using the average amount of DNA in the typical groundwater bacterium, i.e. *D. aespoeensis*, which is 649 Daltons/base pair × 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 $Dalton^{-1}$ = 3.9×10^{-15} g DNA $cell^{-1}$. The DNA then has an average weight of 3.9×10^{-15} g $cell^{-1}$ (Pedersen et al. 2014b). 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 subsequently divided by the total enrichment area to estimate the number of cells per cm^2 in each sample.

2.4.5 Enrichment of biofilms *in situ*

For the *in situ* experiments the FCs were prepared with sterile enrichment materials. Each FC was connected to flowing groundwater from boreholes in the bedrock, either in the Äspö tunnel (Figure 2-9, Figure 2-10) in Sweden or in Olkiluoto, Finland. The groundwater was flowed through each connected FC, 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 FC was noted in regular intervals, in order to identify any factors that could affect the microbes' ability to attach and form biofilms. We hypothesized that the number of attached microbes would correlate positively with the flow rate and the amount of groundwater that passed through the FCs. But a high water flow rate may cause turbulence, with the result that attachment is mitigated. In Finland, the flow rates were fixed by other sampling procedures and they could not be manipulated. In difference, the flow rate and the release pressure in each FC were controlled when operating in the Äspö HRL.

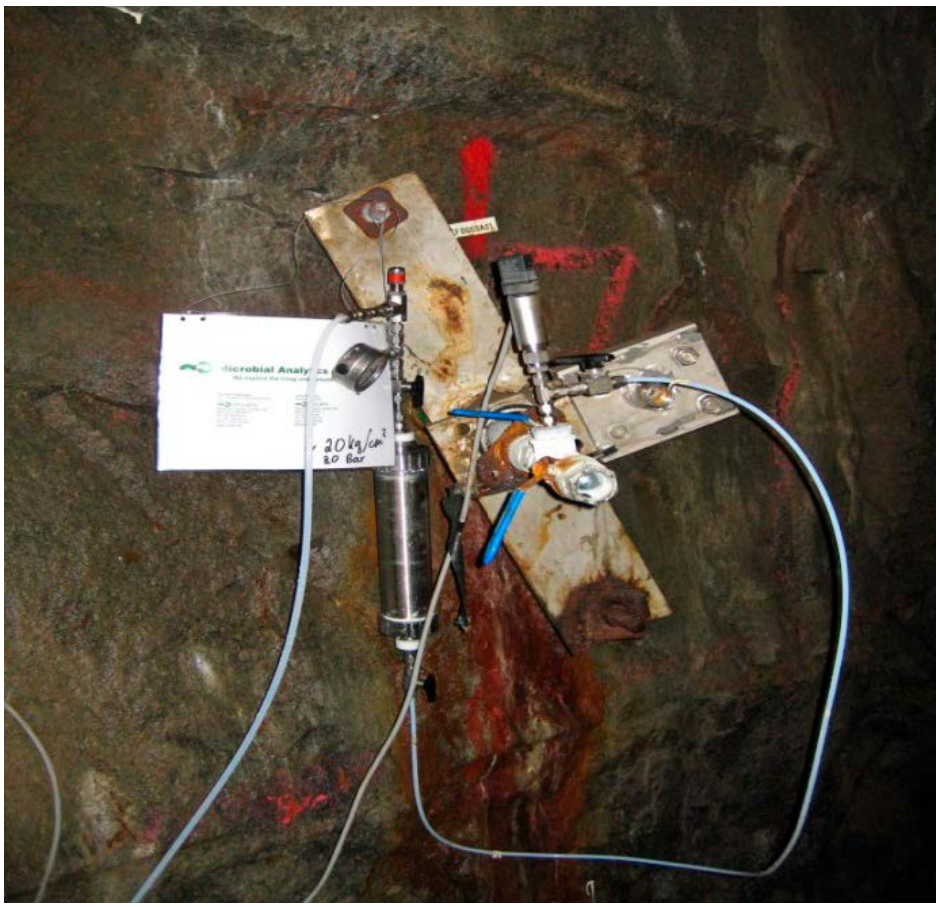

Figure 2-9. *A flow cell connected to borehole KA0069A01 in the Äspö hard rock laboratory tunnel, Sweden.*

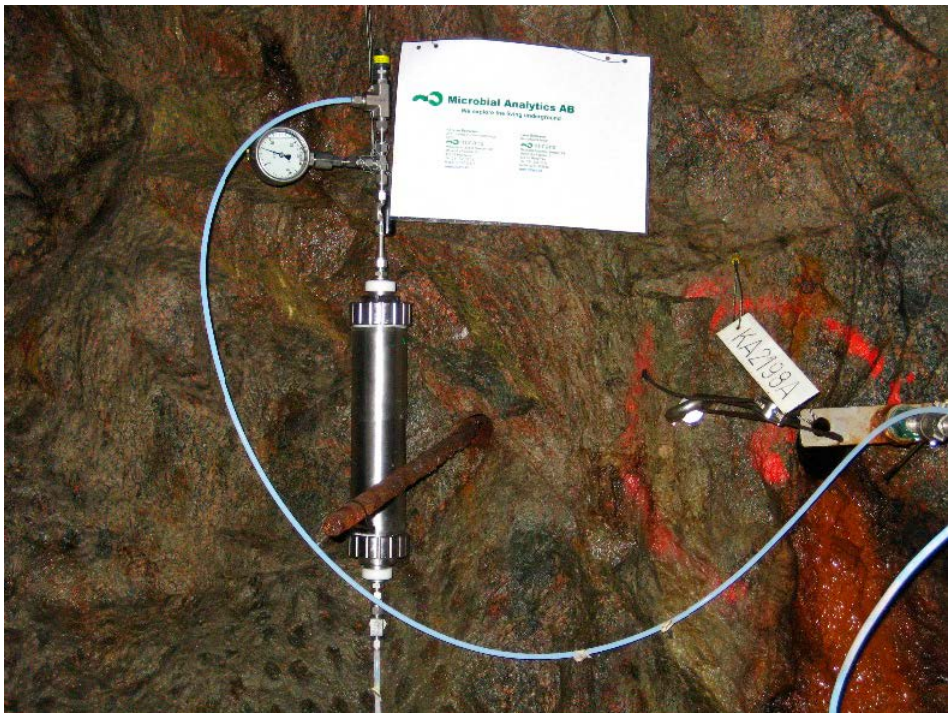

Figure 2-10. *A flow cell connected to the borehole KA2198A in the Äspö Hard Rock Laboratory tunnel, Sweden.*

2.4.6 Sampling methods for *in situ* biofilms

After enrichment in the field, the FCs were detached from the boreholes and transported back to the laboratory for further tests and analysis. Excess groundwater was removed from the FC, leaving only the surface material with attached microorganisms. The materials were divided into garnet and glass samples and washed with PBS to remove any planktonic microbes before proceeding with further analysis.

2.4.7 Analysis of ATP on garnet grains and glass beads from flow cells

Analysis of ATP on garnet grains and glass bead was performed according to the optimization in the laboratory of the method. During sampling, sterile equipment was used and the surfaces of glass respective garnets were kept separated, unless mixed during transport. The sampled beads and grains were washed with sterile PBS to remove any remaining planktonic cells from the solids. The washed sample material was then distributed to Eppendorf tubes and centrifuged and the supernatant was removed with pipette. Approximately 1.5 g of material of glass or garnets were sampled and placed in 1 mL B/S extractant from the ATP Biomass Kit, mixed and placed dark for 30 minutes to lyse the cells before analysed. The materials were washed, dried and weighed in order to calculate their total surface area in cm^2 (2.4.4). The amounts of ATP on garnet grains were compared with ATP on glass beads.

2.4.8 DNA from flow cell garnets grains and glass beads

The extraction efficiency of PowerWater compared to Power Biofilm was tested on garnet grains and glass beads exposed to groundwater from OL-KR57 (57–61m), OL-KR54 (364.5–369 m), OL-KR55 (863–866 m) and OL-KR56 (1154–1158 m). Experiments in the laboratory showed that the best DNA extraction results were obtained with the Power Water DNA isolation kit. Therefore, it was decided to continue using this kit when analysing the materials from the field. It was assumed that even with long exposure to flowing groundwater, there would be small amounts of biofilms.

Throughout the sampling, handling and analysing, the glass and garnets surfaces were consistently kept separated, except for a few samples where the materials had mixed up during transport. Using sterile equipment, the sampled enriched surfaces were washed with sterile PBS to remove remaining

groundwater that may contain planktonic cells from the surfaces. The washed surfaces were distributed in 5 g portions in respective 5 mL Power Water bead beating tubes and stored at −20 °C until DNA extraction. As with the ATP-analysis, the garnet and glass materials were kept separated, washed, dried and weighed in order to calculate their total surface area in cm^2.

2.4.9 Flow cell locations in the field

Äspö

One FC was connected to each of the boreholes KA2198A, KA3110A, KA3385A2 and KF0069A01 in the Äspö Hard Rock Laboratory (HRL) tunnel (Figure 2-9, Figure 2-10). 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 to between 200 and 400 kPa relative to the ambient aquifer pressure. The groundwater was let through the FCs at varying flow rates (Table 2-3). The FCs were connected to groundwater from to 2013-05-23 to 2013-06-24. Immediately after disconnection from the boreholes, the FCs were transported in coolers to the laboratory for sampling and analysis of the biofilms.

Table 2-3. Sampling data for boreholes in Äspö Hard Rock Laboratory tunnel investigated with flow cells for ATP and DNA analysis of biofilms.

Sampled borehole sites in the Äspö tunnel	Depth (m.b.s.l.)	biofilm growth time (days)	flowrate through FCs (ml min^{-1})	Total volume water through the FCs (L)
KA2198A	300	33	293	13 900
KA3110A	400	33	320	15 200
KA3385a2	420	33	285	13 500
KF0069A01	450	33	1635	77 700

m.b.s.l. = meter below sea level.

Olkiluoto

One FC was connected in line with pumped groundwater from a packed off section in each borehole (Table 2-4). Groundwater was let through each FC for attachment and biofilm development of planktonic microorganisms on the glass and garnet surfaces. The groundwater was let through the FCs at ambient pumping flow rates. Immediately after disconnection from the borehole, the FCs were transported in coolers to the laboratory for sampling and analysis of the biofilms.

Table 2-4. Sampling data for boreholes Olkiluoto investigated with flow cells for ATP and DNA analysis of biofilms.

Sampled borehole sites in Olkiluoto	Date sampled	Depth (m.b.s.l.)	biofilm growth time (days)	flowrate through FCs (ml min^{-1})	Total volume water through the FCs (L)
OL-KR57	2013-06-18	57–61	35	225	11 340 000
OL-KR57	2014-10-28	210–212	27	16	622 000
OL-KR54	2013-04-09	364.5–368	30	160	6 910
OL-KR6	2013-08-20	422–425	26	6.2	222 300
OL-KR46	2013-10-29	570.5–573.5	46	0.8	34
OL-KR55	2013-04-09	863–866	27	150	97

m.b.s.l. = meter below sea level.

2.5 Development and testing of a method for sampling and analysis of planktonic microorganisms in groundwater

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 2-11).

2.5.1 Pressure filtration of groundwater for DNA analysis

Groundwater was filtered during 15–16.5 hours at a flow rate of 0.2 L per minute from each of the boreholes KA2198A (Figure 2-10), KA3110A, KA3385A and KF0069A01 in the Äspö Hard Rock Laboratory (HRL) tunnel (Table 2-5). Sample dates were 2012-02-02 for KA2198A and 2012-07-20 for KA3110A, KA3385A and KF0069A01.

Table 2-5. 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 (m.b.s.l.)	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

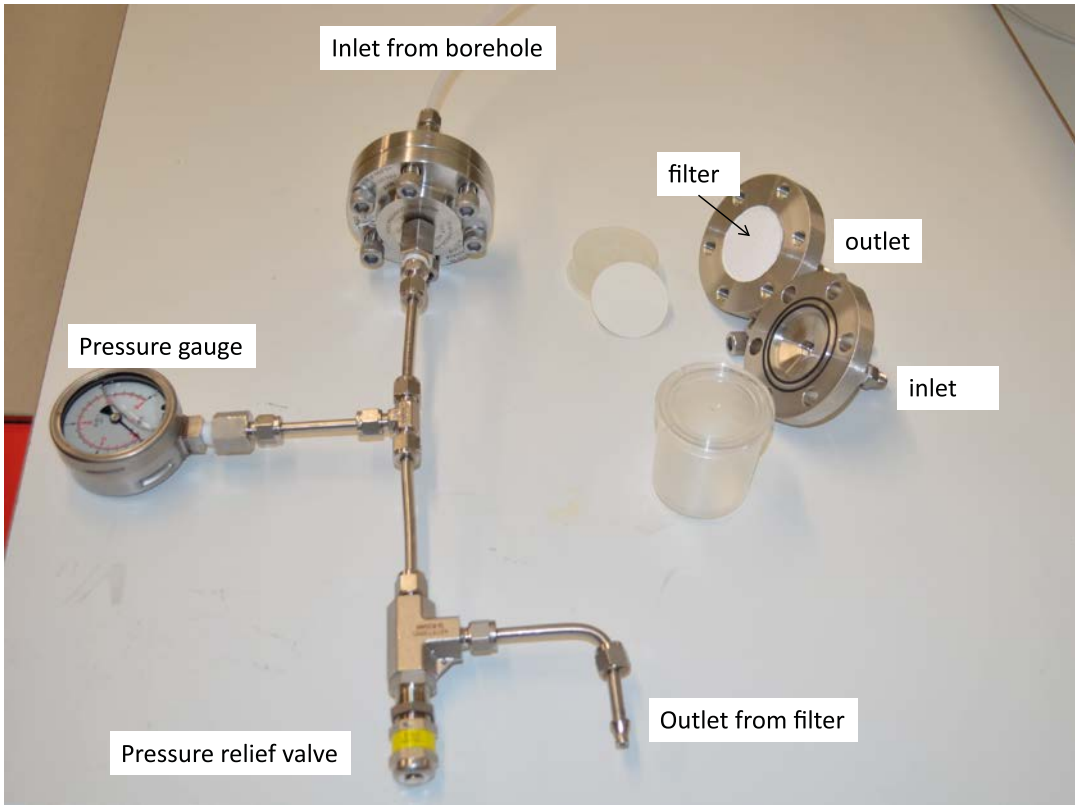

Figure 2-11. *Pressure filtration unit for sampling of microorganisms in groundwater. Fully mounted unit is shown to the left; an opened filter holder with filter is shown to the right.*

3 Results

3.1 Methods for removal of microorganisms from fracture surfaces

3.1.1 Analysis of sterile fracture surfaces and fracture surfaces with known amounts of bacteria

In the laboratory, test trials were performed on natural fracture surfaces and on surfaces made by splitting drill cores in two parts with fresh, sterile fracture surfaces. Two fracture surfaces of the split drill core were spiked with known amount of cells mL^{-1} before sampling and the other surfaces were kept natural as sterile controls. Cells could not be detected on the sterile fracture surfaces using microscopy for TNC and ATP methodology using the Rayon swabs (Table 3-1). When sterile fracture surface material was removed by scraping, a small amount of ATP was registered. It is likely that this reading was generated by auto-fluorescent mineral particles in the ATP luminometer and not by ATP.

The spiked fracture surfaces all had TNC and ATP above the detection limit (Table 3-1). The recovery (analysed/applied) was good for the TNC method but very low for the ATP method irrespective of the sampling method (swab or scrape). Overall, scraping gave about twice the amounts given with swabs.

Table 3-1. Analysed and applied amounts of ATP and TNC with yield ratios.

Sample	Applied ATP (amol)	Analysed ATP (amol)	Analysed/ Applied	Applied TNC (cells)	Analysed TNC (cells)	Analysed/ Applied
Rayon swab on sterile fracture	–	bd	–	–	bd	–
Rayon swab on spiked fracture	7.3×10^9	1.5×10^7	0.002	0.7×10^8	2.0×10^7	0.29
Scalpel on sterile fracture	–	3.7×10^4	–	–	bd	–
Scalpel on spiked fracture	7.3×10^9	3.3×10^7	0.005	0.7×10^8	4.3×10^7	0.61

b.d. = below detection limit. Spiking was done with *P. fluorescens*.

3.1.2 Sampling and analysis of KA3065A01 fracture surfaces

The drillcore from KA3065A01 was analysed on two sides, #1 and #2. Both sides had detectable amounts of ATP and TNC (Table 3-2). The two methods agreed well on both sides. There were more cells after spiking the surfaces, but the recovery was not good with the viscose swabs. There were cultivable, heterotrophic aerobic bacteria (CHAB) above detection limit on the natural surface, but SRB were below detection limit. Spiking with CHAB and SRB increased the CHAB number accordingly and SRB became above the lower limit of detection. The cultivable fungi analysis failed due to swarming of the growing cells on the plates resulting in a smear that could not be quantified (Table 3-3). All DNA analyses were below detection limit as well.

Sampling and analysis of COSC-1 fracture surfaces

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.

ATP analysis of swab samples

The results for ATP in the 17 swab samples are presented in Table 3-4 that displays amounts of ATP in the swabs between 8.92×10^3 up to 1.46×10^5 amol ATP. Since the swabbed surface areas were not exactly registered, the results shown are the total amount of sample in the swabs, which was extracted in 1 ml Extraction BS solution from the ATP Biomass Kit HS.

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 3-4). The amounts of DNA were re-calculated to number of cells for the 5 samples with the largest masses of DNA (Table 3-5).

Drill mud 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 drill mud sample. The drill mud sample was not expected to correlate with swab sample, because the drill mud was diluted with water from a nearby creek on ground. There were detectable amounts of nucleic acids in the drill mud samples (Table 3-6). Two drill mud samples, 401-Z and 427-Z, were extracted for 454 pyrosequencing.

Table 3-2. Analysed and applied amounts of ATP and TNC with yield ratios.

Sample	Applied ATP (amol)	Analysed ATP (amol)	Analysed/ Applied	Applied TNC (cells)	Analysed TNC (cells)	Analysed/ Applied
Viscose swab on fracture #1	–	3.5×10^4	–	–	4.6×10^5	–
Viscose swab on spiked fracture #1	1×10^7	5.9×10^5	0.059	1×10^7	1.4×10^5	0.014
Viscose swab on fracture #2	–	4.9×10^4	–	–	4.9×10^5	–
Viscose swab on spiked fracture #2	1×10^7	1.8×10^5	0.018	1×10^7	2.0×10^5	0.02

Spiking was done with *P. aeruginosa* and *D. aespoeensis*.

Table 3-3. Analysed amounts of cultivable heterotrophic aerobic bacteria (CHAB), cultivable fungal cells, most probable number (MPN) of sulphate reducing bacteria (SRB) and extracted DNA.

Sample	CHAB (cells)	CFC (cells)	MPN SRB	DNA (ng)
Viscose swab on fracture #1	7.0×10^2	n.d.	< 0.2	b.d.
Viscose swab on spiked fracture #1	4.1×10^4	n.d.	> 160	b.d.
Viscose swab on fracture #2	5.4×10^2	n.d.	< 0.2	b.d.
Viscose swab on spiked fracture #2	9.3×10^4	n.d.	> 160	b.d.

n.d. = no data, b.d. = below detection limit.
Spiking was done with *P. aeruginosa* and *D. aespoeensis*.

Table 3-4. Detected amounts of ATP and DNA in the swabs of fracture surfaces.

Sample name	Sampling date	Depth below ground surface (m.b.s.l.)	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.

b.d. = measured concentration was below detection limit of 10 ng per swab.

Table 3-5. 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 3-6. DNA results for analysed drill mud compared with the corresponding swab sample.

Sample	Swab ATP (amol	Drill mud ATP (amol)	Swab DNA (ng)	Drillmud DNA (ng)
401-Z	9.54×10^4	b.d.	14	161
427-Z	4.71×10^4	b.d.	48.4	1230

b.d. = below detection limit.

3.1.3 Comparison of prokaryotic 16S rDNA diversity of COSC-1 drill cores and drill mud

A selection of two fracture samples, 401-Z and 427-Z (Table 3-4) and their corresponding drill mud samples (Table 3-6) were 454 pyro-sequenced. All rarefaction curves showed 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. Sampling intensity was in the range of 12 000 to 24 000 reads (Table 3-7). Rank abundance curves showed a typical pattern for environmental samples were a few species were 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. The total taxa richness (> 0 % abundance) was highest for the fracture surfaces and this difference between fracture and drill mud samples was consistent also at ≥ 0.1 % and ≥ 1 % abundances. The number of taxa at ≥ 1 % abundance was three times larger for fracture surfaces compared to drill mud samples.

A representation regarding which taxa and to which abundance that taxa occurred in respective sample type is shown in Table 3-8. The drill mud libraries were totally dominated by *Hydrogenophaga* and to some extent by *Pseudomonas* while the most frequently occurring sequences in the fracture surfaces libraries were related to *Blastomonas*, Comamonadaceae, *Phyllobacterium, Sphigobium* and *Streptophyta* (Figure 3-1). When comparing fracture and drill mud samples both fracture samples were closely related, but distantly related to the drill mud samples as indicated by the dendrogram generated by an un-weighted pair group method with arithmetic mean (UPGMA) for the tree construction (Figure 3-6). Drilling was done using triple tube technique but not performed with measures against contamination. Still, it is clear that the sampling methodology separated fracture samples from drill mud samples, showing that drill mud did not contaminate the analysed fracture samples. Further, it is also clear that the drill mud diversity was poor compared with the fracture diversity. Only a few different types of microbes survived in the drill mud.

Table 3-7. Samples from COSC-1 fracture surfaces and drill mud. The table shows 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 (m.a.s.l.)	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
401-Z	1242.62	fracture	18 086	81	60	16
427-Z	1320.7	fracture	23 749	107	62	16
401-Z	1242	drill mud	17 188	61	20	7
427-Z	1320	drill mud	11 797	48	14	5

Table 3-8. Percent occurrence of taxa in bacterial 16S rDNA v4v6 sequence libraries from fractures and drill mud in the COSC-1 drill hole at ≥ 1 % abundance.

Taxa	Fracture 401-Z	Fracture 427-Z	Drill mud 401-Z	Drill mud 427-Z
Alicyclobacillus	1.58	2.26		
Blastomonas	8.09	7.67		
Comamonadaceae	8.93	14.99	3.92	1.69
Enterobacteriaceae	3.99	1.41		
Flavobacterium		1.26	4.43	2.45
Hydrogenophaga	1.50	6.62	57.0	85.0
Microbacterium	1.24	1.31		
Novosphingobium	1.71	3.30		
Ochrobactrum	1.72			
Phyllobacterium	6.94	6.57		
Propionibacterium	2.13			
Pseudomonas		22.9	21.4	4.91
Rhodobacter			1.33	
Rhodobacteraceae			1.09	
Sphingobium	14.3	8.64		
Sphingomonadales			5.25	2.16
Staphylococcus	3.00	1.97		
Stenotrophomonas		2.15		
Streptophyta	19.1			
Xanthomonadaceae	1.80	1.01		
Xanthomonas	7.17	4.73		
< 1 %	16.9	16.2	5.53	3.82

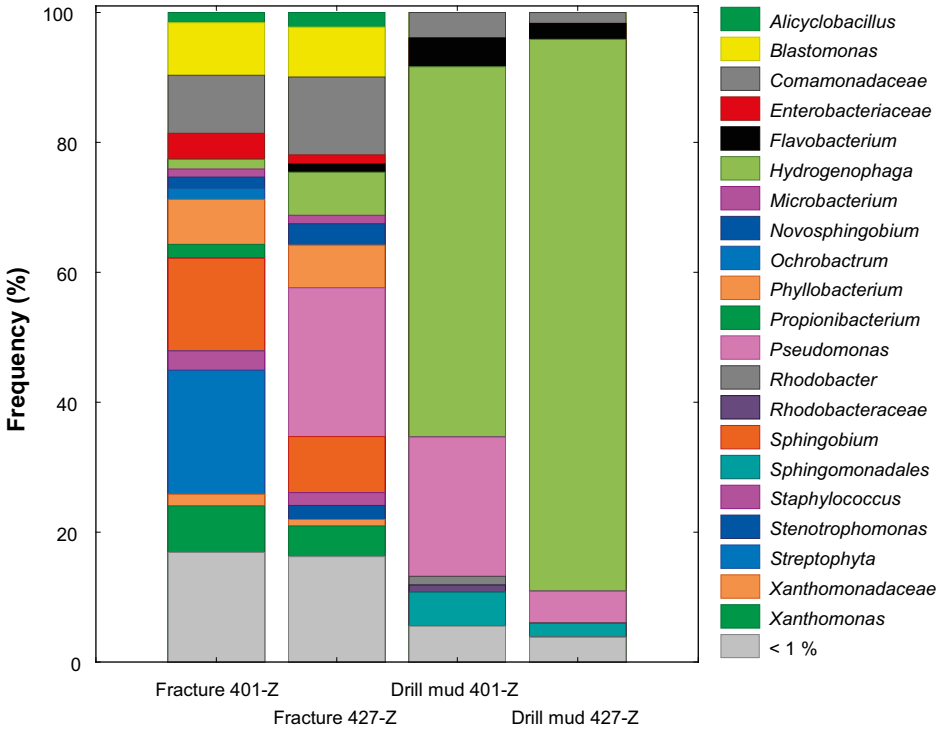

Figure 3-1. *Frequency of taxonomic assignment for 16S Bacteria rDNA v4v6 pyrotag-sequence libraries for samples from the COSC-1 l. Sequences with ≥ 1 % abundance frequency are shown.*

3.2 Flow cell method for microbial attachment and analysis

3.2.1 Laboratory flow cell experiments with pure cultures and field tests

A series of laboratory experiments was performed to evaluate attachment of microorganisms to various materials and the efficiency of methods for analysing biomass and cell numbers on these different materials.

Experiment 1

There were 5×10^5 cells mL^{-1} in the circulating water at start of the experiment that grew to 3.3×10^7 cells mL^{-1} after 20 days (Table 3-9). The ATP analysis detected one order of magnitude larger numbers compared to TNC on glass slides. The numbers did not change after the initial attachment registered at day 4 until day 20 when the TNC number on glass slides increased. On the other hand, the TNC and ATP agreed for the mineral slides. The PowerWater DNA extraction kit detected an amount of DNA that could be re-calculated to cell numbers (2.4.4). The cell numbers obtained on glass slides agreed with the TNC values. The bedrock grains had irregular shapes and the exact surface area per g could not be estimated with good precision. However, the insert supported 110 g of the crushed rock grains (2–4 mm diameter, Figure 2-6), offering a theoretical rock surface area of 895 cm^2 per FC for microbial adhesion and biofilm development assuming spherical rock grains with an average diameter of 3 mm. Consequently, there would be approximately 8 $cm^2\ g^{-1}$ crushed rock. Re-calculating DNA g^{-1} bedrock to DNA cm^{-2} bedrock returned a cell numbers that were somewhat smaller than the values obtained with ATP analysis. In comparison the bedrock grains did attract more biomass than flat glass and mineral surfaces. Taken together, and given the uncertainties of the TNC, ATP and DNA methods and of the constants used to re-calculate amol ATP, all three methods for analysis of biomass report similar values when re-calculated to number of cells cm^2.

Experiment 2

The circulating medium with *Shewanella putrefaciens* contained 2.5×10^5 cells mL^{-1}. When TNC, ATP and DNA numbers are compared, the pattern from experiment 1 was repeated with approximately one order of magnitude lower numbers for TNC than what was observed with ATP and DNA (Table 3-10). Because the constants used for ATP and DNA relate to microorganism in deep groundwater, they may be too small. The microorganisms in experiments 1 and 2 were grown in the laboratory and it has been shown that the amount of ATP can be much larger, at least 10 times, in cultured microorganisms compared to microorganisms from nutrient poor deep groundwater (Eydal and Pedersen 2007). The number of cells calculated from ATP and DNA agreed well, within one order of magnitude, on glass beads and garnet grains. However, it was obvious that garnet grains attracted one order of magnitude more biomass than what glass beads did. PowerWater reported somewhat larger numbers than did PowerBiofilm.

Experiment 3

The analysis of biomass on glass beads and garnet grains exposed to deep groundwater in Olkiluoto corroborated the results from laboratory experiments. Garnet grains attracted approximately one order of magnitude more biomass than did glass beads (Table 3-11). This effect was valid over three orders of magnitude as revealed by three different groundwater types selected over a large depth range: OL-KR54 (365.5–369 mbls), OL-KR55 (863–866 mbls) and OL-KR56 (1154–1158 mbls. Again, PowerWater reported somewhat larger numbers than did PowerBiofilm.

3.2.2 *In situ* flow cell biofilm enrichments

The amount of biomass analysed as ATP decreased over depth in the Äspö HRL tunnel (Table 3-12) which is consistent with data from other laboratories (Hubalek et al. 2016, Wu et al. 2016). Such trend was not found in Olkiluoto. When analysed for, there were amounts of ATP in biofilms of all the used flowing groundwater that corresponded to previously analysed amounts (Pedersen et al. 2008). The garnets and glass materials generally enriched from approximately 10^3 up to 10^6 amol of ATP cm^{-2} with exception for KA2198 that approached 10^8 amol of ATP cm^{-2}. The amounts of DNA

varied from approximately 0.1 to 10 ng cm^{-2} except for three samples that had amounts of DNA below the detection limit for the Picogreen quantification method. Consequently, both methods registered three orders of magnitude ranges.

The amounts of ATP and DNA were re-calculated to numbers of cells according to the constants described in Section 2.4.4 (Table 3-13). The cell numbers obtained from ATP were generally from 1 to 12 times the values obtained from DNA, except for KA2198 that had an extreme value for ATP compared to all other values and KF0069A1 that had a low ratio. Taken together, the over-all mean of ATP/DNA rations for glass and garnets were 4.5. This may indicate that the used quotients for calculating cell numbers were inappropriate. The amount of ATP can vary up to 10 times in a cell depending of the metabolic status. Likewise, can the amount of DNA vary several times in a cell depending on genome size and growth status, a dividing cell may have 2 or more copies of DNA. Further may the extraction efficiencies differ somewhat between the ATP and DNA extraction methods. Given these inherent uncertainties, an average difference of 4.5 can easily be explained.

A comparison of the amount of ATP and DNA on the two different materials shows that garnet grains generally enriched more ATP and DNA than did glass beads (Figure 3-2). The reasons behind this difference can only be speculated at this point. It seems as if the garnets attract cells to attach. In the laboratory experiments, there was no significant difference in attachment to flat glass and mineral surfaces (Table 3-9) which could simply imply 7that the garnets have a specific surface character that attract living cells better than what glass and polished granite do.

There was a large variation in the flow rate of groundwater through the FCs which may have influenced the numbers of attached cells (Table 3-12). However, when comparing the numbers of attached cells calculated from ATP with flow rates there was no correlation (Figure 3-3). It seems safe to conclude that flow rate alone did not influence the attachment and growth on the glass beads and the garnet grains.

Table 3-9. The numbers of cells on glass and mineral slides and crushed bedrock grains re-calculated from values of amounts of ATP and DNA. The slides and grains were exposed in flow cells to a circulating medium with added microorganisms.

Day	TNC cells in medium (cells mL^{-1})	SD (n=3)	TNC on glass slide (cells cm^{-2})	SD (n=3)	ATP on glass slide (cells cm^{-2})	SD (n=3)	DNA with PowerWater glass slide (cells cm^{-2})
1	5×10^5	–	–		–		–
4	–	–	6.0×10^5	4.9×10^4	9.3×10^6	4.9×10^5	–
6	–	–	4.2×10^5	5.6×10^4	8.9×10^6	1.2×10^6	3.0×10^5
13	3.9×10^7	1.5×10^6	6.1×10^5	2.6×10^4	9.0×10^6	3.6×10^5	–
20	3.3×10^7	4.0×10^6	6.5×10^6	7.6×10^4	–		1.2×10^5

Day	TNC on mineral slide (cells cm^{-2})	SD (n=3)	ATP on mineral slide (cells cm^{-2})	SD (n=3)	ATP on bedrock grain (cells cm^{-2})	SD (n=3)	DNA with PowerBiofilm bedrock grain (cells cm^{-2})
1	–	–	–	–	–	–	–
4	–	–	–	–	7.9×10^7	6.2×10^6	–
6	8.6×10^6	3.9×10^5	3.2×10^6	6.2×10^3	n.d.		3.6×10^6
13	6.5×10^6	5.1×10^5	3.0×10^6	2.0×10^5	1.3×10^8	3.7×10^6	1.6×10^7
20	–	–	–	–	–	–	6.8×10^5

n = number of observations.
n.d. = no data obtained.
– = analysis not performed.

Table 3-10. The numbers of cells on glass and mineral slides and glass beads and garnet grains in experiment 2 re-calculated from values of amounts of ATP and DNA.

Site	TNC (cells mL^{-1})	SD (n=3)	TNC glass slide (cells cm^2)	SD (n=3)	ATP glass slide (cells cm^2)	SD (n=3)	PowerWater glass slide (cells cm^2)	PowerBiofilm glass slide (cells cm^2)
Laboratory								
Day 1	2.5×10^5	6.2×10^4	3.1×10^5	5.5×10^4	1.3×10^6	2.9×10^5	4.3×10^6	1.3×10^6
Day 3	–		4.4×10^5	5.4×10^4	–		–	–
Day 8	–		3.2×10^4	3.3×10^3	–		–	–
Site	**ATP Glass beads (cells cm^2)**	**SD (n=3)**	**PowerWater Glass beads (cells cm^2)**	**PowerBiofilm Glass beads (cells cm^2)**	**ATP Garnet grains (cells cm^2)**	**SD (n=3)**	**PowerWater Garnet grains (cells cm^2)**	**PowerBiofilm Garnet grains (cells cm^2)**
Laboratory								
Day 1	–		–	3.6×10^6	–		–	2.5×10^7
Day 3	1.7×10^6	1.1×10^5	7.5×10^5	1.0×10^6	4.4×10^7	2.1×10^7	1.3×10^7	1.3×10^7
Day 8	–		1.6×10^5	6.9×10^4	9.4×10^7	1.9×10^7	8.2×10^6	1.9×10^6

n = number of observations.
n.d. = no data obtained.
– = analysis not performed.

Table 3-11. The numbers of cells on glass and mineral slides and glass beads and garnet grains in experiment 3 re-calculated from values of amounts of ATP and DNA.

Site	TNC in groundwater (cells mL^{-1})	SD (n=3)	ATP in groundwater (cells mL^{-1})	SD (n=3)	ATP Glass beads (cells cm^2)	SD (n=3)	ATP Garnet grains (cells cm^2)	SD (n=3)	PowerWater Glass beads (cells cm^2)	PowerBiofilm Glass beads (cells cm^2)	PowerWater Garnet grains (cells cm^2)	PowerBiofilm Garnet grains (cells cm^2)
Olkiluoto												
OL-KR54	8.5×10^4	6.9×10^3	9.5×10^3	7.0×10^2	8.3×10^3	6.5×10^2	8.3×10^4	2.5×10^3	n.d	n.d	–	–
OL-KR55	1.4×10^5	1.8×10^4	1.8×10^4	2.8×10^3	1.9×10^6	3.5×10^4	1.6×10^7	7.0×10^4	2.7×10^5	2.6×10^5	2.8×10^6	1.8×10^6
OL-KR56	1.0×10^4	4.7×10^3	1.2×10^5	7.1×10^3	3.6×10^4	1.4×10^4	4.5×10^5	1.2×10^4	1.5×10^5	6.5×10^4	9.7×10^5	7.4×10^5

n = number of observations.
n.d. = no data obtained.
– = analysis not performed.

Table 3-12. Borehole and flow data information and amounts of ATP and DNA extracted from in situ enrichments of biofilms in the Äspö tunnel and Olkiluoto, Finland.

Borehole	Depth (m)	Enrichment time (days)	Flow rate (mL min^{-1})	Ground water (L)	Groundwater ATP (amol mL^{-1})	garnet biofilm ATP (amol cm^{-2})	glass biofilm ATP (amol cm^{-2})	garnet biofilm DNA (g cm^{-2})	glass biofilm DNA (g cm^{-2})
Äspö									
KA2198	300	33	293	1.39×10^{4}	-	1.97×10^{6}	8.47×10^{7}	4.30×10^{-9}	1.70×10^{-8}
KA3110A	400	33	320	1.52×10^{4}	-	2.75×10^{5}	6.17×10^{4}	5.40×10^{-10}	3.20×10^{-10}
KA3385A2	420	33	285	1.35×10^{4}	-	9.00×10^{3}	1.00×10^{3}	$< 0.02 \times 10^{-9}$	$< 0.02 \times 10^{-9}$
KF0069A01	450	33	1635	7.77×10^{4}	-	7.95×10^{3}	1.86×10^{3}	2.20×10^{-9}	6.40×10^{-10}
Olkiluoto									
OL-KR57	57–61	35	225	1.13×10^{4}	3.90×10^{3}	9.68×10^{4}	5.68×10^{3}	$< 0.02 \times 10^{-9}$	$< 0.02 \times 10^{-9}$
OL-KR57	210–212	27	17.5	6.80×10^{2}	4.37×10^{6}	3.38×10^{5}	2.91×10^{5}	2.70×10^{-9}	5.46×10^{-10}
OL-KR54	364.5–368	30	160	6.91×10^{3}	3.80×10^{3}	3.31×10^{4}	3.32×10^{3}	$< 0.02 \times 10^{-9}$	$< 0.02 \times 10^{-9}$
OL-KR46	570.5–573.5	28	0.8	3.23×10^{1}	1.16×10^{4}	1.18×10^{5}	4.77×10^{4}	6.86×10^{-10}	3.54×10^{-10}
OL-KR6	422–425	26	6200	2.32×10^{5}	2.40×10^{3}	1.57×10^{6}	4.15×10^{5}	1.77×10^{-9}	3.34×10^{-10}
OL-KR55	863–866	27	150	1.05×10^{3}	7.20×10^{3}	6.35×10^{6}	7.48×10^{5}	1.70×10^{-8}	1.04×10^{-9}

– = analysis not performed.

Table 3-13. Calculated numbers of cells from extracted ATP and DNA on glass beads and garnet grains with estimated numbers of cells cm^{-2} for each surface material.

Sample	Depth (m.b.s.l.)	Garnet ATP (cells cm^{-2})	Garnet DNA (cells cm^{-2})	Ratio garnet ATP/DNA	Glass ATP (cells cm^{-2})	Glass DNA (cells cm^{-2})	Ratio glass ATP/DNA
KA2198A	300	4.93×10^{6}	1.10×10^{6}	4.47	2.12×10^{8}	4.36×10^{6}	48.6
KA3110A	400	6.88×10^{5}	1.38×10^{5}	4.97	1.54×10^{5}	8.21×10^{4}	1.88
KA3385A2	420	2.25×10^{4}	b.d	–	2.50×10^{3}	b.d	–
KF0069A01	450	1.99×10^{4}	5.64×10^{5}	0.04	4.65×10^{3}	1.64×10^{5}	0.03
OL-KR57	57–61	2.42×10^{5}	b.d	–	1.42×10^{4}	b.d	–
OL-KR57	210–212	8.45×10^{5}	6.92×10^{5}	1.22	7.28×10^{5}	1.40×10^{5}	5.20
OL-KR54	364.5–368	8.28×10^{4}	b.d	–	8.30×10^{3}	b.d	–
OL-KR46	570.5–573.5	2.95×10^{5}	1.76×10^{5}	1.68	1.19×10^{5}	9.08×10^{4}	1.31
OL-KR6	422–425	3.93×10^{6}	4.54×10^{5}	8.65	1.04×10^{6}	8.56×10^{4}	12.2
OL-KR55	863–866	1.59×10^{7}	4.36×10^{6}	3.64	1.87×10^{6}	2.67×10^{5}	7.00

b.d. = below detection limit.

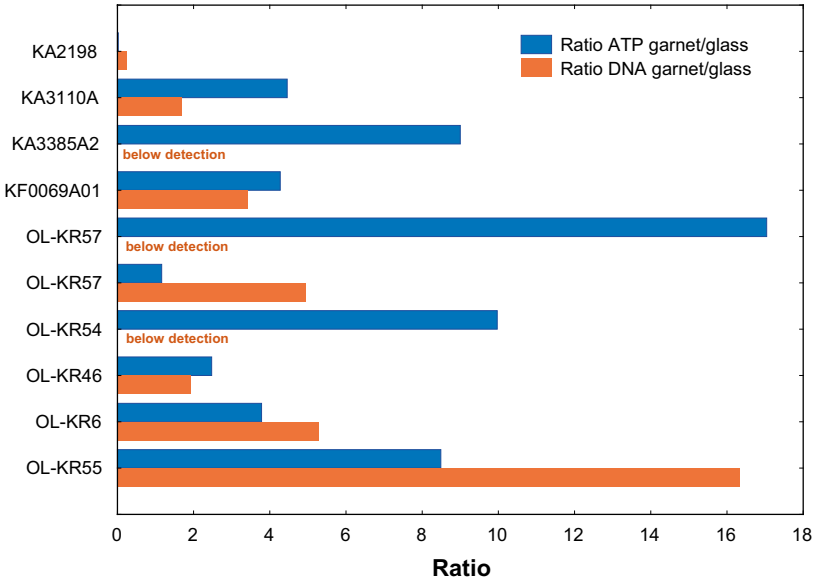

Figure 3-2. *Ratio of analysed ATP and DNA on garnets grains over glass beads for each investigated borehole.*

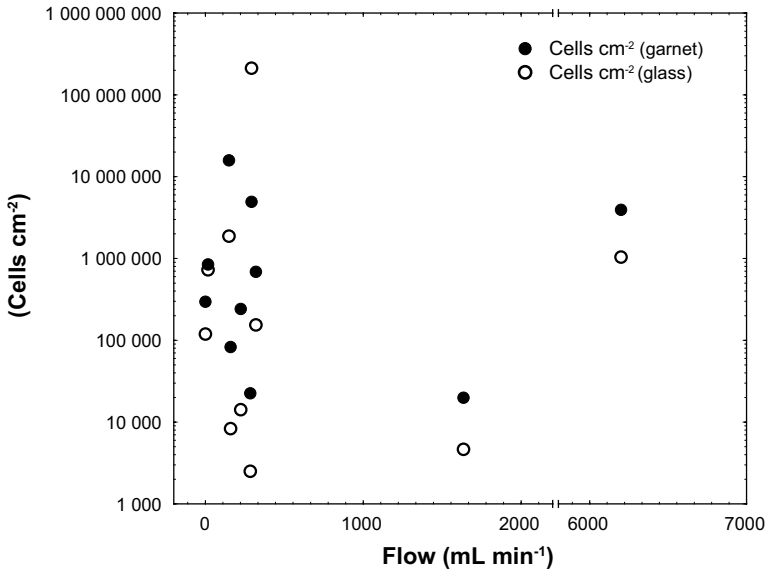

Figure 3-3. *Number of cells calculated from the content of ATP from glass and garnet materials for different flow rates.*

3.2.3 Prokaryotic 16S rDNA diversity in groundwater and flow cell samples from Olkiluoto

In several of the samples, the amount of DNA was small. Therefore, the DNA from glass beads and garnet grains were pooled to increase the total amount of DNA sent for sequencing and thereby minimizing the risk for reagent contamination effects (Salter et al. 2014). 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 (not shown). Sampling intensity was in the range of 15 000 to 41 000 reads (Table 3-14). Rank abundance curves showed a typical pattern for environmental samples were a few species were abundant but most are rare (not shown). A representation regarding which taxa and to which abundance that taxa occurred in respective sample type is shown in Table 3-15. In general, the deeper groundwater samples were more diverse compared to shallow samples with exception for OL-KR6. This sample was found to be distantly related to the other analysed Olkiluoto samples as shown by the distance matrix dendogram generated by using an Unifrac distance measure unweighted pair group method with arithmetic mean (UPGMA) for the tree construction (Figure 3-4). When comparing glass and garnet samples exposed to OL-KR57 from 212 m.b.s.l, they were more similar to each other than to any other samples in the tree.

3.2.4 Comparison of prokaryotic 16S rDNA diversity in groundwater and flow cell samples from Äspö HRL

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 (not shown). Sampling intensity was in the range of 13 000 to 23 000 reads (Table 3-16). Rank abundance curves showed a typical pattern for environmental samples were a few species are abundant but most are rare (not shown). A representation regarding which taxa and to which abundance that taxa occurred in respective sample type is shown in Table 3-17. The biofilm samples KA2198A_glass and KA2198A_garnet had similar numbers of taxa. When comparing glass and garnet samples from the KF0069A01 samples they were closely related as indicated by the dendrogram (Figure 3-5) but the diversity between 0–0.1 % abundance was much larger on the garnet grains. While groundwater samples from KA2198A and KA3110A clustered reasonably well with biofilms from KA2198A; the remaining three groundwater samples did not cluster with biofilm samples. This may be due to that biofilm and groundwater samples were sequenced by different laboratories (Woods Hole, USA and Eurofins, Germany, respectively) and to the fact that one year passed from the collection of groundwater samples to the collection of biofilm samples.

Table 3-14. Biofilm samples from flow cells exposed to flowing Olkiluoto groundwater. The 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 (m.b.s.l.)	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
OL-KR57	57–61	Biofilm garnet/glass mix	20 183	87	26	11
OL-KR57	210–212	Biofilm glass	32 756	85	19	9
OL-KR57	210–212	Biofilm garnet	22 168	77	19	10
OL-KR54	364.5–368	Biofilm garnet/glass mix	21 151	115	30	9
OL-KR6	422–425	Biofilm garnet/glass mix	14 925	31	7	3
OL-KR46	570.5–573.5	Biofilm garnet/glass mix	41 550	131	48	15
OL-KR55	863–866	Biofilm garnet/glass mix	30 289	101	59	12

Table 3-15. Percent occurrence of taxa in bacterial 16S rDNA v4v6 sequence libraries from biofilms from Olkiluoto at ≥ 1 % abundance.

Taxa	OL-KR57 57–61 (m)	OL-KR57 glass 210–212 (m)	OL-KR57 Garnet 210–212 (m)	OL-KR54 365–368 (m)	OL-KR6 422–425 (m)	OL-KR46 571–574 (m)	OL-KR55 863–866 (m)
Acholeplasma						26.9	
Achromobacter						1.17	
Alteromonadaceae							6.11
Alteromonadales		1.02	4.42				
Arenimonas			2.13				
Bacteroidales	3.82	1.11	1.76			1.53	3.47
Caulobacteraceae		1.46				3.20	
Comamonadaceae	1.84	21.8	21.4				3.96
Dechloromonas	3.37	37.4	31.7				
Desulfobacteraceae				10.8			2.35
Desulfobulbaceae	47.6			9.38	22.3		2.35
Dethiosulfatibacter						3.39	
Erysipelotrichaceae	1.26	13.9	7.58	1.96		5.07	
Flavobacteriaceae	4.31	9.15	14.2	12.6		5.54	10.4
Flavobacteriales							16.7
Flavobacterium						2.52	
Fusibacter				6.20		10.3	
Gallionella							22.3
Geobacter	2.81						
Geobacteraceae						8.76	
Hydrogenophaga					1.85		
Nitrospirales				39.3			
OP9				8.93			
Pelobacteraceae	1.43		3.28			1.06	
Pseudomonadaceae		2.72	4.08			9.20	1.37
Pseudomonas		7.29	5.90			5.89	1.47
Rhodocyclaceae	1.27						
Rhodocyclaceae	3.02						1.42
Salinibacterium						1.89	
Soehngenia						1.75	
Sulfuricurvum	23.1						
Sulfurimonas					74.4		
Unknown Phylum				1.26			5.57
> 1 %	6.18	4.12	3.61	9.63	1.41	11.8	22.6

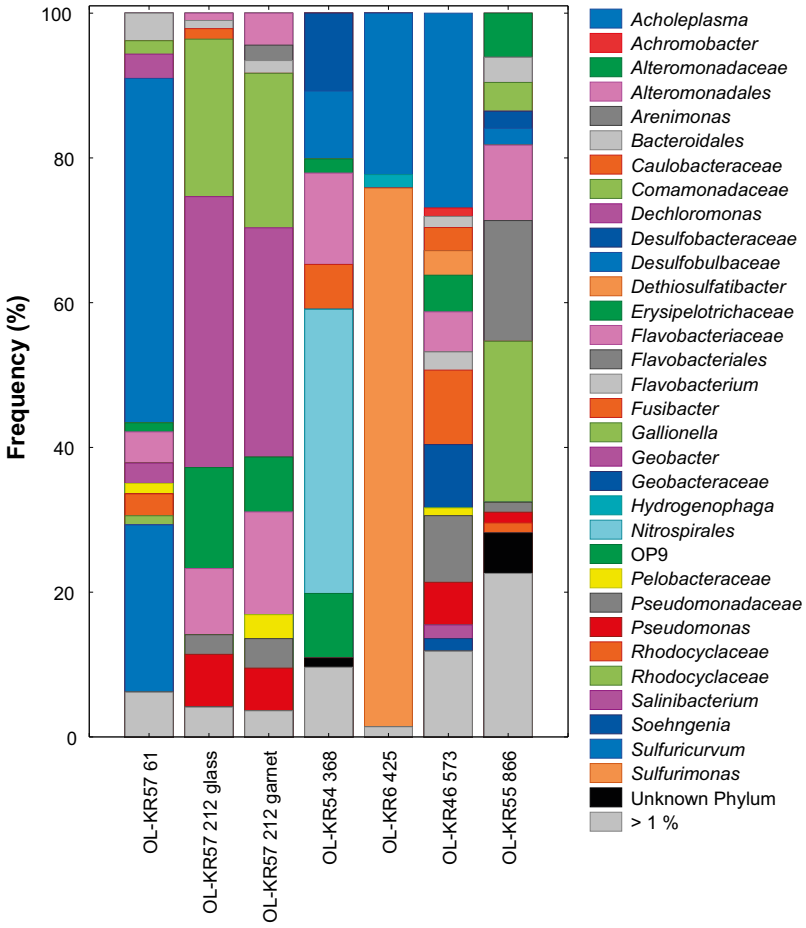

Figure 3-4. *Frequency of taxonomic assignment for 16S Bacteria rDNA v4v6 pyrotagsequence libraries for samples from Olkiluoto. Sequences with ≥ 1 % abundance frequency are shown.*

Table 3-16. Samples from groundwater with the corresponding flow cell biofilm samples. 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 (m.b.s.l.)	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
KA2198A	300	groundwater	15 380	137	51	14
KA2198A_glass	300	biofilm	22 823	107	30	13
KA2198A_garnet	300	biofilm	16 388	108	30	11
KA3110A	400	groundwater	16 227	188	67	17
KA3110A_garnet	400	biofilm	11 890	100	28	7
KA3385A2	420	groundwater	22 830	95	37	14
KF0069A01_garnet	450	biofilm	13 551	102	20	5
KF0069A01_glass	450	biofilm	14 630	39	22	7
KF0069A01	450	groundwater	13 241	34	18	9

Table 3-17. Percent occurrence of Taxa in bacterial 16S rDNA v4v6 sequence libraries from groundwater and biofilms from Äspö HRL at ≥ 1 % abundance.

Taxa	KA2198A	KA2198A garnet	KA2198A glass	KA3110A	KA3110A garnet	KF0069A01	KF0069A01 garnet	KF0069A01 glass	KA3385A2
Acholeplasma									4.54
Actinobacteria						1.59			
Alicyclobacillus					3.21				
Anaerolinaceae									5.59
Bacteroidales									4.32
Betaproteobacteria		4.37	6.11					1.13	
Chromatiales	13.31	8.75	5.12						
Dehalococcoidetes				8.99		1.25			1.27
Deltaproteobacteria				1.06				1.24	
Desulfarculaceae	3.33			3.81					9.55
Desulfobacteraceae									11.96
Desulfobulbaceae		4.49		1.80					11.81
Desulfocapsa							5.37	9.14	
Desulfococcus	1.29			2.10					4.12
Desulfovibrio				4.22					
Elusimicrobiales									1.23
Gallionella		2.72	2.92						
GN02	4.99	9.97	1.68	1.02					
Helicobacteraceae		3.75	8.42						
Ignavibacteria					3.89	8.09			5.16
Methanobacteriales						2.68			
Myxococcales							1.31	1.61	
OD1	8.87			7.27		2.90			
OP3	11.73		1.30	14.24		1.14			
OP8					2.71				
OP9	1.14			2.67	3.89	20.67			8.71
Phycisphaerae									3.98
Rhodocyclaceae		1.64	1.04				15.63	27.54	
Sulfurimonas		48.55	50.69		2.40		68.51	51.64	
Syntrophaceae	3.71			4.99		2.39			
Thermoanaerobacteraceae					1.56				
Thiobacterales			1.22						
Unknown Phylum	35.8	8.19	14.67	30.26	72.86	55.09	2.91	3.39	16.20
< 1 %	15.84	7.58	6.84	17.58	9.48	4.21	6.26	4.30	11.56

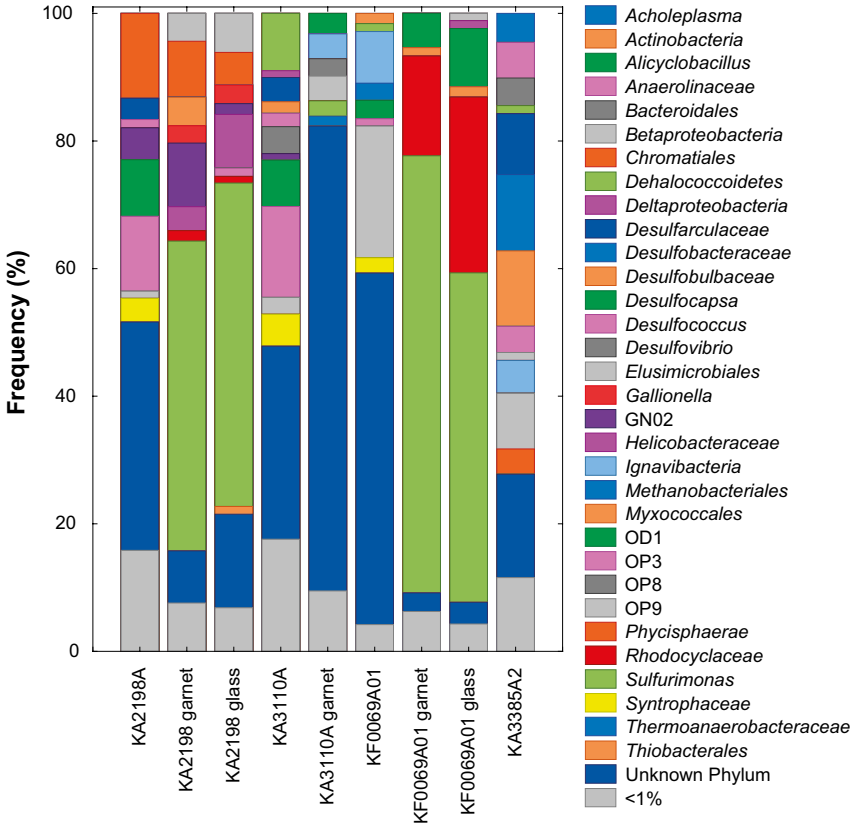

Figure 3-5. *Frequency of taxonomic assignment for 16S Bacteria rDNA v4v6 pyrotag-sequence libraries for samples from the Äspö tunnel. Sequences with ≥ 1 % abundance frequency are shown.*

3.2.5 Diversity analysis of all flow cell biofilm sequence libraries

The phylogenetic relation between all sample sequence libraries was compiled using the UPGMA method for the tree construction (Figure 3-6). Sequences from each of the three different geographical locations clustered together which demonstrates that the microbial populations at the three sites differed significantly. It should be emphasised that the number of libraries was relatively small and, therefore, the analysis shown here is mainly for demonstration of the power of sequence analysis. We refrain from detailed analysis of differences and similarities of diversities at this stage.

With sequence data at hand, combined with meta data on location geochemistry, depth and other selected parameters, relations between diversity and meta data can be analysed. A beta diversity metrics ordination analysis can assess the differences between the microbial communities based on the metadata. Such analyses were performed for geographical location, concentration of methane and conductivity. The influence of geographical location is shown in the phylogenetic tree (Figure 3-6). Sequence library information cluster in three groups, one for each location. This result then shows that the microbial populations differed significantly between the locations. That difference may not be surprising, but it demonstrates the power of using DNA signatures for discrimination of sites. The influence of methane varies between the geographical location groups. The deepest locations in Olkiluoto form a coherent group at the same positions in Figure 3-8 (blue circles). Methane concentration increases exponentially over depth in Olkiluoto (Pedersen et al. 2008) and there is a clear shift in groundwater chemistry at approximately 350 to 400 m.b.s.l. that correlates with disappearance of sulphate and increase in methane. Previously, it was shown that the microbial diversity differs between more shallow groundwater with low concentrations of methane and deeper groundwater with high concentrations of methane (Pedersen et al. 2014a). The UPGMA analysis reflect this shift in diversity properly for the Olkiluoto location group. There was no methane in COSC-1 and methane has, consequently, no significance for the COSC-1 data set. At Äspö, methane does not change much between the sample positions and, therefore, methane does not separate the sample libraries. The third example is on conductivity that did not appear to influence microbial diversity

in Olkiluoto or Äspö (Figure 3-9). Possibly, conductivity reflect a difference between samples in the COSC-1 group, between drill mud that was mixed with diluted surface water and fracture samples that were exposed to higher conductivity in the respective aquifers. However, it should be kept in mind that many other parameters may be nested in the different samples of drill mud and fracture surfaces. The data set from COSC-1 is too small for a proper diversity analysis, mainly because there were very few water conducting fractures.

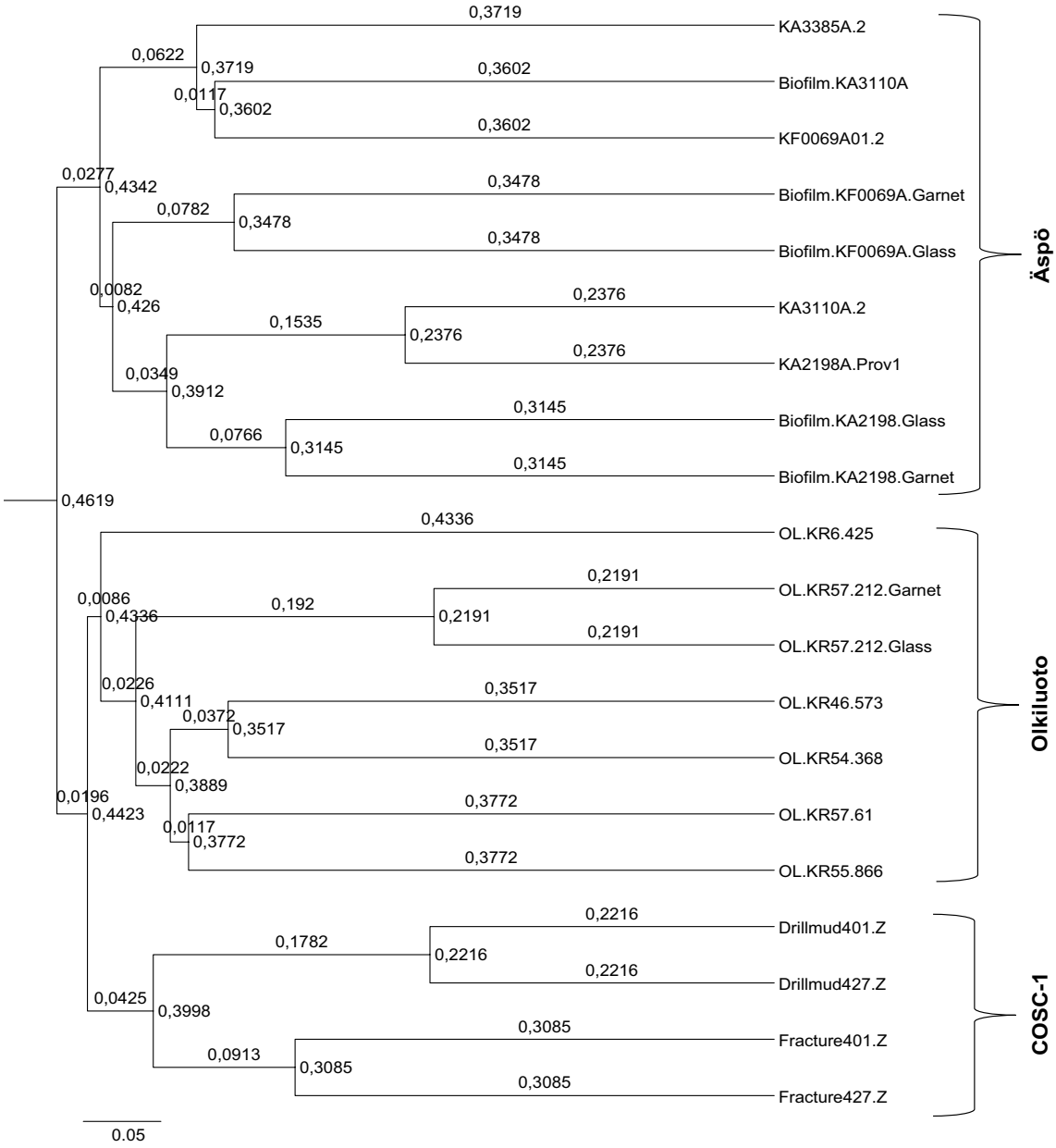

Figure 3-6. *The distance matrix dendogram shows the phylogenetic tree generated by using a Unifrac distance measure unweighted pair group method with arithmetic mean (UPGMA) for the tree construction.*

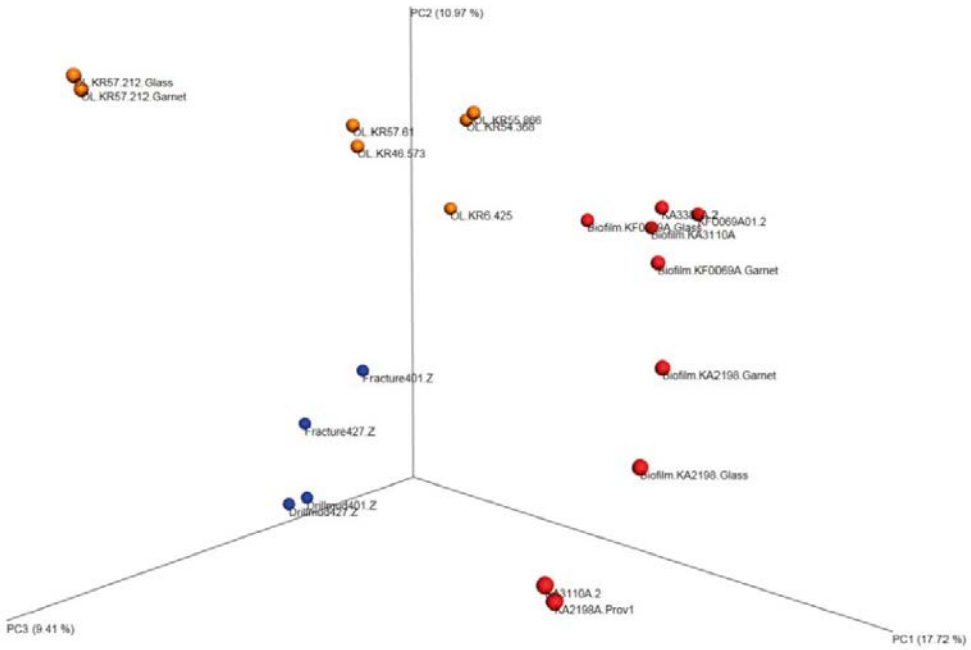

Figure 3-7. *A beta diversity metrics ordination analysis assessing the differences between the microbial communities based on the metadata category Geological location, illustrated by a three dimensional plot of the Principal Coordinate Analysis (PCoA). Blue represent COSC-1 samples, red is Äspö samples and orange is Olkiluoto samples. Some data points overlap because of software limitations.*

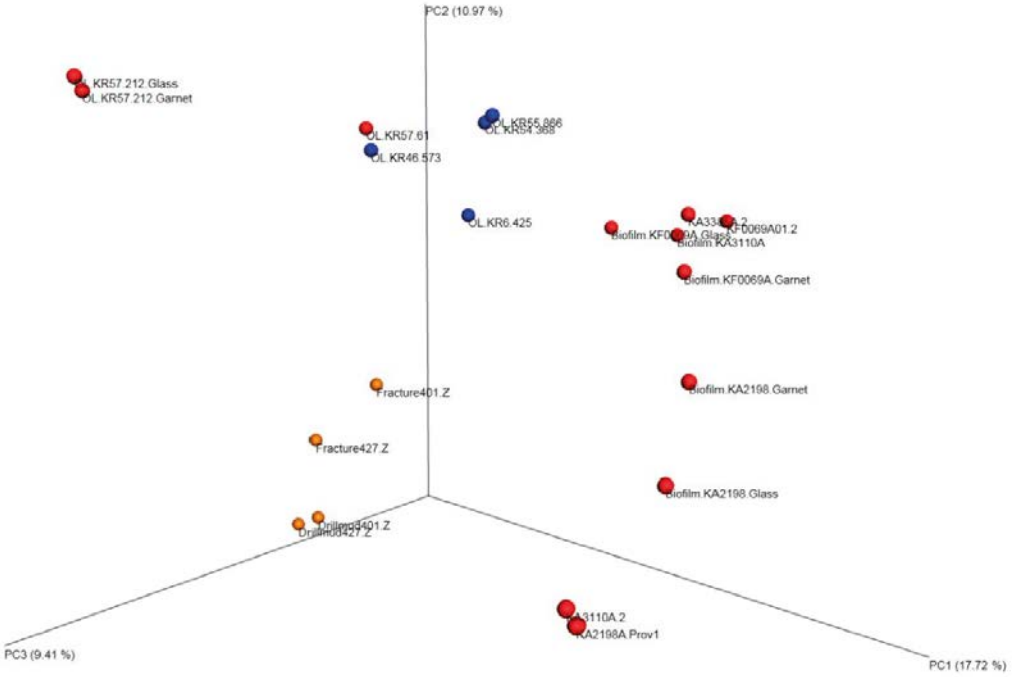

Figure 3-8. *A beta diversity metrics ordination analysis assessing the differences between the microbial communities based on the metadata category Methane, illustrated by a three dimensional plot of the Principal Coordinate Analysis (PCoA). Methane concentrations were distributed into three classes were Blue represent 10^{-2}–10^{0} mol L^{-1}, red is 10^{-5} – < 10^{-2} mol L^{-1} and orange is 0 – < 10^{-5} mol L^{-1}. Some data points overlap because of software limitations.*

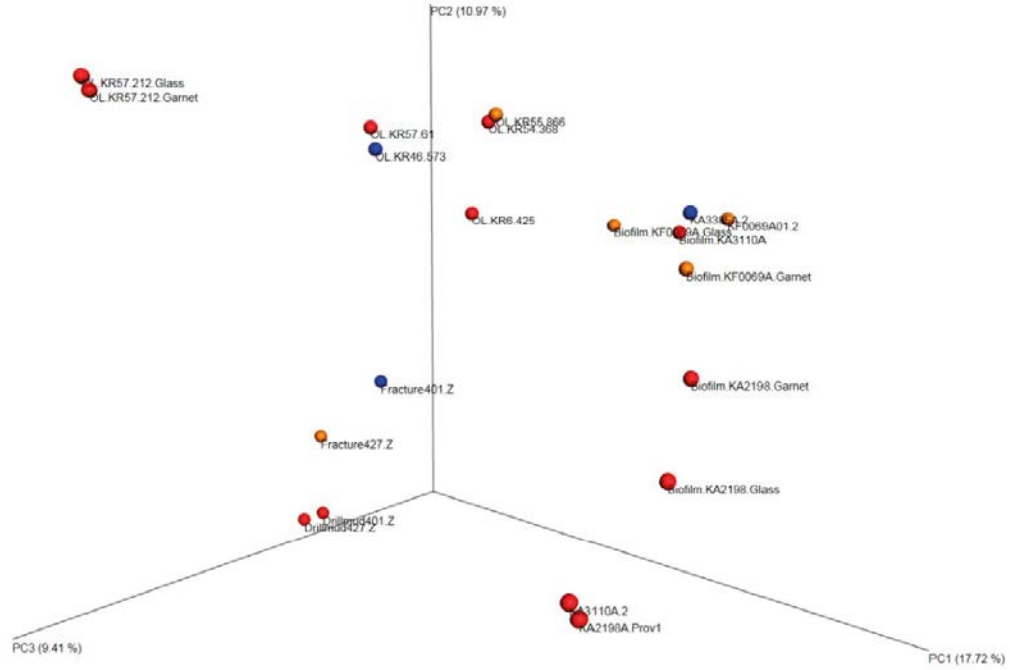

Figure 3-9. *A beta diversity metrics ordination analysis assessing the differences between the microbial communities based on the metadata category conductivity, illustrated by a three dimensional plot of the Principal Coordinate Analysis (PCoA). Conductivities were distributed into three classes were Red represent 0–15 mS cm^{-1}, blue is > 15–35 mS cm^{-1} and orange is > 35–100 mS cm^{-1}. Some data points overlap because of software limitations.*

4 Discussion

4.1 Swab method for sampling and analysis of attached microorganisms on fracture surfaces

4.1.1 Swabs for DNA and ATP collection

Investigation of biofilms spot on fresh bedrock fracture surfaces obtained during drilling is challenging, due to expected low numbers of microorganisms available for sampling. Scraping with scalpels was successfully tested previously for collection of DNA (Jägevall et al. 2011), but the method was here found to interfere with ATP analysis. Several of the tested swabs, such as cotton, rayon and viscose, released fibres that similarly interfered with the subsequent analysis procedures. The flocked swab for forensic use were found to strongly absorb all biomass from the fracture surfaces without releasing interfering material. The selected swabs released cells for ATP and DNA readily in the extraction for analysis of the samples. When tested in the field during the COSC-1 drilling, the developed swab method returned real time data of the microbial biomass and diversity on bedrock fracture surfaces. Sampling was easy to perform and the swab samples for DNA analysis were stored in room temperature while ATP swabs were stored in a freezer. The flocked 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 2-2 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 drill mud, and by the sampling crew, was limited, i.e. below the limit of detection for the DNA quantification analysis. The drill mud and the fracture samples differed significantly in diversity demonstrating that the sampling procedure could be done without drill mud contamination. The analysis results for ATP was less clear. It seems as if rock material that interfered with the analysis was picked up by the swabs. The rock in the COSC-1 drilling is much softer than are granites which may explain why the rayon swabs for ATP function well on granite but less good on the amphibolite facies gneisses of the Lower Seve Nappe in Åre.

The swab method is now ready for next field test, preferably during drilling in Fennoscandian hard rock. A protocol for sampling can be found in Appendix A1.1.

4.2 Filtration method for sampling and analysis of planktonic microorganisms in groundwater

4.2.1 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^{-1}). 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. Further, filtering of collected groundwater in the laboratory generally experience problems with O_2 induced and/or pressure release related precipitates that clog filters. This effect is circumvented in the on-line pressure filtration method. Finally, the use of the filtering method can be advantageous when the access time to groundwater is limited. Filtration of > 100 L groundwater can be performed during less than 24 h if the flow is > 70 mL min^{-1}. The FC method require at least 4–5 weeks of exposure time. While the filtration method will collect all microbial planktonic diversity, the FC will sample attaching diversity as discussed next.

4.3 Flow cell method for sampling and analysis of attached microorganisms on solids

4.3.1 Flow cells for collection of attaching planktonic microorganisms

The use of FCs 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 solid surfaces. With this technique it should possible to investigate microbial diversity in groundwater where pressure filtration is difficult or impossible to achieve, for instance on the ground surface with pumped boreholes. Pumps used in boreholes generally give limited 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 as discussed next, it is possible to investigate preferences by planktonic cells for material with different surface properties. The method requires at least 4–5 weeks of time for microbial attachment and biofilm development to obtain enough biomass for DNA extraction. Therefore, the FC method is best applied on positions where groundwater can be pumped (or flushed in tunnels) for a relatively long time. If the access time is limited, the pressure filtration method may be a better option. 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 present FC method has been tested, used and evaluated previously (Pedersen 2012a, b, 2013, Pedersen et al. 2014a). 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 e.g. Table 3-16) full diversity will be present in flowing groundwater. It will be the conditions in the FCs that determine which of these microorganisms that will prosper and dominate on the surfaces (Jägevall et al. 2011).

4.3.2 Choice of surface material for attachment and growth of microorganisms

Five different types of materials for attachment and growth of microorganisms were tested in laboratory and *in situ* field experiments. They were crushed rock, flat glass and mineral surfaces, glass beads and garnet grains. All have advantages and disadvantages.

Crushed rock can be obtained from drill cores obtained during drilling. Such rock would be the same type as exposed to groundwater and microorganisms in studied aquifers if the drill core is sampled in the vicinity of the aquifer. It will act as a natural substrate and has been used with good results previously (Pedersen 2012a, b, 2013, Pedersen et al. 2014a). However, drill cores may not always be available for crushing and rock surfaces have a tendency to be porous and detach minerals and particles that often disturbs the subsequent analysis of the biofilm. In addition, it is difficult to calculate the surface area available for attachment and growth.

Flat glass surfaces have the advantage of allowing microscopic investigations such as epi-fluorescence microscopy, light microscopy and fluorescent *in situ* hybridisation. Enumeration of attached microorganisms (TNC) can easily be performed with microscopy. Glass surfaces are cheap and easy to sterilize. Glass has been used with good results previously as well (Ekendahl and Pedersen 1994, Pedersen et al. 1996, Pedersen and Ekendahl 1992a, b). On the other hand, extraction of ATP and DNA require a scraping or a swab procedure that should be possible to do without loss of biomass. It can be difficult to swab multiple surfaces in a reproducible way, in particular if the microorganisms have started to produce slime and other attachment substances. Such microorganisms may be difficult to recover. Further, the surface area available for attachment in a FC is limited relative to the use of crushed rock, beads and grains.

Flat mineral surfaces have advantages and disadvantages similar to flat glass surfaces with one important difference. They are very expensive to produce. The cost is motivated for specific research questions such as the sorption of radionuclides to minerals that has been investigated with autoradiography previously with good results (Anderson et al. 2006, 2007).

Glass beads will have surface properties similar to flat glass surfaces but there will be a much larger area for attachment. Theoretically will a FC with glass beads collect much more biofilm for analysis than will a FC with flat glass surfaces. The experiments in this work confirmed that flat glass and mineral surfaces and glass beads collected similar amounts of biomass for the time periods tested.

The extraction of ATP and DNA is done directly on the beads without any swab procedure which reduces the risk for loss of strongly attached microorganisms as discussed for flat glass surfaces.

The garnet grains will, just like the glass beads, offer a large surface area for attachment per FC and the extraction procedures will cover all attached and growing microorganisms. In this work we found that more microorganisms were attracted to the garnet surfaces than any other tested surface material.

Based on advantages and disadvantages with the different surface materials it is recommended to use commercially available enrichment surfaces consisting of garnet grains and glass beads. These two surface materials should be possible to obtain in reproducible batches which will advantageous when microbial biomass and diversity is compared between different sites. However, the other materials, crushed rock, and flat glass and mineral surfaces can be more advantageous for specific methodological applications such as FISH (Fluorescence *in situ* Hybridigation) and autoradiography.

4.4 Choice of method – advantages and disadvantages

4.4.1 Swabbing fracture surfaces

Sampling and analysis of microorganisms in bedrock aquifers can be performed in several steps during drilling. Microorganisms attached to surfaces of water conducting fractures can be sampled during drilling with the swab method. The obvious advantage is that natural attached populations are sampled. However, there are several factors that may jeopardize the correctness of the results. There is always a risk that drill water used to remove drilling debris contaminate the fractures to be sampled. This risk can be mitigated by the use of triple tube drilling and by use of clean drill water with methods similar to those developed during the Swedish site investigations (Pedersen 2005a, b, 2007a, b, Pedersen and Kalmus 2003). Another risk concerns the amount of biomass on a fracture that can be very small. There are always losses in field and laboratory procedures and the final amount of extracted ATP and DNA may be too small for a quality assured analysis. Still, using the swab method will return a first approximation about microbial diversity and amount of biomass in observed fractures as was done in the COSC-1 drilling operation. Later, when discrete fractures can be sealed off and sampled, the results from swabbing may guide in the choice of which fractures should be sampled in more detail with the filtration and the FC methods.

4.4.2 Planktonic microorganisms with filtration

The filtration method samples planktonic microorganisms that freely move in the groundwater. The diversity of these microorganisms may reflect attached diversity as well. However, if quantification methods such as digital droplet PCR is adopted, it is likely that the size distribution of genera and species may differ significantly in attached and planktonic populations as was indicated in this work. Similar observations have been made in Olkiluoto, Finland, where sulphur reducing bacteria almost exclusively were found as attached. However, the number of observations reported here are still small and more data is needed before general conclusions can be drawn about differences and similarities between attached and planktonic microbial diversity. Technically, there can be issues with a too low pump pressure for successful filtration, especially if there are particles in the water that clog the filter and reduce the flow rate through the filter. The filtration method is fast and easy to install and the amount of biomass collected is usually large. Handling time is easy and the extraction processes are also rapid using the PowerWater extraction kit that is developed for filter samples. Sampling and processing of samples is consequently not costly.

4.4.3 Biofilms with flow cells

The FCs select for microorganisms that attach to surfacs. The glass and garnet materials collected approximately similar diversities. The only difference was that garnets collected more biomass than did glass (Figure 3-2). Garnets is consequently the preferred material. It is DNA free and a reproducible material. The only drawback can be the cost as garnets are much more expensive than are glass beads. The use of the FC method will reflect attached diversity and cell numbers. Because the amount of biomass is strongly distributed towards surfaces in relation to groundwater as discussed in the introduction, FCs will reflect the total numbers of cells in aquifers more correctly than will the filter method.

4.4.4 Sampling and analysis process

All three developed methods can be used in a series. Swabbing and analysis of ATP and DNA during drilling will produce a data archive with information on original diversity on aquifer surfaces. The analysis can await data from flow logging and water conducting aquifer samples can be further processed. If such aquifers are packed off and pumped, the filtration method will provide information on planktonic diversity while the use of FCs will provide data on microbial attachment, growth diversity.

4.5 Sequence data analysis and modelling

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 microorganisms 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 briefly 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 20 observations from three locations 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. There is a large array of additional analysis methods than can be applied on DNA from groundwater and biofilms. For instance, 16S sequence libraries were used to infer flow paths in Äspö HRL (Hubalek et al. 2016) and a subglacial lake on Iceland (Marteinsson et al. 2013). Information of metabolic strategies of microbial populations in ecosystems can also be obtained (Gaidos et al. 2009). Genes for specific metabolic processes can be amplified and sequenced which can give insights in potential dominating processes in the deep biosphere (Nyyssönen et al. 2012, Purkamo et al. 2015). Quantitative DNA analysis methods can reveal on-going microbial activity (Rajala et al. 2015). If full genomes are sequenced, detailed information about possible metabolic pathways is obtained as has recently been published for Äspö HRL groundwater samples (Wu et al. 2016). However, it should be noticed that all investigations referred to above have been performed on planktonic microbial populations, often with limited amount of extracted DNA which increases the risk for reagent contamination (Salter et al. 2014). The methods developed, tested and described in this report overcome the risk for reagent contamination because they collect enough large amounts of DNA to overcome reagent contamination issues. They also include the major part of the deep biosphere biota, the attached microorganisms, and that aspect and approach is novel. It remains to collect and analyse more samples to avoid errors in conclusions due to a small dataset. Such work is in progress for the Olkiluoto site in Finland.

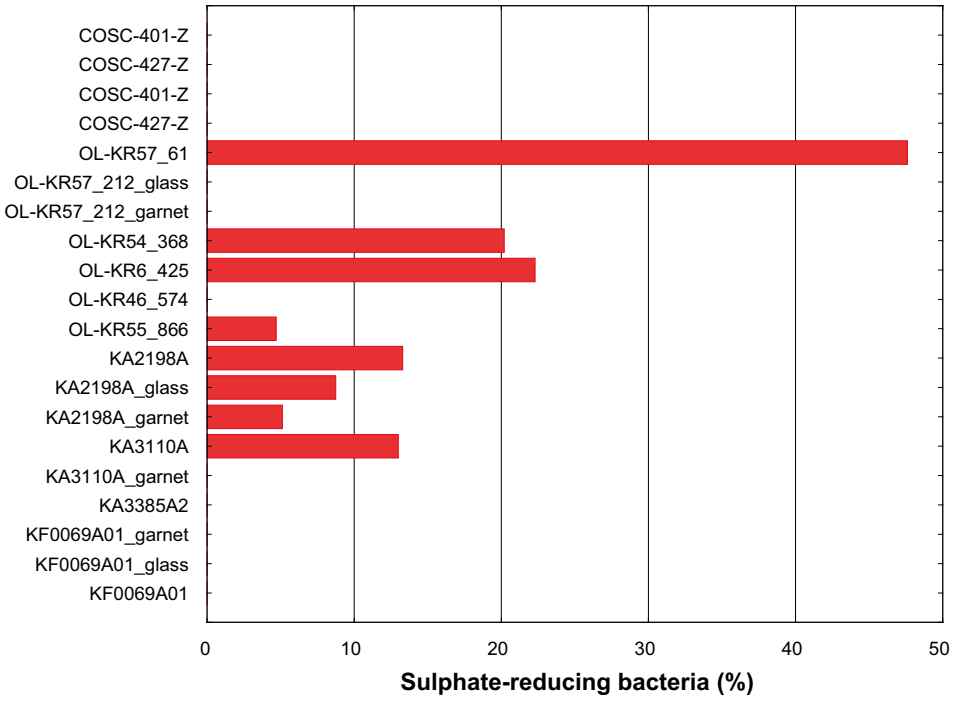

Figure 4-1. *The proportion of 454-pyrosequences belonging to sulphate-reducing taxa. Data are extracted from Table 3-8, Table 3-15 and Table 3-17.*

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Appendix 1

A1.1 Swab method description for ATP and DNA on fracture surfaces

A1.1.1 Materials and equipment for biofilm sampling on fracture surfaces

- Extractant B/S (6.7 mL dropper bottle), art.nr 31-072, Biothema, Sweden.
- Swab Hy-Lite Merck ATP-free, art.nr-1.30103.0001, VWR, Sweden.
- COPAN4N6FLOQSwabs™ Scalpel shaped tip (art.nr-4479438, Life Technologies/ Fischer Scientific, Sweden).
- Sterile pincers and scissors.
- Sterile gloves.

A1.1.2 Material for ATP analysis and DNA extraction

- ATP Biomass kit HS, Biothema, Sweden.
- PowerSoil® DNA Isolation Kit, MO BIO Laboratories, Immuno Diagnostic Oy, Finland.

A1.1.3 Screen each drill core for fractures

1. Evaluate if the fracture appears to have been water conducting, if so is the case there is a need for sampling of ATP and DNA.
2. When the fracture is identified as water conducting, it should be sampled for microorganisms on both corresponding surfaces. If possible, document the fracture appearance with photography.

Figure A-1. *Detection and localisation of water conducting fractures in a drill core.*

A1.1.4 Sampling procedure for ATP

3. Add 2–3 drops from the bottle containing Extractant B/S on the surface. Swab thoroughly all over the surface with the swab marked **ATP**. Place the swab in the provided 1.5 mL Eppendorf tube. Cut off the swab at 2 cm from the tip and close the cap on the tube.
4. Store the ATP samples in a freezer at −20 °C.

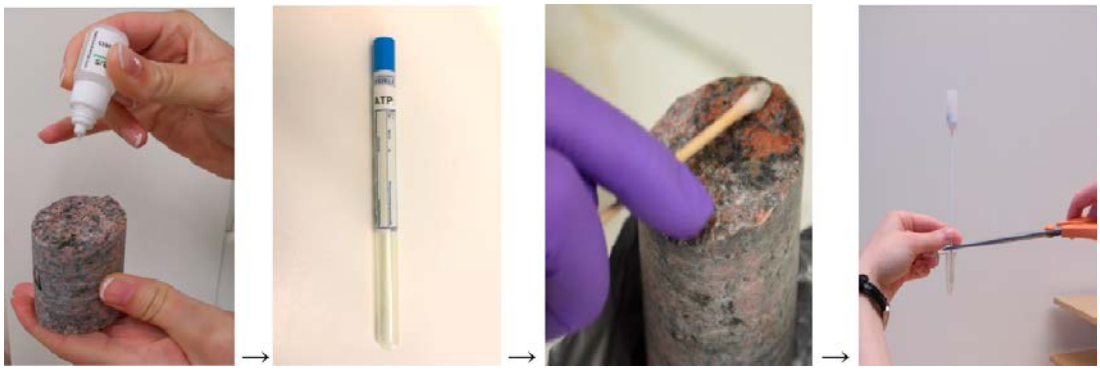

Figure A-2. *Sampling procedure for ATP analysing.*

A1.1.5 Sampling procedure for DNA

5. Sample the opposite fracture surface with the black swab for **DNA**. Put the swab back into the provided tube. Note the sample number on the tube and on the sampling list.
6. Store the DNA samples in room temperature.

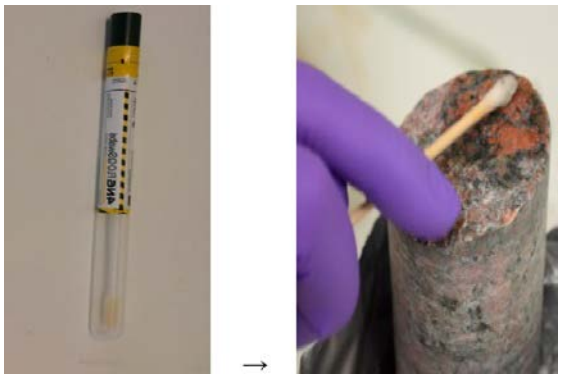

Figure A-2. *Sampling fracture surface for DNA extraction and analysing.*

A1.1.6 Sampling of mud and sand material in fractures

7. When an aquifer fracture surface consists of porous materials of mud and soil minerals, as much as possible of the materials should be collected with a sterile plastic scrape and into a 50 mL polypropylene tube.
8. Sample both opposite surfaces separately to discrete tubes. Scrape material from the fracture surface and place the material into a tube marked “ATP” and repeat on the corresponding surface and place the sample material in a tube marked “DNA”.
9. Note sample id on the tubes and on the sample list. Store these samples in a freezer.

Figure A-3. *Sampling equipment for collecting loose material of mud and sand.*

A1.2 Flow cell ATP and DNA method description

A1.2.1 Materials and equipment for biofilm enrichment and sampling

- Flow cell made of stainless steel with internal container for enrichment surfaces of polyvinyldifluiride (PVDF), equipped with stainless steel grid openings for water at both ends with Swagelok taps at inlet and outlet.
- Stainless steel tubes with hose coupling for groundwater inlet.
- Sterile enrichment surfaces of crushed garnet beads 0.70 mm × Bulk (500 g), (art.no. 13123-05, Immuno Diagnostic Oy, Finland).
- Eppendorf tubes, sterile for PCR applications.
- Sterile cell harvesting scrape (Fischer Scientific art.nr-11371714, Sweden).
- Sterile pincers and scissors.
- Sterile gloves.
- Sterile petri dishes of glass (treated with RNase/DNA away, and dry sterilised in muffle furnace at 450 °C for15 min.)
- FreeBact-20, 2 component disinfectant (225 mg ClO_2, XINIX AB, Sweden).
- RNase/DNA AWAY®, cleansing solution, Ambion (VWR, Sweden).
- RNase/DNase free 1 × PBS 500 mL (Dulbecco's w/o Ca Mg, Fischer Scientific art.nr-11461198).
- RNase/DNase free H_2O, 500 mL (Gibco, Fischer Scientific, Sweden).

A1.2.2 Material for ATP analysis and DNA extraction

- ATP Biomass kit HS, Biothema, Sweden.
- PowerWater® DNA Isolation Kit, MO BIO Laboratories, Immuno Diagnostic Oy, Finland.

A1.2.3 Preparation of flow cells

1. Confer Figure 2-6 for details of the flow cell.
2. Clean and sterilize the flow cell, including the holder for the enrichment surfaces of white plastic, by disassemble all the parts and place in a bath of chlorine dioxide solution, 22 mg L^{-1} for one hour. Rinse thoroughly afterwards with sterile water and let al. parts dry ambient protected with sterile aluminium foil for one day.
3. Assemble the parts keeping all sterile, and fill the container with sterilized enrichment surface garnet material. Surfaces of garnet can be purchased sterile from the supplier. Close the plastic container and seal with the steel grid. Place the container in the flow cell and close with the upper lid.
4. Ensure that all Swagelock taps are closed and wrapped with aluminium foil around the taps and openings to protect against contamination from the environment. The flow cell is now ready to be sent to the sampling area for biofilm enrichment from groundwater.

A1.2.4 Installation of equipment for biofilm enrichment

5. Ahead of the planned sampling the prepared flow cells is sent in a transport case for the current sample location. Responsible for sampling will in advance get al. information how the flow cells should be installed and connected to groundwater from the borehole for investigation.
6. Before flow cells with enrichment material is connected to the borehole, the borehole should be pumped to be remove flushing water from the drilling, and it should be stable in terms of chemistry, approaching pristine groundwater chemistry.

7. Connect the tubing pumping ground water from the borehole to the bottom Swagelock coupling on the flow cell. From the upper coupler is a stainless sterile tube for the outflow of groundwater. When the flow cell is connected to the wellhead, open all taps and check that the groundwater flows through with continuous flow rate and pressure, out of the upper valve outlet.
8. Flow cells should be connected to the borehole for at least 4–5 weeks with a steady flow and pressure of the groundwater to be examined. The given enrichment time is the minimum time for circulating microorganisms to get in contact with and form a biofilm on the enrichment surfaces.
9. Once the enrichment time period is finished, the flow cells valves are closed and disconnected from the borehole. The flow cell is then sent back to Micans laboratory for sampling and analysis of the attached microorganisms.

A1.2.4 Sampling of enrichment material

10. In the laboratory, the enrichment material is collected under sterile conditions, and rinsed with sterile PBS to remove planktonic bacteria, since only the attached microorganisms are to be examined. ATP analysis is performed to determining the amount of living biomass and DNA extraction is used to quantify and determine the diversity of attached microorganisms.
11. ATP analysis should be carried out as soon as possible when the flow cells arrived at the laboratory.
12. Unless the extraction of DNA can be performed on the sampling day, the material must be stored in at −20 °C.
13. To be able calculate the amount of attached microorganisms per surface area, the weight of the material is determined and the average surface area is calculated. Sterile controls for each ATP and DNA extraction are prepared and treated as the samples.

Arkitektkopia AB, Bromma, 2016

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