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Abstract

The discovery of active, growing, and diverse biofilms on solid–water interfaces introduced in deep groundwater suggested that biofilms could influence geochemical rock–water interactions such as dissolution and precipitation. Accordingly biofilms were investigated and found to influence the sorption of trace elements and radionuclides to these surfaces. If such biofilms also form in natural aquifers, they could profoundly influence the mobility of radionuclides escaping from SNF waste repositories. Hence, it was deemed crucial to investigate the presence of biofilms under *in situ* conditions. In this work, the microscopic analysis of total number of cells (TNC) was combined with the analysis of ATP and cultivation methods for aerobic and anaerobic microorganisms. DNA was extracted as well. Positive controls were included by application of known amounts of different microorganisms to natural and sterile rock surfaces. Different methods for sampling of microorganisms attached to rock surfaces were developed and tested.

In previous projects it was found that various swab techniques released fibres and thereby interfered with analyses like TNC and ATP. Swabs were customized for the use in this study with respect to the downstream processing and analysis in which the samples later would end up in. However, we were unable to find a swab material that would hold together sufficiently during sampling. This was especially a problem for the TNC analysis where fibres were released from the swab and limited the sight in the microscope, masking and hiding cells on the filters. The procedure to collect samples by scraping with a scalpel as done previously proved to be a superior method over swabs with better yields and no sight limiting fibres in the microscope. However, the scraping released minerals from the stone which also limited the sight in the microscope to some extent but not as much as did fibres from the swabs. There appeared to be interference from rock mineral with the ATP analysis and the DNA extraction.

This work demonstrated that it was possible to detect microorganisms on a natural fracture surface from a drill core retrieved during drilling at 400 m depth in the Äspö hard rock laboratory tunnel. At present, cultivation of microorganisms on fracture surfaces had the best results with a very low limit for detection. ATP analysis did reflect the biomass on the surfaces, but it appeared as if this method was very sensitive to interference from rock minerals and from stress on the microorganisms. However, ATP analysis should be continued. DNA analysis needs further development and testing before a full understanding of drawbacks and advantages can be given. This method is very strong once it is operational, but interferences during extraction and amplification of the nucleic acids must be optimized because the amount of DNA available on most natural fractures is minute. It was concluded very important that all sampling should commence directly after drill core retrieval; sending cores may be very detrimental to the viability and extractability of biofilm microorganisms.

Contents

1 Introduction

An interface is the boundary between two phases in a heterogeneous system. Solid–liquid interfaces are very important in microbial ecology as they influence microbial life in various ways (Marshall 1976). Microorganisms attach within minutes to most inanimate solid surfaces immersed in natural water (Bitton and Marshall 1980), where they may grow to form biofilms (Characklis and Marshall 1990). Microorganisms have been found in most subsurface environments where life is possible in terms of temperature and water availability (Fredrickson and Fletcher 2001). Like most other investigated underground environments, groundwater from deep aquifers in Fennoscandian Shield granitic rock is populated by significant numbers of microorganisms of which sulphate reducing bacteria (SRB) constitute a significant part (Pedersen 1993, 2000, Motamedi and Pedersen 1998). The solid-to-liquid ratio of subsurface environments is strongly displaced to the solid side, with very large surface areas relative to the available volumes of water. The existence of subsurface aquifers populated by attached microorganisms is very likely.

When investigated for biofilm formation, microorganisms were found to attach and grow on glass slides installed in laminar flow reactors (LFRs) connected to anoxic groundwater from drill hole depths ranging from 830 to 1,240 m (Pedersen and Ekendahl 1992a, b). Further investigation using 16S rRNA gene sequencing and scanning electron microscopy revealed that these biofilm populations comprised many species (Ekendahl et al. 1994, Pedersen et al. 1996). The discovery of active, growing, and diverse biofilms on solid–water interfaces introduced in deep groundwater suggested that biofilms could influence geochemical rock–water interactions such as dissolution and precipitation. Accordingly, biofilms on glass and rock surfaces in LFRs containing groundwater from a depth of 450 m were investigated and found to influence the sorption of trace elements and radionuclides to these surfaces (Anderson et al. 2006, 2007). This was an important observation, because the significant sorption and retardation of radionuclides at the rock–groundwater interface is accounted for in the safety analysis of geological deep repositories for spent nuclear fuel (SNF) waste. If such biofilms also form in natural aquifers, they could profoundly influence the mobility of any radionuclides escaping from SNF waste repositories (Keith-Roach and Livens 2002). Hence, it is crucial to investigate the presence of biofilms under *in situ* conditions.

Pressure-resistant circulating systems with LFRs were constructed to enable the investigation of attached and unattached microbial populations under *in situ* pressure (2.5 KPa), diversity, dissolved gas, and chemistry conditions (Pedersen 2005). The activity of biofilms was studied under different conditions. It was found that nitrate was reduced by microorganisms when introduced together with the carbon source lactate (Nielsen et al. 2006). Later, three parallel LFR cabinets were configured to allow observation of the effect on microbial metabolic activity of adding 3 mM hydrogen or 2.3 mM acetate, compared with an untreated control (Pedersen 2012). Hydrogen addition reduced the generation time four-fold to two weeks, doubled the sulphide production rate, and increased acetate production by approximately 50%. The acetate addition induced acetate consumption. The studied subterranean microbial processes appeared to proceed very slowly in terms of volume and time, though the results suggest that individual cells could be very active. Lytic bacteriophages are hypothesized to have caused this contradictive observation. Phages may consequently significantly reduce rates of subterranean microbial processes. Furthermore, the results suggest that hydrogen from corroding underground constructions could induce significant local microbial activity and that the low concentrations of hydrogen often observed in pristine subterranean environments may support slow but sustainable microbial activity in deep groundwater.

Drilling and retrieving cores that intersect natural aquifers would make it possible to investigate fracture surfaces for natural biofilms. Evidence of fossilized biofilms was obtained from such cores using transmission electron microscopy (Pedersen et al. 1997). Unfortunately, due to strong auto-fluorescence from most minerals, the detection of living biofilm microorganisms using fluorescence methods is impossible or severely hampered. However, the applicability of nucleic acid analysis was recently investigated as a method to reveal the presence and community composition of fracture surface biofilms (Jägevall et al. 2011). In that study, six cores were drilled and retrieved from 186 m depth in the Äspö Hard Rock Laboratory (HRL) tunnel to investigate whether indigenous biofilms developed on fracture surfaces in groundwater-conducting aquifers in granitic rock. A clone

library was constructed from fracture surface material (FSM), for community composition analysis. Quantitative PCR (qPCR) was applied to quantify gene copies using the 16S rRNA gene for domain *Bacteria* and the adenosine-phosphosulphate reductase gene (*apsA*) for sulphate-reducing bacteria (SRB). Results were compared with three groundwater systems with biofilms in laminar flow reactors (LFRs) at 450 m depth in the Äspö HRL. The total number of cells, counted microscopically, was approximately 2×10^5 cells cm^{-2} in the LFR systems, consistent with the obtained qPCR 16S rRNA gene copies. qPCR analysis reported ~1×10^2 up to ~1×10^4 gene copies cm^{-2} on the FSM from the drill cores. In the FSM biofilms, 33% of the sequenced clones were related to the iron-reducing bacterium *Stenotrophomonas maltophilia*, while in the LFR biofilms, 41% of the sequenced clones were affiliated with the SRB genera *Desulfovibrio*, *Desulforhopalus*, *Desulfomicrobium*, and *Desulfobulbus.* The community composition of the FSM biofilms differed from the drill water community, excluding drill water contamination. This and previous work discussed above consequently reports significant numbers of microorganisms on natural hard rock aquifer fracture surfaces with site-specific community compositions. The probability that biofilms are generally present in groundwater-conducting aquifers in deep granitic rock is consequently great.

The use of nucleic analyses was reasonably successful, but it was clear that the numbers obtained was in the lower range of what has been observed using LFR (Jägevall et al. 2011). A very crucial step in all nucleic acid analyses is the extraction of DNA from the environmental sample. Many of the commercial kits for extraction have been demonstrated to often have a low recovery percentage (Lloyd et al. 2010). In addition, the extraction and analysis of DNA does not indicate viability of the microorganisms. It was, therefore, judged important to investigate other, non-nucleic acid based methods for the quantification of attached microorganisms and their viability on natural fractures in groundwater conducting aquifers. In this work, the microscopic analysis of total number of cells (TNC) was combined with the analysis of ATP and cultivation methods for aerobic and anaerobic microorganisms. DNA was extracted as well. Positive controls were included by application of known amounts of different microorganisms to natural and sterile rock surfaces. Different methods for sampling of microorganisms attached to rock surfaces were developed.

2 Materials and methods

2.1 Total number of cells

The total number of cells (TNC) mL^{-1} was determined in 1-mL samples using the acridine orange direct count (AODC) method as devised by Hobbie et al. (1977) and modified by Pedersen and Ekendahl (1990).

2.2 ATP analysis

The ATP Biomass Kit HS (no. 266-311; BioThema, Handen, Stockholm, Sweden) was used to determine total ATP in living cells (Lundin 2000). The ATP biomass method used here has been described, tested in detail, and evaluated for use with Fennoscandian Shield groundwater (Eydal and Pedersen 2007).

2.3 Cultivable heterotrophic aerobic bacteria

Petri dishes containing agar with nutrients were prepared as described elsewhere (Pedersen and Ekendahl 1990) for determining the CHAB numbers in the flushing water used for drilling and in groundwater samples. 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.

2.4 Cultivable fungal cells

For determination cultivatable 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 (VWR, Prolabo, art no AX0222456) were prepared to Petri dishes. 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 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.

2.5 Preparation of medium, inoculations, and analysis for most probable number of culturable sulphate-reducing bacteria

The procedures described by Widdel and Bak (1992) for preparing anoxic media were modified and used to determine the most probable number (MPN) of microorganisms, as described elsewhere (Hallbeck and Pedersen 2008). Five tubes were used for each 10-times dilution, resulting in an approximate 95% confidence interval lower limit of 1/3 of the obtained value and an upper limit of three times the value (Greenberg et al. 1992). Media were prepared for SRB. The cultivation time was about eight weeks to ensure that slow-growing microorganisms were included in the results. The specific media compositions were based on previously measured chemical data of groundwater from 400 m depth in the Äspö HRL. The sodium chloride concentration was adjusted to obtain a medium salinity corresponding to the 15‰ salinity of the sampled borehole water. This allowed the formulation of artificial media that closely mimicked *in situ* groundwater chemistry for optimal microbial culturing, as was previously found to be successful (Haveman et al. 1999, Hallbeck and Pedersen 2012).

2.6 DNA extraction

Genomic DNA from each sample was extracted using PowerSoil® DNA Isolation Kit (no. 12888-50; Immuno Diagnostics Oy, Finland) according to the manufacturer's protocol. The DNA extractions were then stored at –20°C.

2.7 PicoGreen quantification of extracted dsDNA

To quantify extracted dsDNA the fluorescent nucleic acid stain Picogreen® Quanti-iT™ DNA Assay Kit *2000 assays* (no.P7589, Invitrogen, Sweden) was used as advised by manufacturer.

2.8 Sampling methods tested

Two sampling methods were evaluated for the ability to efficiently collect cells from the core surfaces.

2.8.1 Swabs

The swab-collection method involved swabbing the fracture surface with a swab soaked in isotonic phosphate solution. The swab was then put in a tube with 1 mL isotonic phosphate solution and vortexed to release the cells from the swab. Three different swab materials were used depending on the downstream analysis; DNA/RNA-free cotton, rayon and ATP-free polyamide (viscose).

2.8.2 Scraping

The scraping method 2 mL isotonic phosphate solution was applied to the fracture surface. The surface was then thoroughly scraped with a sterile scalpel and the solution collected with a pipette and transferred to Eppendorf tubes. This sampling method has previously been described and evaluated by Jägevall et al. (2011) with good results.

2.9 Test bacteria

2.9.1 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 solution to approximately 10^7 cells mL^{-1}.

2.9.2 Pseudomonas fluorescens

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

2.9.3 Desulfovibrio aespoeensis

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

2.10 Analysis of sterile fracture surfaces – negative controls

Sterile fracture surfaces were prepared as follows. Dry drill cores with a diameter of 76 mm were put in a sterile plastic container filled with deionised water (analytical grade) for approximately one week to allow the cores to become water-saturated. At the time of analysis the cores were crack in the middle, generating two sterile fracture surfaces. The two pieces of the cracked core were mounted in a vice so that the fractures surfaces became horizontal. Without touching the surfaces a sterile plastic film were wrapped around the top of each core to prevent liquids from running off the surface.

Half of one surface was thoroughly swabbed with a rayon swab soaked with an isotonic phosphate solution. The swab was then put in a tube with 1 mL isotonic phosphate solution and analysed for TNC. The other half 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 other surface was treated with 2 mL isotonic phosphate solution whereon 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.

2.11 Analysis of fracture surfaces with known amounts of bacteria

Sterile fracture surfaces were prepared, sampled and analysed as in 2.10. However, before swabbing and scraping, the surfaces were treated with 1 mL of a *P. fluorescens* culture (10^7 cells mL^{-1}) which was given time to be almost completely absorbed by the rock of the core.

2.12 Obtaining a core with fracture surfaces from KA3065A01

Drilling and sampling was performed according to the following activity plan ‘AP TD VERK-DETU-11-063 Provtagning av sprickytor vid borrning inför utbyggnad av Äspölaboratoriet’.
A brief description of the procedure is given here:

Drilling 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 18 m per day where drilled. The task was to give access to drill cores of water-bearing fracture surfaces immediately after removal. In the original plan sampling of fracture surface material should have taken place in the tunnel. This type of sampling was previously tested and did prove to be successful (AP TD F82P1-08-050). When one core with water-bearing fractures was obtained is was sampled by performing staff in 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 more 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.

2.13 Sampling 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 isotonic phosphate solution 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 depth and #2 to 23.97 m. Each fracture was divided into four equally large sectors, one for each downstream analysis, according to Table 2-1.

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 isotonic phosphate solution
3	ATP-analysis	Extraction solution B/S
4	DNA extraction	Extraction solution Power Soil (MO BIO Laboratories)

All four sectors were thoroughly scraped with a sterile scalpel to release the biofilm from the surface (Figure 2-1). After scraping each sector was swabbed with a DNA/RNA-free swab which was subsequently transferred to 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 a *D. aespoeensis* and a *P. aeruginosa* culture, 10^7 cells mL^{-1}. 4 mL of this mix was added to the fractures by pipette and was allowed to dry for 20 min. When the fractures still were moist the sampling procedure stated above was repeated.

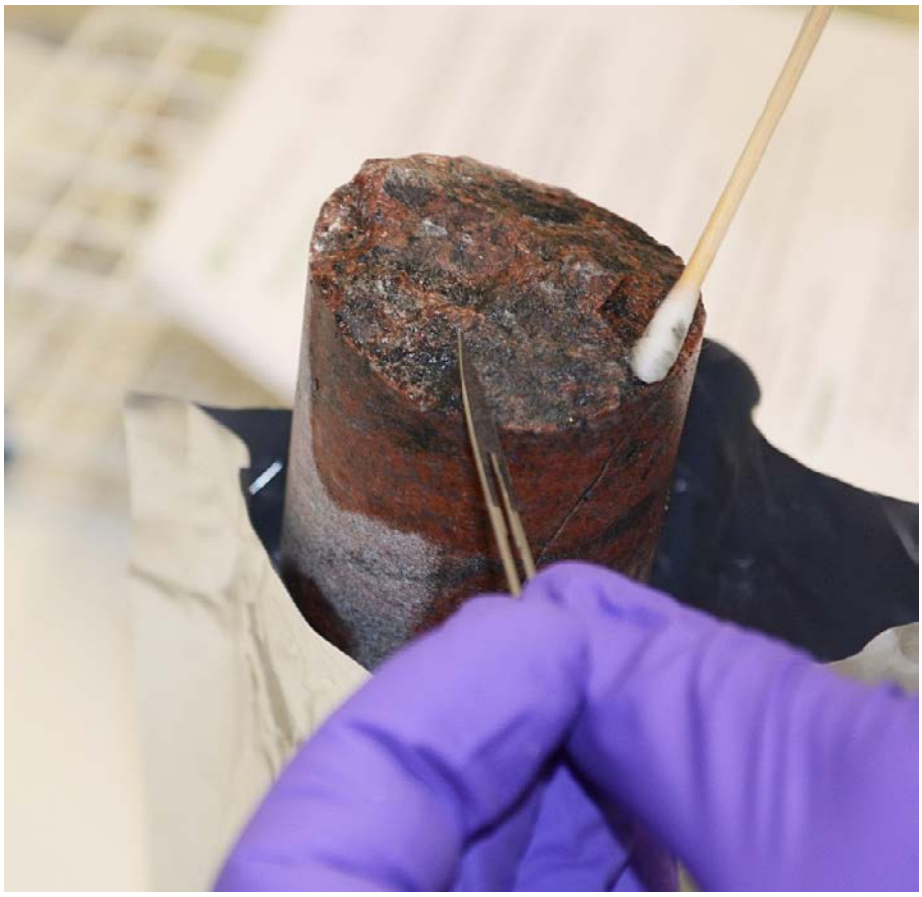

Figure 2-1. *Sampling of one of the KA3065A01 fracture surfaces.*

3 Results

3.1 Analysis of sterile fracture surfaces – negative controls, and surfaces with known amounts of bacteria

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 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. The spiked samples all had TNC and ATP above the detection. The recovery was good for the TNC method but bad 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. bd = below detection limit. Spiking was done with *P. fluorescens* (p.f).

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 P.f 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 P.f spiked fracture	7.3×10^{9}	3.3×10^{7}	0.005	0.7×10^{8}	4.3×10^{7}	0.61

3.2 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 total numbers (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 CHAB above detection on the natural surface, but SRB were below detection. Spiking with CHAB and SRB increased the CHAB number accordingly and SRB became above the upper limit of detection. The CFC analysis failed due to swarming of the growing cells resulting in a smear that could not be quantified. All DNA analyses were below detection as well.

Table 3-2. Analysed and applied amounts of ATP and TNC with yield ratios. bd = below detection limit. Spiking was done with *P. aerunginosa* and *D. aespoeensis*.

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

Table 3-3. Analysed amounts of cultivable heterotrophic aerobic bacteria (CHAB), cultivable fungal cells (CFC), most probable number of sulphate reducing bacteria and extracted DNA. nd = no data. Spiking was done with *P. aerunginosa* and *D. aespoeensis*.

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

4 Discussion

4.1 Sampling methods

4.1.1 Swabs

In previous projects we have had problems with swabs that released fibres and thereby interfered with analyses like TNC and ATP. In an attempt to circumvent this problem, we tried to customize the swabs used in this study with respect to the downstream processing and analysis in which the samples later would end up in. However, since the swabs are not designed and manufactured for uneven and sharp surfaces like those of a core fracture surface we were unable to find a swab material that would hold together sufficiently during sampling. This was especially a problem for the TNC analysis where fibres were released from the swab and limited the sight in the microscope, masking and hiding cells on the filters. When samples were diluted 100–1,000 times this problem was circumvented but when working with samples with naturally low cell numbers such a dilution was not an option because the results became below detection.

4.1.2 Scraping

To collect sample by scraping with a scalpel as done previously (Jägevall et al. 2011) proved to be a superior method over swabs with better yields and no sight limiting fibres in the microscope. However, the scraping released minerals from the stone which also limited the sight in the microscope to some extent but not as much as did fibres from the swabs. There appeared to be interference from rock mineral with the ATP analysis. This analysis is based on a light emitting enzyme reaction (Lundin 2000) and the luminometer in use is very sensitive and do give a reading signal from only a few photons. Auto-fluorescent material may, therefore, interfere with the analyses. It is likely this was the case for the sterile surfaces (Table 3-1). However, such problems can be dealt with by the use of sterile controls

4.2 Microbiological analysis methods

The investigations of microbiological processes in deep groundwater environments can employ many methods (Table 4-1). As each method has characteristic strengths and weaknesses, it is advisable to use several methods when investigating process-related parameters such as microbial biomass, diversity, and activity. This multi-pronged approach has been applied previously in microbiological investigations. For example, biomass analyses (i.e. the amount of living organisms) have utilized microscopic counts, analysis of biochemical components, cultivation, and nucleic acid methods, while microbial diversity has been examined using cultivation and nucleic acid analyses and microbial process activity has been examined using nucleic acid analyses. Next follows a review of the microbiological analysis methods applied in this work, and also methods that will be applied after further development of the methodologies. The strengths and weaknesses of each method are presented, together with information about reproducibility, resolution, detection range, and uncertainties.

Table 4-1. List of methods that can be used for the investigation of drill core surfaces with reference to the main parameter(s) analysed, i.e. biomass, diversity and activity.

Method	Name	Biomass	Diversity	Activity
Total number of cells	TNC	X		
Adenosine triphosphate	ATP	X		X
Cultivation of aerobic, heterotrophic bacteria	CHAB	X	X	
Cultivation of metabolic groups of microorganisms, MPN	MPN	X	X	
Quantitative PCR of DNA	qPCR DNA	X	X	
PCR, cloning, and sequencing	Sequencing		X	

4.2.1 Total number of cells

The total number of cells (TNC) was determined using the acridine orange direct count (AODC) 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 TNC method is used to determine biomass (Table 4-1). The acridine orange method is described in detail in several papers (e.g. Hallbeck and Pedersen 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 is counted under blue light (390–490 nm), using a band-pass filter for orange light (530 nm), in an epifluorescence microscope at 1,000 × 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 was 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.

Strengths:

The method is straightforward and technically simple to perform. Results can be obtained within one day of sampling. Alternatively, samples can be preserved in the field and analysed later. The method gives a good overview of the size and morphology of the sampled microorganisms, supporting reliable estimates of cell numbers. Acridine orange usually gives very good contrast in the prepared filters.

Weaknesses:

The method is fairly time consuming and therefore costly. It can be difficult to use acridine orange for turbid samples containing non-living particles and colloids and swab fibres that may take up the stain. This can result in unspecific staining that makes it difficult to distinguish bacteria from particles. If cells adhere to particles in the sample, they may not be observed.

Reproducibility:

The method is highly reproducible between samples. Typically, three subsamples of a groundwater taken in series reproduce a mean with 15–30% standard deviation.

Detection range:

The detection limit depends on the filtered volume. Typically, a volume of 1–10 mL can be filtered without filter clogging problems. There should be at least 20 cells per counted microscopic field for good statistics and precision, which sets 10^4 cells mL^{-1} as the detection limit. This detection limit can be forced towards 10^3 cells mL^{-1} if the accepted number of cells per field is lowered to 2, in which case up to 300 microscopic fields must be counted to reach the 600 cells needed for good reproducibility. Samples with numbers of cells above approximately 10^6 cells mL^{-1} should be diluted; the upper range will then easily exceed typical groundwater TNC numbers.

4.2.2 Adenosine triphosphate

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, and 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 on average 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 (Table 4-1). The ATP Biomass Kit HS (no. 266-311; BioThema, Handen, Sweden) was used in the Olkiluoto investigations to determine total ATP in living cells. This analysis kit was developed based on the results of Lundin et al. (1986) and Lundin (2000). This kit, which includes enzymes and reagents, is used, with a luminometer, to determine ATP, as the photon counts are proportional to the amount of ATP in the groundwater sample. The ATP method has been described, tested in detail, and evaluated for use with Fennoscandian groundwater, including Olkiluoto 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. This uncertainty can be mitigated by concomitant evaluation of TNC results.

Strengths

The ATP method is very sensitive and can detect as little as a few cells in a sample. Very small sample volumes are needed (i.e. <0.1 mL), which is an advantage when the sample size is small. Only living cells contains ATP, so this method only captures living biomass.

Weaknesses

High salt concentrations, like those found in deep groundwater, may disturb the analysis and significantly reduce the reported values. Samples should preferably be analysed immediately after sampling, because storage of samples, including extracted samples, tends to reduce the ATP content. Auto-fluorescent material may cause interference with the analytical instrument.

Reproducibility

The method is highly reproducible between samples. Typically, three subsamples of a groundwater taken in series reproduce a mean with 5–15% standard deviation.

Detection range

The lower detection limit is approximately 10 cells and the typical sample volume in the reaction tube is 0.1 mL, resulting in a sample detection limit of 10^2 cells mL^{-1}. Samples with concentrations approaching the internal ATP standard must be diluted, which is easy to do; the upper range will then easily exceed typical groundwater ATP concentrations.

4.2.3 Plate counts of heterotrophic bacteria and fungi

Petri dishes with a diameter of 9 cm are prepared using agar and nutrients as described elsewhere (Pedersen and Ekendahl 1990) for determining the numbers of cultivable heterotrophic aerobic bacteria (CHAB) in groundwater samples. This method mainly reports a biomass-related value (Table 4-1). Ten-times dilution series of samples are made in a sterile dilution liquid and 0.1 mL portions of each dilution are spread with a sterile glass rod on plates in triplicate. The plates are incubated for 7–9 days at 20°C in air, after which the number of colony forming units (CFU) is counted; plates with 10–200 colonies can be counted. The type of medium used can be varied almost infinitely. Here we also used a medium adapted for fungi with a pH of about 5.0.

Strengths

This is a very basic and well-known methodology that has been in use for more than a century. The results can be compared with those of many other investigations of similar or different environments. This method is effective when a specific group of bacteria is sought, provided they can be cultivated using this method.

Weaknesses

This method does not detect obligate anaerobic microorganisms or microorganisms living on inorganic energy sources and using carbon dioxide as the source of cell carbon. Not all aerobic, heterotrophic bacteria are cultivable on given media. The diversity span is fairly limited for each combination of nutrients and incubation conditions.

Reproducibility

The method displays very good CFU reproducibility between plates and over dilutions. Typically, plates in the same dilution reproduce a mean with 5–15% standard deviation.

Detection range

The lower detection limit is 10 CFU and the typical sample volume of a plate is 0.1 mL, resulting in a sample detection limit of 10^2 cells mL^{-1}. This detection limit can be forced towards 10^1 cells mL^{-1} if the accepted number of CFU per plate is lowered to 1, but then the reproducibility decreases significantly.

4.2.4 Cultivation of metabolic groups of microorganisms

Anaerobic media for determining the most probable number (MPN) of various anaerobic and aerobic microorganisms in groundwater can be prepared according to the procedures described by Hallbeck and Pedersen (2008). The specific media details are commonly formulated based on previously measured chemical data the sampled environment. This was previously fond to allow the formulation of artificial media that most closely mimicked *in situ* groundwater chemistry for optimal microbial cultivation (Haveman and Pedersen 2002). Media for the nitrate-reducing bacteria (NRB), iron-reducing bacteria (IRB), manganese-reducing bacteria (MRB), sulphate-reducing bacteria (SRB), autotrophic acetogens (AA), heterotrophic acetogens (HA), autotrophic methanogens (AM), heterotrophic methanogens (HM), and aerobic methane-oxidizing bacteria (MOB) can be autoclaved and anaerobically dispensed according to the formulations described elsewhere (Hallbeck and Pedersen 2008). The MPN procedures result in protocols with tubes that scored positive or negative for growth. The results of the analyses are rated positive or negative compared with control levels. Three dilutions of five parallel tubes were used to calculate the MPN of each group, 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. In this work, we only used a medium for SRB, but the application of all other types of media described above can be equally easy to apply.

Strengths

The method is robust with a low detection limit and produces good data from small sample volumes. Many metabolic groups can be analysed simultaneously. It is subsequently possible to identify and describe the dominant cultured species in a sample using PCR, cloning, and sequencing.

Weaknesses

The method requires a significant amount of advanced equipment in the laboratory, including systems for gas distribution and mixing, anaerobic chambers, gas chromatographs, and anaerobic cultivation equipment. The results are obtained long after sampling, because the cultivations need to be incubated for up to two months. The sample may contain microorganisms that do not grow under the various conditions offered.

Reproducibility

Several reproducibility tests using groundwater samples have been performed. Groundwater samples taken simultaneously in a deep drillhole indicated very good reproducibility (Hallbeck and Pedersen 2008). The difference between the two samples was generally represented by a difference of only a single tube in the MPN analyses. The maximum difference between the two samples was 1.6, so

the two samples were not statistically different. In comparison, groundwater samples differed significantly between drillholes, indicating the heterogeneity of microbial populations in fractured rock. These reproducibility tests demonstrated that the analytical protocols for microbiological analyses were reproducible between samples. The reproducibility of the methods when used on groundwater from drillhole sections over a 3.5-month interval was also tested. It was found that the results per drillhole section were reproducible over time, indicating that the microbiological diversity in the groundwater from the analysed drillholes did not change over time. When applied to shallow drillhole groundwater from Olkiluoto, most of the groundwater in the ten analysed drillholes reproduced well on three sampling occasions distributed over one year (Pedersen et al. 2008). MPN procedures and the analyses have consequently been found to be very robust and reproducible both over samples and sampling times and between drillholes.

Detection range

The detection limit for the methods using five parallel tubes with 1 mL of sample per first tube results in a detection limit of 0.2 cells mL^{-1}. The upper limit is determined by the number of dilutions, and this number can be set to reach the TNC of a sample (see Section 4.2.1). The detection range will then include all possible combinations of numbers of microorganisms in the sample.

4.3 Molecular methods for characterizing microbial communities

Studies of environmental microorganisms have demonstrated that many microorganisms do not grow on any known growth media (Amann et al. 1995). Therefore, molecular methods have been increasingly used in environmental microbiology to study the diversity and function of microbial communities. Total DNA or RNA can be extracted from an environmental sample, resulting in an extract containing the genomes of all organisms in the sample. Using various PCR techniques combined with sequencing techniques can provide information about the community structure of the microorganisms, and their metabolic potential and interactions.

Microorganisms can be identified by certain evolutionally conserved gene sequences, such as the genes for the ribosomal RNA subunits (rRNA). These genes are present in all cellular organisms. One of the most commonly used genes is the one for the 16S subunit of a microorganism's ribosomes. The 16S rRNA gene does not, however, reveal the function of the detected microbial communities. For this purpose, genes encoding enzymes involved in specific functions, such as methanogenesis or sulphate reduction, may be used. Methanogens have successfully been detected by their genes for methyl coenzyme M reductase alpha subunit (*mcr*A) in many different environments, while sulphate reducers can be detected by their dissimilatory sulphite reductase (*dsr*AB) genes.

Polymerase chain reaction (PCR) is one of the most widely used techniques for examining uncultured microorganisms. Using this technique, the gene of interest can be amplified (copied) to provide a large number of copies of the gene for further examination. By separating the gene copies derived from different organisms inhabiting the same sample, for example, by means of cloning, DGGE, or high-throughput sequencing techniques, it is possible to determine the composition of a microbial community as well as the presence of genes encoding specific functions in a sample.

PCR-based methods are generally very sensitive, and genes present in low numbers in a community may be detected. The concentration of microbial cells in deep groundwater often ranges from 10^3 to 10^5 cells mL^{-1}, which is very low. Therefore, there is a need to concentrate the microbial biomass, for example, by filtration, which allows the water to be extracted by vacuum suction through 0.22-µm pores while the bacterial cells are trapped on the membrane. Solid material need to be collected in amounts that ensure cell numbers above the detection limits for the extraction protocols. The cells are subsequently mechanically and chemically disintegrated to release the DNA and RNA for use in various analyses. DNA is fairly resilient to degradation, but RNA is generally rapidly degraded. Though rRNA may survive for many hours in a sample, the mRNA often lasts only a few minutes. RNA is therefore often used as an indicator of active microorganisms while DNA indicates the presence of all microorganisms, some of which may not be active but may nevertheless be activated and start sulphate reduction due to changing environmental conditions.

4.3.1 Quantitative PCR of DNA

The numbers of various types of microorganisms in a sample can be determined based on known genetic information (Ginzinger 2002). Cells are first collected from the sample, disintegrated using biochemical methods, and the genetic material, DNA, is extracted. The DNA is quantitatively PCR (qPCR) amplified using pairs of short DNA sequences (i.e. primers) specific to various microorganisms.. Known amounts of DNA from the analysed genes are included as standards and analysed concomitantly with the samples. The output of the method is the number of gene copies related to the primer pair in a sample. As the number of gene copies can vary depending on the type of genes and the species, it becomes important to use standard DNA closely related to the microorganisms being analysed for. Internal controls must be included to test the DNA extraction efficiency and map the possible inhibiting effects of sample contaminants on the amplification process

Strengths

The results can be obtained within a couple of days. The sensitivity is good, but is related to the available sample volume. All types of microorganisms are detectable provided proper primers are available. The method is independent of culturing, which implies that microorganisms difficult or impossible to culture can be detected.

Weaknesses

Extraction efficiency may differ significantly between types of microorganisms, samples, and methods (Lloyd et al. 2010), meaning that considerable time and resources must be invested to optimize the extraction procedure. The selected standard gene sequences may differ from those of the sampled microorganisms. Discrepancies in numbers may arise due to differences in gene copy numbers between the standards and the sampled microorganisms. Genetic analysis is severely influenced by sample quality; for example, traces of metals, salts and organic material may disturb the amplification process, thereby influencing the detection limit.

Reproducibility

This method consists of several parts. The reproducibility of qPCR is very good once DNA has been extracted and purified. The sensitive part of this method, with respect to reproducibility, is the DNA extraction, where the efficiency is sensitive to small variations in handling and in the sample volume. Therefore, standard microorganisms should be included, to enable compensation for variations in extraction efficiency; doing so will enhance the reproducibility of the results.

Detection range

Typical analysis extractions concentrate the nucleic acids from the sample into volumes of 100–200 µL. The qPCR amplification usually includes 2 µL of this extraction in the qPCR reaction mix. These proportions then set a theoretical detection limit of 100 gene copies in the extraction volume. The amount of sample that can be extracted can be large, i.e. the more groundwater (or any other sample such as fracture surface material) that is concentrated, the lower the detection limit. However, it is often found that large sample volumes increase the amount of PCR-inhibiting substances that will counteract the effect of increasing the sample volume on the detection limit. If the sample volume is 100 mL, then the theoretical detection limit would be 1 gene copy mL^{-1}. However, in reality, losses due to efficiency issues in the DNA extractions (Lloyd et al. 2010) and inhibiting effects from the sample on the PCR reactions often reduce the detection limit to 100–1,000 gene copies mL^{-1}. The proper way to deal with this is to always report data together with the detection limits based on internal standards. Several quality controls should preferably be reported as well, including qPCR reaction efficiency, the intercept of the standard curve for one copy and negative control and master mix results (which should be significantly lower than the sample and standard values).

4.3.2 PCR, cloning, and sequencing

This method aims at the identification of microorganisms in a sample by determining a partial DNA gene sequence. The target gene can be a universal type that is present in all microorganisms, such as the 16S rRNA gene in domain Bacteria and Archaea, or a gene that is specific to a group of microorganisms, such as the *apsA* and *dsrAB* genes that encode enzymes involved in sulphate reduction. Cells are collected and disintegrated and the genetic material, DNA, is extracted and purified. The DNA is then PCR amplified using pairs of short DNA sequences (i.e. primers) specific to various microorganisms. Amplified DNA is cloned into a plasmid vector that is transformed into *Escherichia coli* for multiplication. The target sequences are subsequently extracted and sequenced. The obtained DNA sequences are compared against international databases for identity to deposited genes; the most closely related sequence in the database will reveal species-related information about the analysed microorganisms.

Strengths

The method reveals the diversity of sample microorganisms within a week, including organisms difficult or impossible to cultivate.

Weaknesses

The method tends to be biased in favour of the dominant microorganisms in the sample. Often, many more than 100 clones may need to be sequenced for a full diversity assessment, which is expensive and time consuming. The method is sensitive to contamination, particularly when analysing samples containing few microorganisms

Reproducibility

The obtained information can be reproduced, provided that the full diversity is analysed.

Detection range

Not relevant.

4.4 Analysis of sterile fracture surfaces – Negative controls, and surfaces with known amounts of bacteria

The hardest and best ‘hold-together’ swab that we could find was made of a material called rayon which is a semi-synthetic fibre. Hence, this type of swab was used to collect samples for TNC analysis. However, when observing the samples in the microscope it was concluded that this type of swab. Like the other tested swabs, released too much fibres for optical cell counting on natural fractures samples. On the other hand, when the cell numbers were high as it was on the *P. fluorescens* spiked fractures, this swab method may be possible to use since the samples can be diluted, thereby diluting the fibre effect, and still produce statistically confident results.

A loss of 71% of the applied cells according to the TNC analysis was observed with the swab method, which may be insufficient for natural samples. The ATP analysis presented similar results where almost all applied cell ATP was lost. An explanation for low results with the swab method could be that cells adhere to strong to the swab material and are not released with vortexing. Or, it is also possible that the application of cells in surfaces induced stress responses in the cells that could shut down metabolisms with a rapid decrease in the ATP content as a result. The half live of ATP in cells is only minutes (Eydal and Pedersen 2007). ATP determination will consequently reflect the living and metabolizing part of a total biomass determination, such as cell number, protein, or DNA analysis. Active metabolism is necessary to maintain a high ratio of ATP to ADP+AMP in cells, commonly denoted energy charge (Lundin 2000). It has been suggested that ATP concentration reflects

the biovolume of metabolizing cells, because the cytoplasmic ATP concentration is approximately equal in living, metabolizing cells. Comparing analysed ATP concentrations with the results of various enumerations will consequently reveal how well such enumerations reflect the volume and activity of the biomass of a studied system.

The scraping method, as mentioned above, proved to work better than the swab method with a loss of 39% of applied cells. This can be considered as acceptable for the TNC analysis when working with natural samples. However, the ATP-analysis did not produce equally satisfying results with a much larger loss of almost 100%. This could be due to a number of reasons. *P. fluorescens* was in retrospect found to be an unsuitable positive control bacterium for this study since it produces and secretes pyoverdin, a fluorescent iron chelating compound (siderophore). Pyoverdin can potentially disturb ATP-analysis which is based on luminescent readings. Minerals released from the fracture upon scraping could also be a source of error since they reflect light like crystals, thereby also interfering with ATP-analysis. This could also be the reason for the positive ATP result (and negative TNC) on the scraped sterile fracture.

4.5 Analysis of KA3065A01 fracture surfaces

For the KA3065A01 fracture surface we used a sample collection method where the surface first was scraped with a sterile scalpel and then the material was collected using a viscose swab. For the ATP and TNC analysis the scraping-swab method gave rise to the same problem as discussed in Section 4.3.2, resulting in very low yields. To spike the surfaces, a 4 mL mix of *D. aespoeensis* and *P. aeruginosa* were used. However, in this experiment the core surface was not secured for liquid run-off (cf. Figure 2-1) which resulted in a substantial loss of applied cells were approximately 1 mL of the applied culture remained on the fracture surface. Hence, the 1×10^7 applied cell value (Table 3-2). The CHAB and CFC analysis both produces positive results. However, only CHAB was quantifiable. The CFC-plates had a very smeary, greenish growth with no individual colonies.

The swab problem described in Section 4.3.2 seemed to not affect the MPN analysis which produced strong, over range, positive results on the spiked surfaces. This sampling method did, however, not detect any SRB on the natural fracture surfaces. This most probably was due to the absence of SRB on the natural core surfaces. For future the scraping sampling method described in 2.8.2 is more effective on natural surfaces than the one used in this experiment.

DNA could not be detected using fluorescent labelling with PicoGreen after extraction on any surface. The downstream processing with qPCR, cloning and sequencing could not be performed here. The DNA extraction seemed to have been either inhibited by mineral substances in the spiked samples (Section 4.3), or, was the extraction protocol not efficient enough to collect DNA (Lloyd et al. 2010). Further tests and development is needed for DNA extraction and analysis. Previous work did succeed with extraction, but that work was performed on the drilling site (Jägevall et al. 2011).

4.6 Further development of the methodology

The scraping sampling method described in 2.8.2 should be further developed and evaluated since it produces the best results in this method development study. To get a more accurate understanding of the biology of fracture surfaces the sample collection should be done at site, directly after retrieval of the rock surfaces. Samples for TNC could be preserved and analysed at the laboratory while samples for cultivation and ATP should be analysed directly. DNA samples should be preserved on site

MPN culture proved to be an excellent method that showed applied cells on natural fracture surfaces (Table 3-3). On the other hand, the cell numbers on this particular sample was too small to be above the detection limit on untreated surfaces. A more likely explanation is that the core was exposed to air for a long time and SRB are very sensitive to oxygen and may, consequently have been killed off before analysis. This interpretation further argues for analysis directly after drill core retrieval.

4.7 Conclusions

- It was possible to detect microorganisms on a natural fracture surface from a drill core.
- The method with scraping for the collection of fracture surface material was superior to the swab methodology.
- Cultivation of microorganisms on fracture surfaces had the best results with a very low limit for detection.
- ATP analysis did reflect the biomass on the surfaces, but it appeared as if this method was very sensitive to interference from rock minerals and from stress on the microorganisms. However, ATP analysis should be continued.
- DNA analysis needs further development and testing before a full understanding of drawbacks and advantages can be given.
- Sampling should commence directly after drill core retrieval.

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