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Forsmark site investigation

Numbers and metabolic diversity of microorganisms in boreholes KFM06A and KFM07A

Results from sections 353.5–360.6 and 768–775 m in KFMA06A and section 848–1,001.6 m in KFM07A

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June 2005

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Keywords: ATP, Groundwater, Microorganisms, Nitrate, Iron manganese, Sulphate, Reduction, Acetogen, Methanogen, Total number, Forsmark, AP PF 400-05-53, AP-PF-400-05-66.

Contents

1 Introduction

This document reports performance and results of microbe investigations in borehole KFM06A and KFM07A within the site investigation programme in Forsmark /1/. The work was conducted according to the activity plans (SKB internal controlling documents) listed in Table 1-1. The report presents microbiological data from three borehole sections:

- KFM06A, 353.5–360.6 m.
- KFM06A, 768.0–775.1 m.
- KFM07A, 848.0–1,001.6 m.

The sampling was carried out in February, March and April 2005 within the hydrochemical characterisation activities in KFM06A and KFM07A according to the activity plans AP PF 400-04-110 and AP PF 400-05-012 respectively (SKB internal controlling documents), Table 1-1. In KFM06A, 353.5–360.6 m, two PVB samplers were collected simultaneously and analysed for microbiology. The sampling and the down hole sampling equipment is described in /2, 3/. Subsequent laboratory work was performed during 8–10 weeks after the samples reached the laboratory.

The flushing water used during core drilling of the boreholes may cause contamination by foreign bacteria and thereby affect the in situ microbiological conditions. Control of the microbe content in the flushing water was performed during drilling of borehole KFM06A but not KFM07A. The results from drilling of KFM06A are reported in /4/.

Table 1-1. Controlling documents for performance of the activity.

Activity plan	Number	Version
Undersökning av mikroorganismer i KFM06A	AP PF 400-05-53	1.0
Undersökning av mikroorganismer i KFM07A och KFMA08A	AP-PF-400-05-66	1.0

2 Objective and scope

Microorganisms have been demonstrated in every groundwater investigated in the Fennoscandian shield rocks, at depths ranging from surface to 1,700 m /5/. Active microorganisms influence the groundwater geochemistry /6/ and the redox potential /7/. Therefore, a full understanding of the geochemical situation in deep groundwater requires knowledge about presence, diversity and activity of microorganisms.

The microbiological analysis program was carried out according to protocols developed during previous investigations of Finnish groundwater /8, 9/. They include determination of the total number of cells in the groundwater (AODC), the concentration of adenosine-tri-phosphate (ATP) and a statistical cultivation method for estimating the most probable number of cultivable metabolic groups of micro-organisms (MPN). These are nitrate, manganese, iron and sulphate reducing bacteria, autotrophic and heterotrophic acetogens and autotrophic and heterotrophic methanogens.

A PVB sample container was filled with groundwater /2, 3/ and sent to the laboratory in Göteborg within 4–6 h. Sub-sampling for analysis was performed immediately at arrival of the PVB sampler.

3 Equipment and methods

3.1 Equipment for transfer of sample from the PVB sampler

The transfer of sample from the PVB sampler to the culturing tubes required a procedure that did not expose the sample to oxygen. This was solved by the design of an adapter (no 4 in Figure 3-1) that could be attached to the PVB sampler (at no 3 in Figure 3-1). Portions of 10 ml sample were distributed to nitrogen flushed Hungate tubes as shown in no 5 in Figure 3-1. The pressurized PVB sampler automatically ejected sample when the sampling valves were opened (nos 6 and 7 in Figure 3-1).

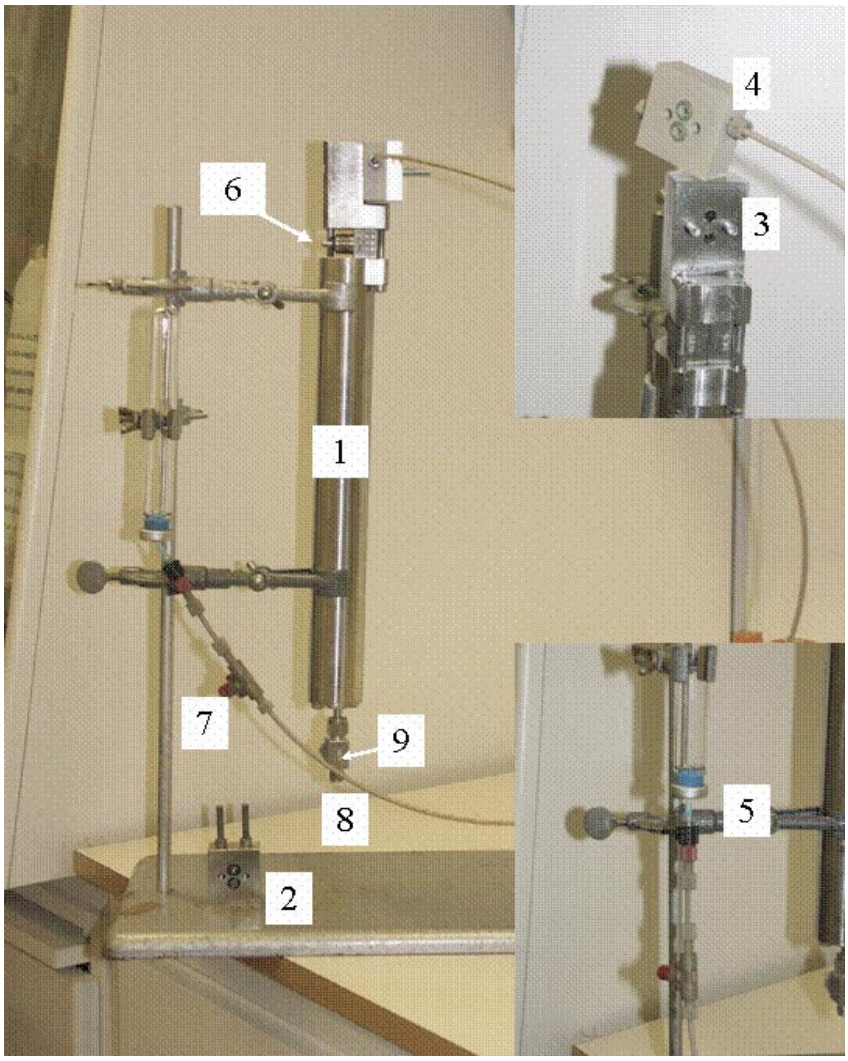

Figure 3-1. *This setup was designed for oxygen-free transfer of samples from the PVB sampler (1) to nitrogen flushed, stopped Hungate tubes (5). 1, PVB sampler; 2, transportation seal; 3, inlet/outlet of the PVB; 4, PEEK sampling device; 5, transfer of sample to Hungate tubes, 6, PVB valves; 7, PEEK sampling valve; 8, PEEK sampling tube; 9, PVB pressure valve.*

3.2 Equipment for most probable number determination

The preparation of anaerobic media required an anaerobic box and a gas bench for mixing and delivery of gas mixtures and gases for growth as described in detail in the activity plans. Typically, the preparation time for one sample delivery corresponded to about two weeks full-time work in the laboratory. The dilution and inoculation of samples for analysis of metabolic groups followed a well-defined procedure, depicted in Figure 3-2. One set of between 32 to 47 tubes was used for each analysis. Incubation at about 17°C was performed next. Finally, each tube was analyzed for presence of metabolic products typical for the respective metabolic group cultivated. Those were: nitrate reducing bacteria: consumption of NO_3^-, manganese reducing bacteria: Mn^{2+}, iron reducing bacteria: Fe^{2+}, sulphate reducing bacteria: S^{2-}, autotrophic and heterotrophic acetogens: acetate and autotrophic and heterotrophic methanogens: methane. The analysis for nitrate reducing bacteria was introduced for KFM07A.

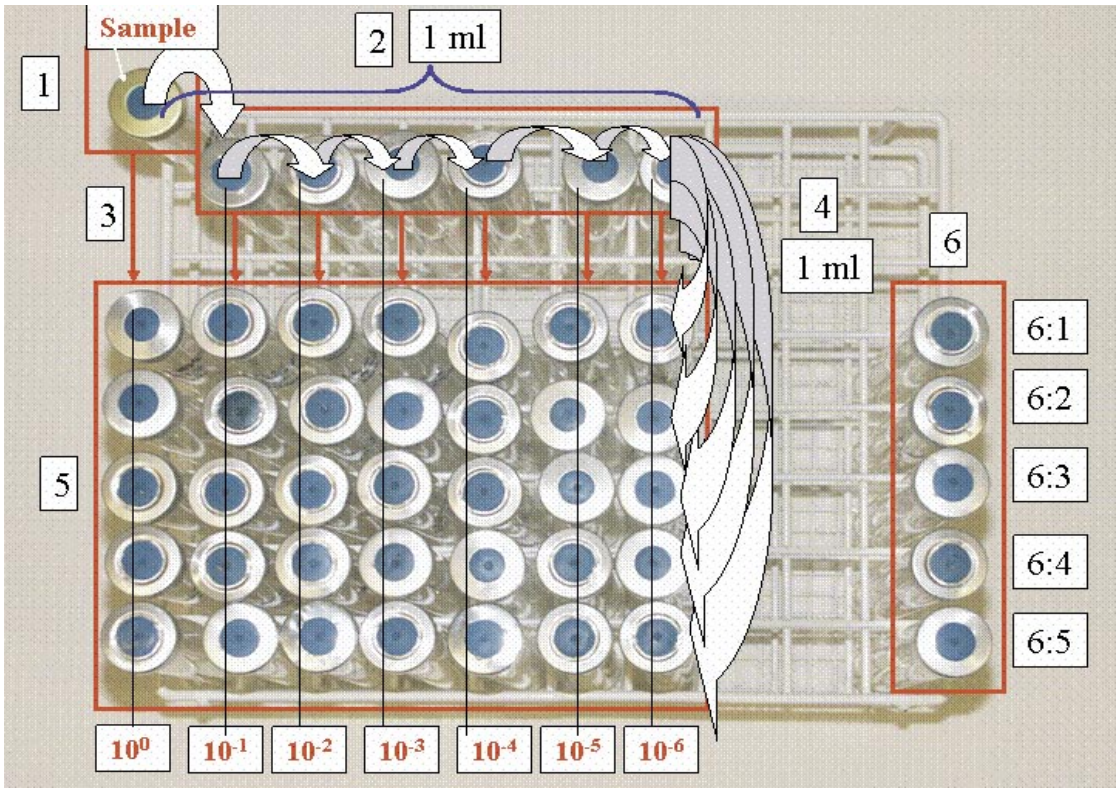

Figure 3-2. *The procedure for a "most probable number" determination. The Hungate tube with sample is used as the source for inoculation (1). A serial dilution was performed first (2). Thereafter, sub-samples were transferred (3–4) to the growth tubes (5) and to control tubes (6).*

3.3 Tests for stability and reproducibility of the methods

The methods used for MPN have been developed and tested since 1997. Results from Finnish investigations have been published /8, 9/. Quality control procedures have continuously been applied to the analyses, also under the investigations reported here. The decontamination procedures and the reproducibility of the analysis methods have been tested and the detailed results are presented in Appendices 2 and 3. The main conclusions are given below.

3.3.1 Decontamination

The PVB system is presently decontaminated with 70% ethanol. This procedure worked relatively well but was not optimal. Bacteria could still be cultivated in fairly large numbers in both decontamination tests (Appendix 2). It is recommended that the system is decontaminated with a 10 ppm solution of chlorine dioxide (XINIX FREEBACT-20 gives 22.5 L with 10 ppm). The FREEBACT disinfectant should be prepared fresh and pumped through the PVB system. This procedure is in operation at the POSIVA OY Onkalo investigations and gives a very good result. It will minimize the risk for irrelevant contaminants in the microbiology samples, compared to the use of 70% ethanol. In addition, ethanol remnants may compromise the organic carbon concentration in the sample.

3.3.2 Reproducibility of the analytical procedures

The reproducibility of the analytical procedures has been extensively tested and the results are reported in Appendix 3. The main finding is that the methods have an extremely good reproducibility over samples. Repeating the sampling and analytical procedures on a specific borehole level gave two data sets that were very close to identical. The MPN analyses never differed in more than one tube (confer Figure 3-2).

The reproducibility over time was demonstrated to be good as well. Two boreholes were analysed with about a 3.5 month interval. The two boreholes showed very different signatures that did reproduce very well.

In conclusion, the analytical procedures reported here are reliable, reproducible and they distinguish different boreholes and borehole levels. The obtained results can be regarded as a borehole/section specific signature that gives the required information about what biogeochemical processes were dominant at the time for sampling.

4 Performance

The microbial characterizations were performed according to activity plans AP AP PF 400-05-53 (KFM06A) and AP PF 400-05-66 (KFM07A). Details can be obtained from the appendices attached to those plans.

4.1 Sample transport

Samples were rapidly transported to the laboratory with car and air cargo. They reached the laboratory the same day of sampling, before 15:00.

4.2 Preparations of media

The media were prepared under two weeks before each sample date. They included a redox indicator that turns pink if the redox potential goes above –40 mV (relative a H_2 electrode). Such tubes are not used or analysed if they appear. This guarantees anoxic cultivation conditions. Controls for the media and the inoculation procedure were included.

4.3 Start of analyses

All analyses were started the day of arrival of the samples. ATP was measured at the arrival day and the results were obtained directly. Total number of sub-samples were preserved and counted during the following weeks. The MPN analyses were inoculated according to specific instructions for up to 8 weeks.

4.4 End of analyses

After inoculation, various analyses were started to measure the number of positive MPN tubes with growth. A positive reading was taken as at least the double value compared to sterile filtered controls, controls with medium only, and adjacent, negative MPN values /10/.

4.4.1 Nitrate consumed by nitrate reducing bacteria

A cadmium reduction method (0.3–30 mg/L NO_3^--N) was used according to HACH DR/2500, Method 8039 for water, waste water and seawater.

4.4.2 Ferrous iron from iron reducing bacteria

A phenanthroline method (0.02–3 mg/L Fe^{2+}) was used according to HACH DR/2500, method 8146 for water, waste water and seawater.

4.4.3 Manganese (II) from manganese reducing bacteria

A periodate oxidation method (0.2–20 mg/L Mn^{2+}) was used according to HACH DR/2500, Method 8034 for soluble manganese in water and waste water.

4.4.4 Sulphide from sulphate reducing bacteria

Sulphide was measured as copper sulphide with a spectrophotometer and compared with standard curve /11/. The main reagent was $CuSO_4 \times 5H_2O$, 1.25 g and concentrated HCl, 4.14 ml dissolved in double distilled H_2O to 1,000 ml. Detection limit is 0.01 mg L^{-1}.

4.4.5 Acetate from acetogens

A kit from Boehringer Mannheim / R-Biopharm Enzyme BioAnalyis was used, Cat No 10 148 261 035. UV-methods for the determination of acetate. The detection limit for this method is about 0.15 mg/L.

4.4.6 Methane from methanogens

A Varian 3400 gas chromatograph was used with a 2 m stainless steel HayeSep A column attached to a flame ionization detector (FID). Detection limit is 0.2 ppm.

4.5 Nonconformities

The microbial analyses from the investigated boreholes were conducted according to the activity plan with one deviation:

- The MRB rack from KFM07A 735.5 m was unfortunately tilted and could not be analysed correctly. The tubes analysed were, however, negative, suggesting a very low or below detection MPN for this analysis.

5 Data handling

5.1 Analyses and interpretation

Two samplers were analysed from KFM6A 353.5–360.6 m. In this report, the results were pooled and given as averages of the obtained results. The reproducibility task is treated in Appendix 3.

The total numbers of microorganisms were counted on duplicate filtration filters from three sample tubes. Each filter is regarded as one independent observation. The mean of 6 filters from 3 tubes was calculated and reported with the standard deviation (SD) and the number of observations (n).

The ATP measurements were performed three times for each sample from the different depths. The mean of the three samples was calculated and reported with the standard deviation (SD).

The MPN procedure results in a scheme with tubes that score positive or negative for growth. Combinations of three dilutions (15 tubes) were used to calculate the most probable number of respective group, as described elsewhere /10/.

6 Results

The detailed results are given in Appendix 1.

6.1 Total numbers of microorganisms

The acridine orange direct count (AODC) gives the total numbers of microorganisms in a sample. This number does, by definition, include living, inactive and dead cells. A dead microbe can still appear in the AODC analysis even if it has been dead for some time. To complement this uncertainty of the AODC count, a new analysis was introduced during 2004. The measurement of adenosine-tri-phosphate (ATP) reflects the living bio-volume. This is because all living cells contain a relatively constant concentration of ATP. A detailed analysis of the relation between AODC and ATP in deep groundwater is presently being executed and the results will be published during 2005. A present approximation gives that 1 amol (10^{-18} mol) corresponds to 1 healthy cell.

Figure 6-1 shows that the total number of cells decreases with depth and that the amount of ATP relative to the AODC numbers increases in KFM07A relative to KFM06A. This indicates that the living biomass was approximately similar in all three examined borehole sections. If the present relation between ATP and cell based on bio-volume given above is used, the ATP indicates 15,000, 5,100 and 9,500 cells ml^{-1} in KFM06A 353 m, KMF06A 768 m and KFM07A 848 m, respectively.

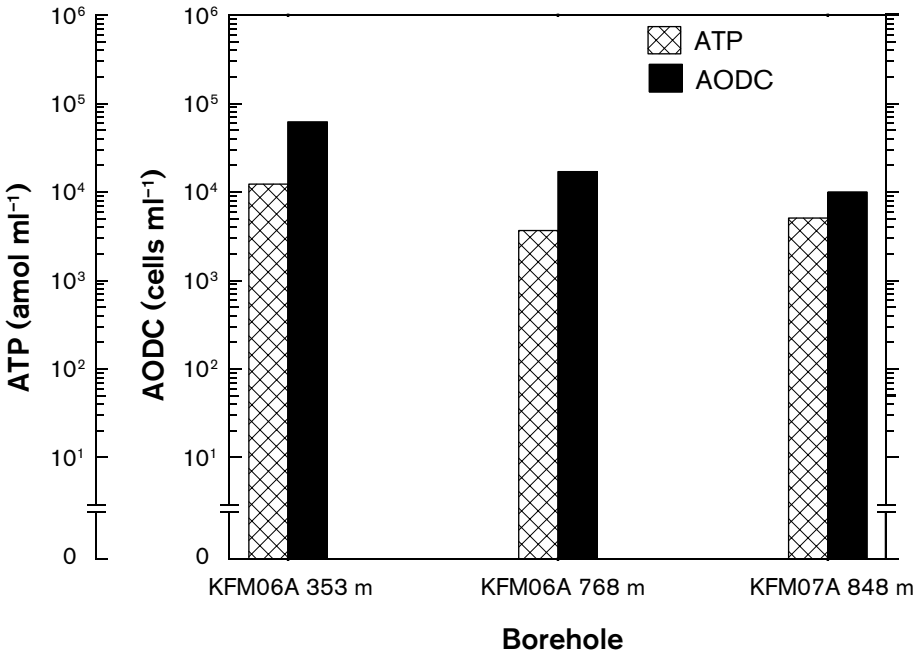

Figure 6-1. *The total number of cells (AODC) and the concentration of ATP in the analysed boreholes. KFM06A gives the average of two analysed PVB samplers.*

6.2 Numbers of cultivable microorganisms

The MPN determination gives a signature of what metabolic types of microorganisms are present in the examined sections. Far from all viable cells, as judged from the AODC and ATP measurements are cultivated (Table A1-6), which is a common, well known and accepted result throughout the scientific literature. Many microorganisms just cannot be cultivated. This is not as surprising as it first may seem. There are many animals – fishes, birds, insects – and plants on Earth than can only be studied in their natural environment. If we capture them, they will soon die because we do not understand how to give them suitable living conditions. It is the same for many microorganisms and the only way is to develop skills to cultivate – which is a continuous process and done during the site investigations as well as during other investigations of our laboratory.

The MPN signature is very reproducible as presented in Appendix 3. The percentage cultivated during the site investigations varies from more than 10% down to about 0.001%, i.e. a 10,000 times range. At the MICROBE site, the two boreholes studied reflect the upper and mid MPN number of this range. In KJ0052F01, about 5% of the ATP number is cultivated, while only 0.2% is cultivated from KJ0052F03. The borehole sections reported here were all below the mid range (Table A1-6).

Each separate MPN analysis (Figure 6-2) is briefly commented below. Detailed discussion and modeling of relations to depth, hydrology, geology and geochemistry is performed elsewhere and is not dealt with here.

6.2.1 Nitrate reducing bacteria

This analysis is more sensitive to sampler contaminations than any of the other MPN analyses. This is because nitrate is the most favourable electron acceptor next to oxygen for bacteria. Facultative anaerobic bacteria generally can switch from oxygen to nitrogen when oxygen disappears. It is possible that contaminating microorganisms show up here and it is, therefore, very important that the decontamination protocols are efficient. The sample from KFM07A 848–1,001.6 m was analysed for cultivable aerobic heterotrophic bacteria (CHAB) (confer Appendix 2). Apparently, the NRB detected in this sample was not contaminating bacteria, because the CHAB was at the detection limit of 10 CHAB ml^{-1}.

This analysis is new and therefore, it is premature to draw general conclusions from the result, else that that cultivable nitrate reducing bacteria are present in much larger amounts in KFM07A than any of the other analysed metabolic groups. This analysis is presently tested for reproducibility at the MICROBE site on Äspö, following the procedures presented in Appendix 3.

6.2.2 Iron and manganese reducing bacteria

Iron and manganese reducing bacteria are generally observed in larger numbers at shallow depths where the redox potential is above about –200 mV. The data obtained here follows that trend and the deepest, most saline and reduced borehole section did not have IRB above the detection limit.

6.2.3 Sulphate reducing bacteria

The numbers of sulphate reducing bacteria (SRB) are somewhat lower than expected. It may indicate that stagnant groundwater has been sampled. In such groundwater, the renewal rate of organic carbon will be very slow, resulting in very low numbers of SRB.

6.2.4 Acetogens

This group of microorganisms produces acetate from small organic compounds (one carbon) or from hydrogen and carbon dioxide. They have always been detected during the site investigations and in deep groundwater from Olkiluoto. This is consequently a very versatile and common group, present in the boreholes investigated here, with one exception for the deepest section.

6.2.5 Methanogens

This group of microorganisms produces methane from small organic compounds (one carbon) and acetate or from hydrogen and carbon dioxide. They are commonly detected above the detection limit during the site investigations. However, they have only been sparsely detected (except for KFM03A 448-453 m) in the Forsmark area in earlier investigated boreholes /12, 13, 14/ (KFM01A, 02A and 03A) and that trend is sustained also for the sections investigated here.

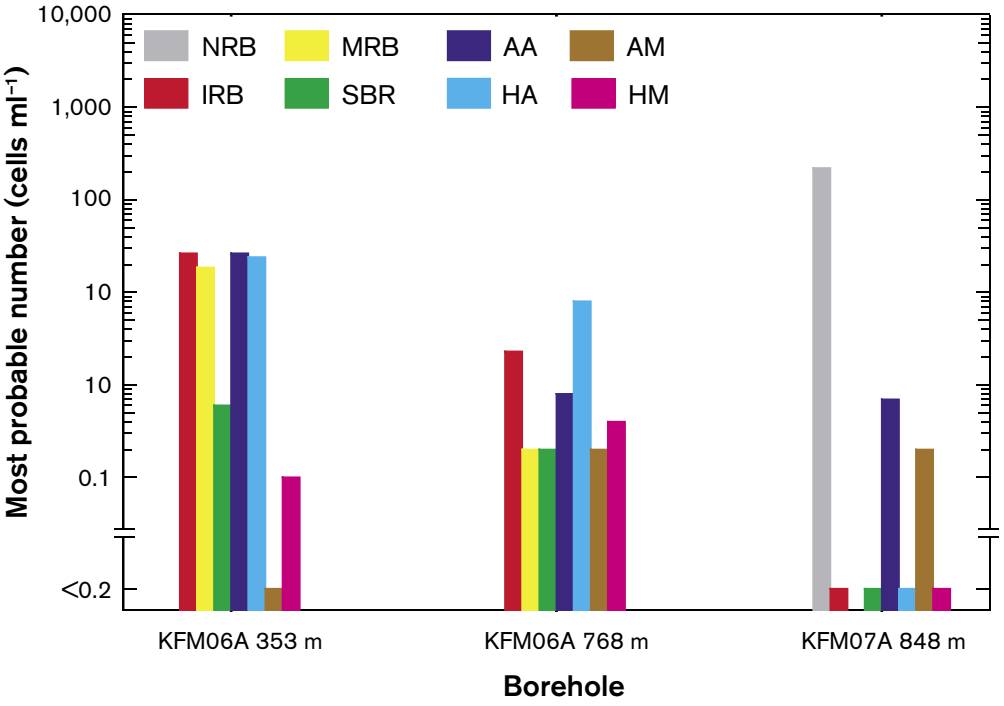

Figure 6-2. *Most probable number (MPN) of analysed physiological groups in KFM06A and KMF07A. MRB was not analysed for KFM07A. Abbreviation: NRB (Nitrate Reducing bacteria), MRB (Manganese Reducing bacteria), AA (Autotrophic Acetogens), AM (Autotrophic Metanogens), IRB (Iron Reducing Bacteria), SRB (Sulphate Reducing Bacteria), HA (Heterotrophic Acetogens), HM (Heterotrophic Metanogens).*

7 Conclusions

- The present use of 70% ethanol for decontamination should be replaced by decontamination with 10 ppm chlorine dioxide after a steam cleaning of the PVB sampler.
- The analytical methods have been demonstrated very reproducible and reliable.
- MPN measurements give a reproducible signature of representative metabolic groups which relates to dominating processes in the investigated section.
- The total numbers of microorganisms in the analysed borehole sections were between 10^4 and 10^5 cells ml^{-1}, which is in the average range of what is usually found in deep groundwater.
- The introduction of ATP measurements demonstrated that 10% or more of the total numbers (AODC) obtained is biologically active.
- The MPN numbers obtained for KFM06A and KMF07A ranged from below detection (0.2 cells ml^{-1}) up to 30 cells ml^{-1}, which is in the lower regime compared to the typical range covered by this analysis.

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Appendix 1

Data

Table A1-1. Total number of cells and concentration of ATP in the analysed sections of KMF06A and KMF07A.

Borehole (section m)	Total counts (cells ml^{-1})			ATP amol ml^{-1}		
	AODC	Standard deviation	Number of observations	ATP	Standard deviation	Number of observations
KFM06A:1 (353.5–360.6)	7.2×10^{4}	$\pm 1.7\times10^{4}$	6	1.5×10^{4}	$\pm 6.7\times10^{2}$	3
KFM06A:2 (353.5–360.6)	5.2×10^{4}	$\pm 1.7\times10^{4}$	6	0.9×10^{4}	$\pm 5.2\times10^{2}$	3
KFM06A (768.0–775.1)	1.7×10^{4}	$\pm 0.3\times10^{4}$	6	0.37×10^{4}	$\pm 1.7\times10^{3}$	3
KFM07A (848.0–1,001.6)	6.0×10^{4}	$\pm 0.5\ 10^{4}$	6	$0.51\text{x}\ 10^{4}$	$\pm 0.5\times10^{4}$	3

Table A1-2. Most probable number (MPN) of metabolic groups of microorganisms in KMF06A:1, section 353.5–360.6 m.

Metabolic groups	Cells ml^{-1}	
	MPN	Lower – upper 95% confidence limits
Iron reducing bacteria	30	10–120
Manganese reducing bacteria	13	5–39
Sulphate reducing bacteria	0.8	0.3–2.4
Autotrophic acetogens	30	10–130
Heterotrophic acetogens	24	10–94
Autotrophic methanogens	< 0.2	–
Heterotrophic methanogens	0.2	0.1–1.7

Table A1-3. Most probable number (MPN) of metabolic groups of microorganisms in KMF06A:2, section 353.5–360.6 m.

Metabolic groups	Cells ml^{-1}	
	MPN	Lower – upper 95% confidence limits
Iron reducing bacteria	23	9–86
Manganese reducing bacteria	24	10–94
Sulphate reducing bacteria	0.4	0.1–1.7
Autotrophic acetogens	24	10–94
Heterotrophic acetogens	24	10–94
Autotrophic methanogens	0.2	0.1–1.1
Heterotrophic methanogens	0.4	0.1–1.7

Table A1-4. Most probable number (MPN) of metabolic groups of microorganisms in KMF06A, section 768.0–775.1 m.

Metabolic groups	Cells ml^{-1}	
	MPN	Lower – upper 95% confidence limits
Iron reducing bacteria	2.3	0.9–8.6
Manganese reducing bacteria	0.2	0.1–1.1
Sulphate reducing bacteria	0.2	0.1–1.1
Autotrophic acetogens	0.8	0.3–2.4
Heterotrophic acetogens	8	3–25
Autotrophic methanogens	< 0.2	–
Heterotrophic methanogens	< 0.2	–

Table A1-5. Most probable number (MPN) of metabolic groups of microorganisms in KMF07A, section 848.0–1,001.6 m.

Metabolic groups	Cells ml^{-1}	
	MPN	Lower – upper 95% confidence limits
Nitrate reducing bacteria	220	100–580
Iron reducing bacteria	< 0.2	–
Manganese reducing bacteria	–*	–
Sulphate reducing bacteria	< 0.2	–
Autotrophic acetogens	0.7	0.2–2.1
Heterotrophic acetogens	< 0.2	–
Autotrophic methanogens	0.2	0.1–1.1
Heterotrophic methanogens	< 0.2	–

* not analysed due to a nonconformity.

Table A1-6. The percentage of the total number of cells (Table A1-1) cultured with MPN (Table A1-2 to A1-5) in KMF06A and KFM07A.

Borehole (section, m)	Cells cultured (%)	
	MPN/AODC	MPN/ATP
KFM06A:1 (353.5–360.6)	0.13	0.64
KFM06A:2 (353.5–360.6)	0.18	0.63
KFM06A (768.0–775.1)	0.06	0.12
KFM07A (848.0–1,001.6)	0.0015*	0.018*

* For consistency, the MPN of nitrate reducing bacteria is omitted from this calculation.

Appendix 2

Control of disinfection procedure

Background

The decontamination procedures for the POSIVA OY forerunner to the PVB sample system, the PAVE sample system, was tested 1998 /1/. The PAVE was rinsed with a 10 ppm chlorine solution. Subsequently, it was filled with de-ionized, filtered water (ELGA). The ELGA water was sampled and inoculated into MPN tubes. Growth was not detected. The decontamination procedures were deemed satisfactory.

A program was set up for testing the PVB sampler in a way, similar to the PAVE test briefly described above. The decontamination procedure in use for the PVB sample system was applied. This test was made in two steps:

1. A tube that simulated a borehole was constructed at the SKB instrument storage in Oskarshamn. A PVB system from the storage was decontaminated with 70% ethanol, flushed with sterile de-ionised water and finally filled with sterile de-ionized water and sampled.
2. The procedure in 1 above was repeated at the site in Forsmark directly after retrieval of the PVB system from a borehole. This was to test that the decontamination procedures were satisfactory also with a newly retrieved sampler from deep ground water.

Equipment disinfection test

Set-up

A tube that simulates a borehole was constructed (Figure A2-1A) with a valve in one end (Figure A2-1B). The valve was attached to a typical packer pressure tank (Figure A2-1C) which was filled with sterile water after careful disinfection with 70% ethanol. The PVB sampler was sterilised with 70% ethanol and installed in the borehole tube. A packer was attached to the lower end of the PVB sample unit (Figure A2-1D, E). Finally, the PVB sample unit was installed in the borehole tube and the packer was inflated using a hydraulic pump (Figure A2-1F). Then, water was passed through the PVB-shell through the PVB samplers 1 and 2 and then out. One tank volume was allowed to flow through. After a second tank volume was passed, the PVB samplers were closed and brought back to the laboratory for analysis. This activity was performed 2003-08-12.

Results and conclusions

All MPN analyses were negative, i.e. < 0.2 cells ml^{-1} for iron, manganese and sulphate reducing bacteria, autotrophic and heterotrophic acetogens, autotrophic and heterotrophic methanogens. The total numbers decreased somewhat from flush water to PVB:2 (Figure A2-2). The numbers of cultivable bacteria increased in numbers from flush to PVB:2.

It was expected that the MPN results should be negative. The microbes analysed in the MPN are generally sensitive to oxygen, and this test was run under oxygenic conditions to simulate normal decontamination in air. The total numbers varied (Figure A2-2), but this test gives information about the total amount of living and dead microbes. The other test of counting cultivable bacteria (CHAB) showed numbers between 300 and 900 cells

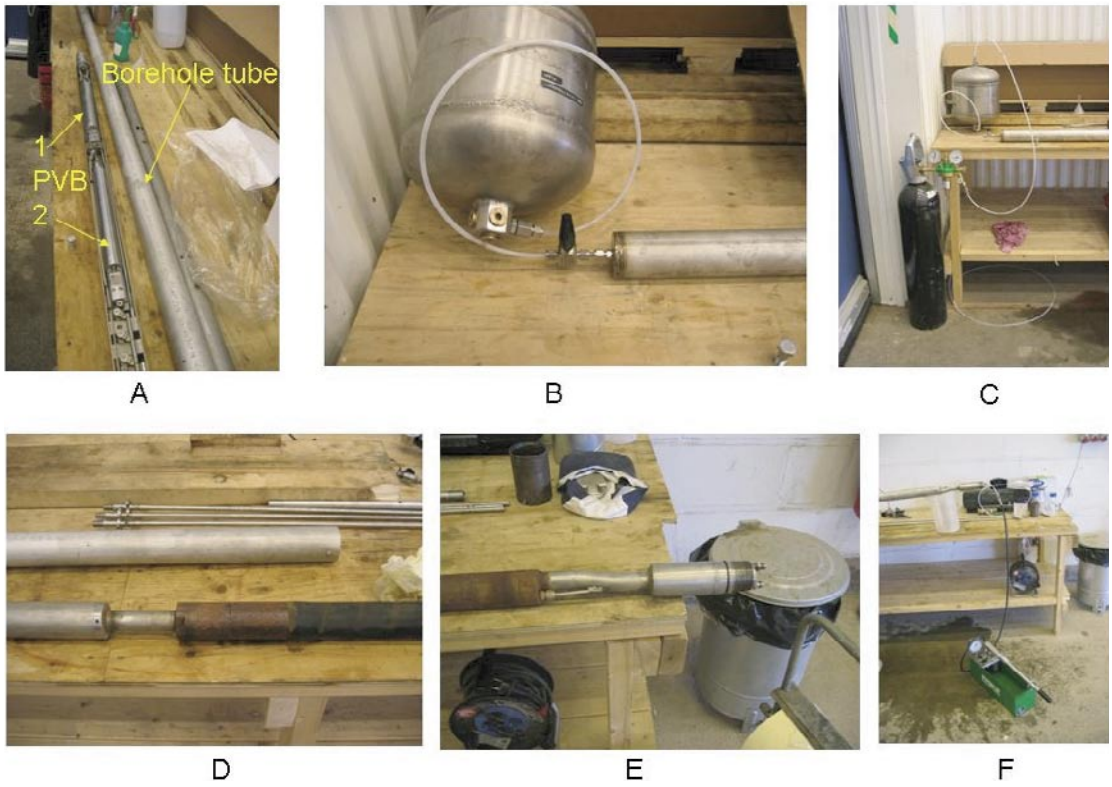

Figure A2-1A–F. *The setup of the decontamination test. See text for details.*

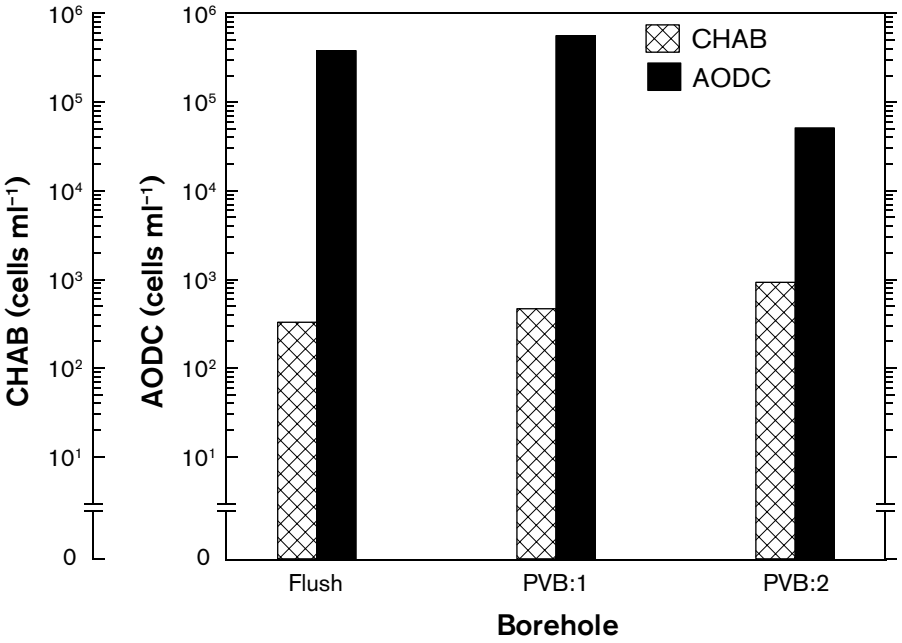

Figure A2-2. *Total number of cells (AODC) and the number of cultivable, heterotrophic, aerobic cells (CHAB) in the analysed PVB samplers.*

per ml. This is not perfect, but it is within the recommendation for large systems like this one. It is very difficult to totally sterilise this equipment with ethanol. There may always be small cavities and places where microbes survive. The decontamination procedure was set-up and performed exactly as done in the field. New tests using chlorine dioxide as used for the PAVE tests, and improved cleaning procedures, may give a better result with less microbes, but that disinfectant has yet not been used at the SKB drill sites. However, the most important tests, the MPN tests, were all negative.

Disinfection in situ test

Set-up

The test described above was applied again in Forsmark 2004-05-12. This time, a newly used PVB system was tested. This system had recently been exposed for deep groundwater, and could possibly have carried microbes that would grow in the MPN analysis.

Results and conclusions

All MPN analyses were again negative, i.e. < 0.2 cells ml^{-1}. The total numbers were lower than in the equipment test (Figure A2-3). The CHAB was about equal to the equipment test. As for the equipment test, the results are below recommended levels for drill water contamination (1,000 cells ml^{-1}). However, the results from the PAVE tests show that a better decontamination agent (chlorine dioxide instead of ethanol) and cleanness procedures will give more satisfactory results with very low numbers of contaminating microbes.

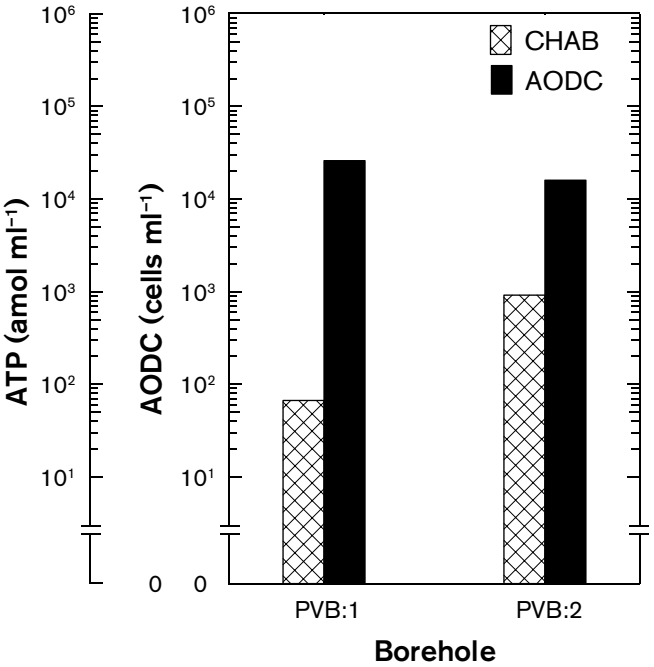

Figure A2-3. *Total number of cells (AODC) and the number of cultivable, heterotrophic, aerobic cells (CHAB) in the analysed PVB samplers.*

Reference

/1/ **Haveman S H, Pedersen K, Routsalainen P, 1999.** Distribution and metabolic diversity of microorganisms in deep igneous rock aquifers of Finland. Geomicrobiol. J. 16, 277–294.

Appendix 3

Reproducibility tests

Reproducibility over samples

KFM06A 353.5–360.6 in Forsmark was sampled 2005-03-14 with two PVB-samplers installed at the same location. This was done to test the reproducibility of the sampling and analysis procedures. Table A3-1 and Figure A3-1 show the results. The MPN analyses reproduced very well. Maximum discrepancy between the samples was equal to a factor of two (Table A3-1), which is well within the 95% confidence intervals of the MPN analysis (confer Tables A1-2 and A1-3). The total number determinations and the ATP analysis also showed excellent reproducibility, as depicted in Figure A3-2.

In conclusion, this test demonstrated remarkable reproducibility over samples. This includes the sampling procedure, transportation logistics and inoculation, cultivation and analysis of the MPN of respective physiological group of microorganisms. The total number determination and the ATP analysis showed similarly an eminent reproduce-bility.

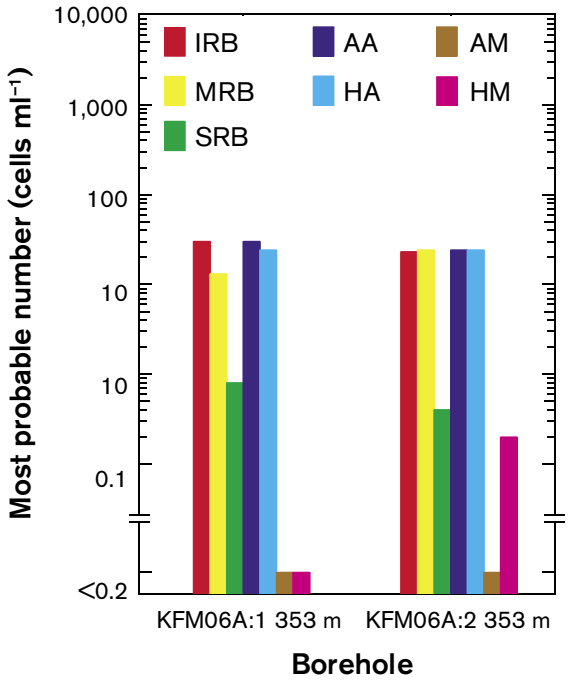

Figure A3-1. *MPN of analysed physiological groups in KFM06A 353.5–360.6 m using two PVB-samplers in the same sampling location, 2005-03-14.*

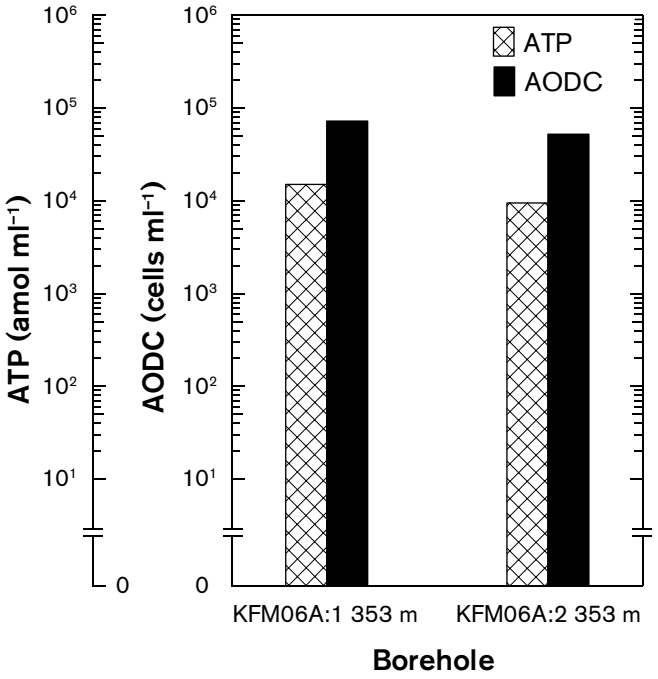

Figure A3-2. *Total number of cells and concentration of ATP in KFM06A 353.5–360.6 m using two PVB-samplers in the same sampling location, 2005-03-14.*

Table A3-1. The most probable number of analysed physiological groups of microorganims in two different PVB-samplers, taken at the same location in KFMA06 353.5–360.6 m, 2005-03-14.

Metabolic groups	MPN (cells ml^{-1})		
	KFM06A:1	KFM06A:2	KFM06A:1/KFM06A:2
Iron reducing bacteria	30	23	1.3
Manganese reducing bacteria	13	24	0.54
Sulphate reducing bacteria	0.8	0.4	2
Autotrophic acetogens	30	24	1.3
Heterotrophic acetogens	24	24	1
Autotrophic methanogens	< 0.2	0.2	< 1
Heterotrophic methanogens	0.2	0.4	0.5

Reproducibility over time

Two boreholes, KJ0052F01 and KJ0052F03, at the MICROBE site /1/ in the Äspö tunnel were sampled at two different occasions to test the reproducibility of the MPN procedure over time. The MICROBE site is situated 450 m underground in the Äspö hard rock laboratory. This site is stable with respect to the groundwater composition over months, although it is drifting towards a deeper signature over years due to up-coning of deep groundwater to the tunnel /1/.

PVB samplers were attached to respective circulation from each borehole and groundwater was circulated overnight at in situ pressure, temperature and chemistry. In the early morning, they were closed, detached and transported to the laboratory in Göteborg. Analysis started the same afternoon, before 14:00. This procedure is in all parts similar to the

sampling of site investigation boreholes, except for the fact that here, personnel is standing next to the PVB samplers and they are operated manually with an adjustable spanner and not from the ground surface.

This test explores the reproducibility of two completely different analytical rounds with in several cases different personnel involved and whole new sets of chemicals and media.

There was a very good reproducibility between the samples for each borehole (Figure A3-3). The reproducibility was however not as good as for the over sample test. There are several possible reasons for this. The main uncertainty is the groundwater conditions. It was not possible to control the stability of the microbial populations in the boreholes. Rather, it was anticipated that a stable situation was prevailing. This seems also to have been the case. However, at the last sampling time, it was revealed that drilling for an experiment at Äspö, called MINI-CAN, had punctured the groundwater zone from which MICROBE extracts its groundwater. A pressure drop of 0.3 MPa and a large drain of groundwater at the MINI-CAN site was the result. This disturbance started in the end of January 2005, meaning that the effect on the MICROBE site populations probably was limited at the time for the second sampling. Still, some of the observed discrepancy may be due to this disturbance.

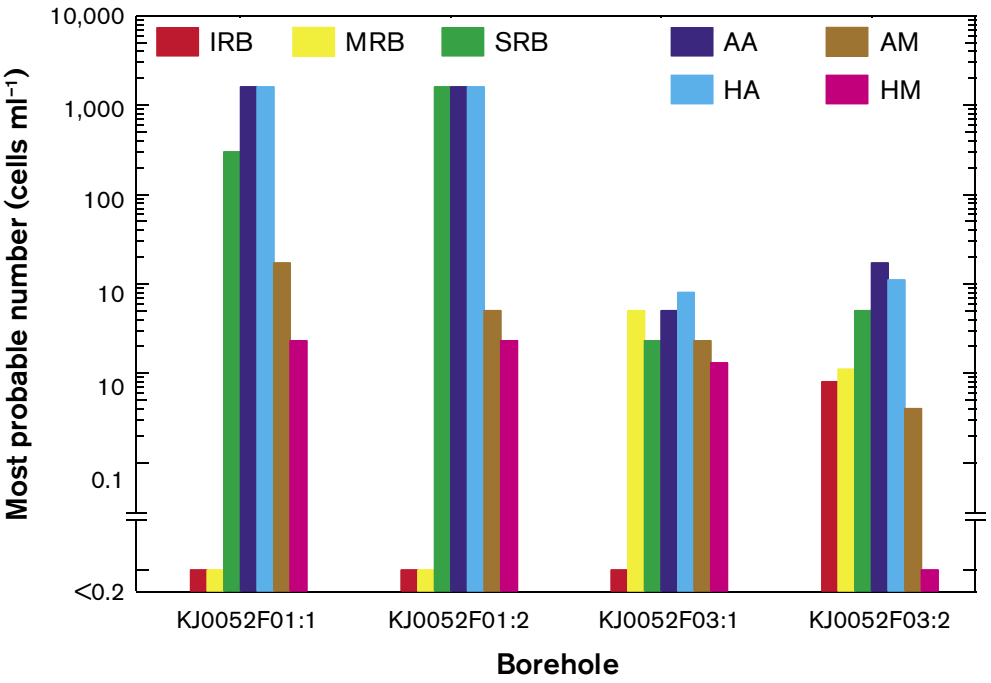

Figure A3-3. *MPN of analysed physiological groups in two boreholes at the MICROBE site at two different occasions, 2004-10-26 (1) and 2005-02-09 (2). Abbreviations: MRB (Manganese Reducing bacteria), AA (Autotrophic Acetogens), AM (Autotrophic Metanogens), IRB (Iron Reducing Bacteria), SRB (Sulphate Reducing Bacteria), HA (Heterotrophic Acetogens), HM (Heterotrophic Metanogens).*

In this over time test, there was a long time passing between the tests, 3.5 months, and there was a possible disturbance from the MINI-CAN drilling and finally, there was most probably a general variability in biological populations present in the sampled aquifers. Taking all those possible factors of variability into consideration, it must be concluded that the over time test did extremely well. The MPN procedures and the analyses appear to be very robust and reproducible both over samples as demonstrated in Appendix 2 and over sample times as demonstrated here.

Reference

/1/ **Pedersen K, 2005.** The MICROBE framework: Site descriptions, instrumentation, and characterization Äspö Hard Rock Laboratory. International Progress Report IPR-05-05, pp 1–85. Svensk Kärnbränslehantering AB.