P-07-53

Forsmark site investigation

Numbers, viability, and metabolic diversity of microorganisms in groundwater from boreholes KFM01D and KFM08A

Results from section 683.5–690.6 m in KFM08A and sections 428.5—435.6 and 568.0—575.0 m in KFMA01D

Karsten Pedersen, Microbial Analytics Sweden AB

March 2007

Svensk Kärnbränslehantering AB
Swedish Nuclear Fuel
and Waste Management Co
Box 5864
SE-102 40 Stockholm Sweden
Tel 08-459 84 00
+46 8 459 84 00
Fax 08-661 57 19
+46 8 661 57 19

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Abstract

The geochemical characterisation of groundwater is included in the site investigation of Forsmark, and microbial numbers and processes are part of in this characterisation. The microbial investigation consists of determining the total numbers of microorganisms, the concentration of adenosine-tri-phosphate (ATP), and the number of culturable heterotrophic aerobic bacteria (CHAB); also included is a method to determine the numbers of organisms that belong to different physiological groups, the most probable number (MPN) method. This investigation included eight different groups, namely nitrate-, iron-, manganese-, and sulphate-reducing bacteria, auto- and heterotrophic acetogens, and auto- and heterotrophic methanogens. The reproducibility of the MPN method has been tested using groundwater from a depth of 450 m at the Äspö Hard Rock Laboratory and was found to be excellent.

Groundwater samples were taken from boreholes KFM08A – section 683.5–690.6 m, KFM01D – section 428.5–435.6 m, and KFM01D – section 568.0–575.0 m. The acridine orange direct count (AODC) test indicated that the total number of cells was higher in groundwater from KFM01D than from KFM08A. The numbers found in groundwater from KFM01D were the highest so far found in a total of 14 analysed samples from different borehole sections in the Forsmark area; in contrast, groundwater from the analysed section in KFM08A had the fourth lowest number. A high amount of ATP per cell indicates high activity and large cells. The average of all ATP/AODC ratios ($n \cong 100$) in deep groundwater has been determined to be 0.43. It was higher than this average value in KFM01D but very low in KFM08A. The sample from the KFM01D-568 m section had the highest ATP/AODC ratio so far found in Forsmark. Groundwater from this section had the highest values for most of the bacteriological parameters analysed, suggesting that the microorganisms in the groundwater from this section were more viable and active than those in groundwater from the two other sections studied.

The CHAB determinations indicated an increasing range of numbers consistent with increasing ATP and AODC values: 2,100 cells mL^{-1} in section KFM08A-683 m, 3,300 cells mL^{-1} in KFM01D-428 m, and 32,000 cells mL^{-1} in KFM01D-568 m. High numbers of CHAB, above 1,000 cells mL^{-1}, are rare in pristine deep groundwater, and may indicate a contamination problem. Such contamination can be caused by 1) a drawdown of shallow water while pumping the section, 2) drill water with high numbers of CHAB, 3) drill water with a high concentration of organic carbon (> 5 mg L^{-1}), which may support the growth of CHAB, and 4) a combination of the previous three cases. The groundwater in borehole KFM01D had higher levels of CHAB than did KFM08A, with a very high number in the sample from the deepest section. It is not possible to properly evaluate the reasons for these high numbers of CHAB here, as the control for contaminating bacteria in the drill water was abandoned in Forsmark after drilling KFM06A. However, indications of a drawdown to the deepest section in KFM01D have been noted, and the drill water had high TOC values of approximately 7 mg L^{-1}. It is thus probable that a combination of contamination problems did generate the high numbers of CHAB found in KFM01D.

The percentages of the AODC culturable using MPN during the site investigation in Forsmark ranged from 0.0015% to 36.6%; the borehole sections reported here were in the 0.13–36.6% range. The numbers of NRB were significantly lower than those of CHAB in KFM08A and KFM01D-428 m but much higher than the CHAB in KFM01D-568 m. This number correlates with the high number of CHAB, thus providing an additional indication of surface-related contamination of the groundwater in this section. The numbers of sulphate-reducing bacteria (SRB) in the sample from KFM08A were the second highest of all obtained numbers (14 data points) from Forsmark. The highest numbers of SRB were found in groundwater from the KFM01D-568 m section, where the number of acetogens was very high as well. Acetogens are very versatile and common in shallow and deep groundwater. They were present in the groundwater investigated here in numbers that were the highest detected in Forsmark. Methanogens

have commonly been found above the detection limit in the Forsmark and Oskarshamn site investigations. However, they were only sparsely detected in groundwater from the Forsmark area in the earlier-investigated KFM01A, 02A, 03A, and 06A boreholes; this finding was upheld in the sections investigated here, all of which were below the detection limit.

Sammanfattning

I den geokemiska karakteriseringen av grundvatten i samband med platsundersökning i Forsmark ingår en mikrobiell del. Denna del omfattar bestämning av den totala mängden mikroorganismer (AODC), mängd adenosintri-fosfat (ATP), antalet odlingsbara heterotrofa aeroba bakterier (CHAB) samt en metod för analys av fysiologiska grupper av mikroorganismer. Metoden kallas ”most probable number” (MPN). I MPN-analysen ingick de åtta olika grupperna nitrat-, järn-, mangan- och sulfat-reducerande bakterier, auto- och heterotrofa acetogener samt auto- och heterotrofa metanogener. Metodens reproducerbarhet har befunnits utmärkt vid tester på grundvatten från 450 m djup vid Äspölaboratoriet.

Provtagningarna gjordes i följande tre borrhålssektioner KFM08A: 683,5–690,6 m, KFM01D, 428,5–435,6 m och KFM01D, 568,0–575,0 m. Totalantalet celler var högre i grundvatten från KFM01D än i vatten från KFM08A. Totalantalet i de två proverna från KFM01D var högst av alla hittills analyserade grundvattenprov i Forsmarkområdet. Provet från KFM08A hade däremot det fjärde lägsta antalet. En förhållandevis stor mängd ATP per cell tyder på hög aktivitet och på att cellerna är stora. Medelvärdet av förhållandet mellan ATP och totalantalet celler i alla analyserade djupa grundvatten (N ≅ 100) är bestämt till 0,43. Detta förhållande var högre än medelvärdet i proverna från KFM01D men lågt i KFM08A. Provet från KFM01D-568 m hade dessutom det högsta medelvärdet av alla hittills analyserade prov i Forsmark. Grundvattnet från den här sektionen hade också de högsta värdena för merparten av alla övriga analyserade bakteriologiska parametrar, vilket indikerar att mikroorganismerna i denna sektion var mer aktiva än mikroorganismerna i grundvattnet från de andra två sektionerna.

Bestämningar av odlingsbara aeroba heterotrofa bakterier (CHAB) korrelerar positivt med värdena på ATP och AODC enligt: 2 100 celler mL^{-1} i KFM08A-683 m, 3 300 celler mL^{-1} i KFM01D-428 m och 32 000 celler mL^{-1} i KFM01D-568 m. Höga antal av CHAB, över 1 000 celler mL^{-1}, är sällsynta i djupa grundvatten och kan därför betraktas som en indikator på ett kontaminationsproblem. Det kan vara orsakat av att 1) ytligt grundvatten dras ner mot den pumpade sprickan, 2) ett spolvatten med ett högt antal CHAB, 3) ett spolvatten med en hög halt organiskt material (> 5 mg L^{-1}) som kan främja tillväxt av CHAB, 4) en kombination av alla tre orsaker. Borrhålet KFM01D hade högre CHAB än KFM08A och antalet var mycket högt i grundvatten från den djupaste sektionen. Det är inte möjligt att fullt ut avgöra vilken eller vilka av orsakerna som låg bakom de höga antalen av CHAB. Det beror på att rutinmässig bakteriologisk kontroll av spolvatten inte utfördes på spolvattnet under borrningen av KFM08A och KFM01D. Emellertid observerades en ovanligt hög avsänking av grundvattennivån under pumpning av den djupaste sektionen i KFM01D och spolvattnet vid borrning hade en hög TOC-halt på omkring 7 mg L^{-1}. Det är således troligt att det fanns ett eller flera kontaminationsproblem som orsakade de höga antalen CHAB i framförallt KFM01D-568 m.

Procentsatsen av totalantalet bakterier (AODC) som har odlats med MPN-metoden under platsundersökningarna i Forsmark (14 prov) varierar från 0,0015 % upp till 36,6 %. De prov som rapporteras här låg i området mellan 0,13 % och 36,6 %. Antalet nitratreducerande bakterier (NRB) var signifikant lägre än CHAB, utom i KFM01D-568 m, där antalet låg i samma höga område som NRB vilket stärker antagandet att denna sektion hade problem med kontamination av grundvatten från ytliga system.

Antalet sulfatreducerande bakterier (SRB) var näst högst i KFM08A av alla analyserade prov (totalt 14 stycken) från Forsmark. Det högsta antalet SRB uppmättes i grundvatten från KFM01D-568 m där antalet acetogener också var mycket högt. Acetogener är en mycket anpassningsbar och vanlig grupp bakterier. De förekom i de här analyserade och rapporterade proverna i de högsta antal hittills uppmätta i Forsmark. Metanogener påträffas i de flesta djupa grundvatten. I Forsmark förekom de dock bara sparsamt i grundvatten från de tidigare undersökta borrhålen KFM01A, 02A, 03A och 06A. Den trenden vidhölls även i grundvattnet från de aktuella borrhålen och borrhålssektionerna och förekomsten av metanogener låg i samtliga fall under detektionsgränsen.

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1 Introduction

This document reports the performance and results of microbiological investigations in boreholes KFM08A and KFM01D as part of the site investigation programme in Forsmark /1/. Microbiological data from groundwater from the following three borehole sections are presented:

- KFM08A, 683.5–690.6 m
- KFM01D, 428.5–435.6 m
- KFM01D, 568.0–575.0 m

The sampling was carried out in November 2005 and in June and August 2006 as part of the hydrochemical characterisation activities for KFM08A and KFM01D, according to the AP PF 400-05-063 and AP PF 400-06-053 activity plans, respectively (SKB internal control documents; see Table 1-1). The sampling process and the down-hole sampling equipment are described elsewhere /2, 3/. Subsequent laboratory work was intermittently performed over the 12 weeks after the samples reached the laboratory AP PF 400-05-066.

The flushing water used when core drilling the boreholes may have caused contamination with foreign bacteria, thus affecting the in situ microbiological conditions. Proper routine control of the microbe content of the flushing water requires analysis of culturable bacteria and ATP twice while drilling a deep borehole approaching a depth of 1,000 m. The microbe content of the flushing water was, however, not determined while drilling the boreholes analysed and reported here. It was concluded that the results for the previously reported borehole, KFM06A /4/, convincingly indicated that the cleaning procedure works well, so it was decided that microbe analysis during drilling was no longer necessary /2/.

Original data from the reported activity are stored in the primary data base SICADA where they are traceable by the activity plan number (AP PF 400-05-066). Only data in databases are accepted for further interpretation and modelling. The data presented in this report are regarded as copies of the original data. Data in the databases may be revised, if needed. Such revisions will not necessarily result in a revision of the P-report. Minor revisions are normally presented as supplements, available at www.skb.se.

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

Activity plan	Number	Version
Fullständig kemikaraktärisering med mobilt fältlaboratorium i KFM08A	AP-PF-400-05-063	1.0
Fullständig kemikaraktärisering med mobilt fältlaboratorium i KFM01D	AP-PF-400-06-053	1.0
Undersökning av mikroorganismer i KFM07A, KFM08A och KFM01D	AP-PF-400-05-066	1.0

2 Objective and scope

The presence of microorganisms has been demonstrated in every investigated groundwater from Fennoscandian shield rocks, from depths ranging from the surface to 1,700 m /5/. Active microorganisms influence the groundwater geochemistry /6/ and redox potential /7/. Therefore, a full understanding of geochemical conditions in deep groundwater requires knowledge of the presence, diversity, and activity of microorganisms. In their metabolisms, microorganisms oxidise electron- and energy-rich compounds with a variety of electron acceptors. The preferred electron acceptor of many, but far from all, microorganisms is oxygen. This is why the oxygen content of groundwater diminishes rapidly with depth: it is continuously being reduced by microorganisms, organic carbon from surface ecosystems being the electron donor. Once oxygen is consumed, the next group of microorganisms is the nitrate-reducing bacteria, which will be active until the system is depleted of nitrate. Thereafter, manganese and/or iron reducers will flourish. These groups use ferric iron and manganese oxides as electron acceptors. The last group of respiring organisms, to which all of the above microorganisms above belong, is the sulphate-reducing bacteria; they reduce sulphate to sulphide in their metabolisms. The energy and electron donors in these metabolisms are organic material that eventually becomes oxidised to carbon dioxide. Concomitant with aerobic and anaerobic respiration, fermenting organisms degrade organic material without the use of an external electron acceptor. These organisms split organic molecules into one or more reduced species and one or more oxidised species. The oxidised compounds can be organic acids, ketones, and carbon dioxide, while the reduced species can be alcohols, and, more commonly, gaseous hydrogen. Hydrogen can be used as an energy and electron source by autotrophic methanogens and acetogens. Methanogens oxidise hydrogen gas and reduce carbon dioxide to produce methane; acetogens convert the same compounds to acetate. In addition, heterotrophic methanogens and acetogens can utilise organic one-carbon compounds, such as methanol and methylamine, as well as the two-carbon compound acetate. The major objective here was to enumerate all physiological groups of microorganisms that, through their growth and metabolising activities, may influence groundwater geochemistry.

The microbial communities occurring in granitic rock from the surface to a depth of at most 1,700 m, have been studied for almost two decades /5/. It has been found that the total numbers of microbial cells in granitic groundwater range from 10^6 mL^{-1} in shallow waters to 10^4 mL^{-1} at greater depths, down to approximately 1,000 m. It has also been demonstrated that specific groups of microorganisms in deep groundwater can utilise all the electron acceptors mentioned above /6/. These results have been used to formulate a conceptual model of microbially catalysed geochemical reactions in granitic groundwater in the Fennoscandian shield. An important objective of this investigation was to enumerate the different microorganisms present in the analysed boreholes.

The microbiological analysis programme reported here was carried out according to protocols developed in previous investigations of Finnish groundwater /8, 9/. These protocols cover the determination of the total number of cells in groundwater (AODC), number of culturable, heterotrophic aerobic bacteria (CHAB), concentration of adenosinetri-phosphate (ATP), and a statistical cultivation method for estimating the most probable number (MPN) of culturable metabolic groups of microorganisms. 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 from each borehole section /2, 3/ and sent to the laboratory in Göteborg within 4−6 h; subsampling for analysis was performed immediately at arrival of the PVB sampler.

3 Equipment and methods

3.1 Equipment for transfer of samples from the PVB sampler

The transfer of samples from the PVB sampler to the culturing tubes required a procedure that did not expose the samples to oxygen. This was done using a specially designed adapter (no. 4 in Figure 3-1) that could be attached to the PVB sampler (no. 3 in Figure 3-1). Sample portions 10-mL in size were distributed to nitrogen-flushed anaerobic tubes via butyl rubber stoppers, as indicated by no. 5 in Figure 3-1. The pressurised PVB sampler automatically ejected the sample when the sampling valves were opened (nos. 6 and 7 in Figure 3-1).

3.2 Equipment for most probable number determination

Preparing anaerobic media required an anaerobic box and a gas bench for mixing and delivering gas mixtures and gases for growth, as described in detail in the activity plan. Typically, preparing one sample for delivery required the equivalent of approximately two weeks of full-time laboratory work. Diluting and inoculating samples for the analysis of metabolic groups followed a well-defined procedure, depicted in Figure 3-2. One set of 30–45 tubes was used for each analysis, and incubation was done at approximately 17°C. Finally, each tube was analysed

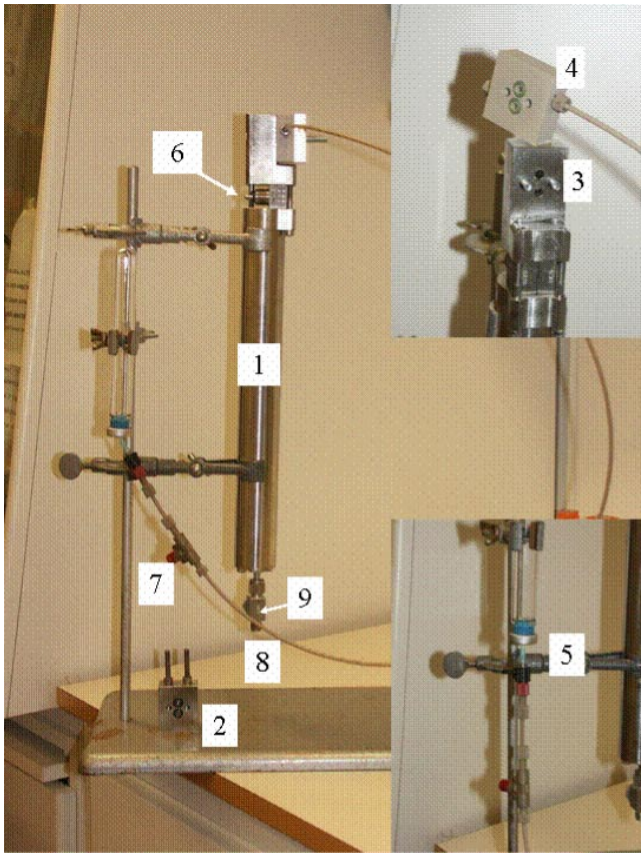

Figure 3-1. *This setup was designed for the oxygen-free transfer of samples from the PVB sampler (1) to nitrogen-flushed, anaerobic tubes stoppered with butyl rubber stoppers (5). (1) PVB sampler, (2) transportation seal, (3) inlet/outlet of the PVB, (4) PEEK sampling device, (5) transfer of sample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

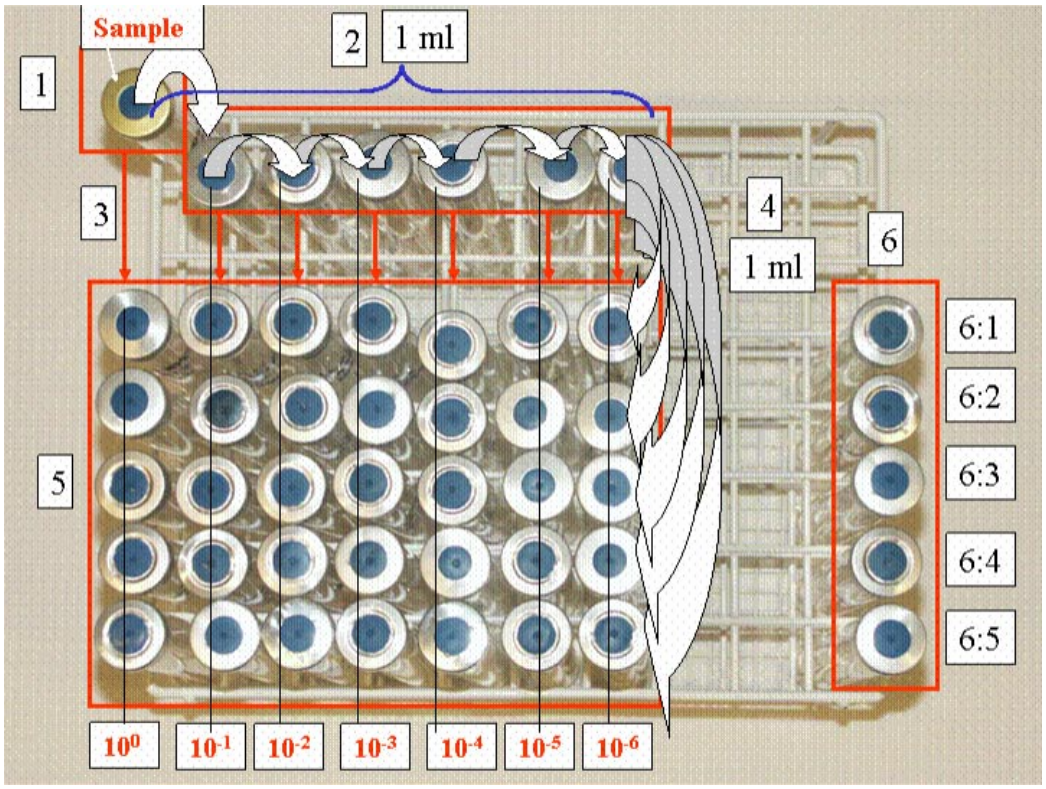

Figure 3-2. *The procedure for most probable number determination. The tube containing the sample is used as the inoculation source (1). Serial dilution is performed first (2); thereafter, subsamples are transferred (3−4) to the growth tubes (5) and control tubes (6).*

for the consumption of the electron donor or the presence of metabolic products typical of the following cultivated metabolic groups: nitrate-reducing bacteria – consumption of nitrate, manganese-reducing bacteria – manganese(II), iron-reducing bacteria – ferrous iron, sulphate-reducing bacteria – sulphide, autotrophic and heterotrophic acetogens – acetate, and autotrophic and heterotrophic methanogens – methane.

3.3 Method for total number enumeration

The total number of cells was determined using the acridine orange direct count (AODC) method. All solutions used were filtered through sterilised 32-mm diameter, 0.2-µm pore size Filtropur S syringe filters (Sarstedt, Nümbrecht, Germany). Prior to filtration, stainless steel analytical filter holders, 13 mm (no. XX3001240; Millipore, Billerica MA, USA), were rinsed with sterile filtered 2% HCl in double-distilled water (DDW) for use with groundwater samples (to remove possible iron oxide precipitates) and with sterile filtered DDW for use with laboratory samples. Samples of 1 mL were suction filtered (−20 kPa) onto 0.22-µm pore size Sudan black-stained polycarbonate filters, 13 mm in diameter (Osmonics, Minnetonka, MN, USA). The filtered cells were stained for 5 min with 200 µL of an acridine orange (AO) solution (SigmaAldrich, Stockholm, Sweden). The AO solution was prepared by dissolving 10 mg of AO in 1 L of a 6.6 mM sodium potassium phosphate buffer (pH 6.7). The filters were mounted between microscope slides and cover slips using fluorescence-free immersion oil (Olympus). 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 (Axioskop 20, Carl Zeiss, City, Germany). Between 400 and 600 cells, or a minimum of 30 microscopic fields (1 field = 0.01 mm^2), were counted on each filter.

3.4 Method for cultivation of aerobic, heterotrophic bacteria

Petri dishes containing agar with nutrients were prepared for determining the CHAB. This agar contained 0.5 g L^{-1} of pepton (Merck), 0.5 g L^{-1} of yeast extract (Merck), 0.25 g L^{-1} of sodium acetate, 0.25 g L^{-1} of soluble starch (Merck), 0.1 g L^{-1} of K_2HPO_4, 0.2 g L^{-1} of $CaCl_2$ (Merck), 10 g L^{-1} of NaCl (Merck), 1 mL L^{-1} of trace element solution, and 15 g L^{-1} of agar (Merck). The medium was sterilised in 1-L batches by autoclaving at 121°C for 20 min; after this they were cooled to approximately 60°C in a water bath, and finally distributed in 20-mL portions in 9-cm-diameter plastic Petri dishes (Sarstedt, Landskrona, Sweden). Ten-times dilution series of culture samples were made in DDW with 0.9 g L^{-1} of NaCl; 0.1-mL portions of each dilution were spread with a sterile glass rod on NA plates in triplicate. The plates were incubated for between 5 h and 7 d at 20°C, after which the number of colony-forming units (CFU) was counted. Plates with between 10 and 300 colonies were counted.

3.5 Method for ATP determination

The ATP Biomass Kit HS (no. 266-311; BioThema AB, Handen, Sweden) was used to determine total ATP in living cells. Sterile, PCR Clean epTIPS with filters (Eppendorf, GTF, Göteborg, Sweden) were used in transferring all solutions and samples, to prevent ATP contamination of pipettes and solutions. Light may cause the delayed fluorescence of materials and solutions, so all procedures described below were performed in a dark room and all plastic material, solutions, and pipettes were stored in the dark. A new 4.0-mL, 12-mm diameter polypropylene tube (no. 68.752; Sarstedt AB, Landskrona, Sweden) was filled with 400 µL of the ATP kit reagent HS (BioThema, Handen, Sweden) and inserted into an FB12 tube luminometer (Sirius Berthold, Pforzheim, Germany). The quick measurement FB12/Sirius software, version 1.4 (Berthold Detection Systems, Pforzheim, Germany) was used to calculate light emission as relative light units per second (RLU s^{-1}). Light emission was measured for three 5-s intervals with a 5-s delay before each interval, and the average of the three readings was registered as a measurement. The background light emission (I_{bkg}) from the HS reactant and the tube was monitored and allowed to decrease to below 50 RLU s^{-1} prior to registration of a measurement. ATP was extracted from 100-µL aliquots of sample within 1 h of collection, by mixing for 5 s with 100 µL of B/S extractant from the ATP kit in a separate 4.0-mL polypropylene tube. Immediately after mixing, 100 µL of the obtained ATP extract mixture was added to the HS reactant tube in the FB12 tube luminometer, and the sample light emission (I_{smp}) was measured. Subsequently, a volume of 10 µL of an internal ATP standard was added to the reactant tube, and the standard light emission (I_{std}) was measured. The concentration of the ATP standard was 10^{-7} M; samples with ATP concentrations close to or higher than that of the ATP standard were diluted with B/S extractant to a concentration of approximately 1/10 that of the ATP standard. Mixtures of HS reactant and B/S extractant were measured at regular intervals to control for possible ATP contamination. Values of 500 ± 400 amol ATP mL^{-1} (n = 10) were obtained using clean solutions, while solutions displaying values above 500 amol ATP mL^{-1} were disposed of. The ATP concentration of the analysed samples was calculated as follows:

$$\text{amol ATP mL}^{-1} = (I_{smp} - I_{bkg}) / ((I_{smp+std} - I_{bkg}) - (I_{smp} - I_{bkg})) \times 10^9 / \text{sample volume} \quad (1)$$

where I represents the light intensity measured as relative light units, s^{-1}, smp represents sample, bkg represents the background value of the HS reagent, and std represents the standard (all referring to a 10^{-7} M ATP standard). The ATP measurements were performed three times each for the samples from the different depths; the mean reading for the three samples was calculated and reported along with the standard deviation (SD).

3.6 Method for most probable number analysis

Media for the MPN determination of microorganisms in groundwater were formulated based on chemical data from the site. This allowed, for optimal microbial cultivation, the creation of artificial media with what very closely resembled in situ groundwater in terms of chemistry /9/. Media for the metabolic groups of iron-reducing bacteria (IRB), manganese-reducing bacteria (MRB), sulphate-reducing bacteria (SRB), autotrophic acetogens (AA), heterotrophic acetogens (HA), autotrophic methanogens (AM), and heterotrophic methanogens (HM) were prepared anaerobically in 27-mL anaerobic tubes (no. 2048-00150; Bellco Glass Inc., Vineland, NJ, USA) fitted with butyl rubber stoppers and sealed with aluminium crimps (nos. 2048-117800 and 2048-11020, respectively; Bellco Glass Inc.), as described elsewhere /9/. All culture tubes were flushed with 80/20% N_2/CO_2 gas and then filled with 9 mL of their respective media. Inoculations for IRB, MRB, SRB, AA, HA, AM, and HM were performed in the laboratory within 6 h of sample collection from all boreholes. After inoculation, the headspace of only the AA and AM tubes was supplied with 80/20% H_2/CO_2 to an overpressure of 2 bars. All MPN tubes were incubated in the dark at 17°C for 8−12 weeks. Confirmation of growth in the MPN tubes after incubation was done by detecting either metabolic products or electron acceptor consumption. The MPN method produced results according to a scheme with tubes that score positive or negative for growth, when analysed (see sections 3.6.1–4.6.6). Combinations of three dilutions (15 tubes) were used to calculate the most probable numbers of all microbial groups, as described elsewhere /10/.

3.6.1 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, wastewater, and seawater.

3.6.2 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, wastewater, and seawater.

3.6.3 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 wastewater.

3.6.4 Sulphide from sulphate-reducing bacteria

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

3.6.5 Acetate from acetogens

A model 10-148-261-035 kit (Boehringer Mannheim/R-Biopharm Enzyme BioAnalyis, Food diagnostics, Göteborg, Sweden) and UV methods were used for the determination of acetate; the detection limit of this method was approximately 0.15 mg/L.

3.6.6 Methane from methanogens

A Varian 3400 gas chromatograph (Varian, Palo Alto, CA, USA) with a 2-m stainless steel HayeSep A column (VICI AG, Schenkon, Switzerland) attached to a flame ionisation detector (FID) was used to determine the methane produced by methanogens; the detection limit was 0.2 ppm.

3.7 Tests for stability and reproducibility of the methods

The methods used for MPN determination have been under development and subject to testing since 1997 /8, 9/. Quality control procedures have continuously been applied to the MPN analyses, and also to the investigations reported here. The decontamination procedures and the reproducibility of the analysis methods used here have previously been tested, and detailed results have been presented /12/. The main conclusions regarding the stability and reproducibility of the methods are given below.

3.7.1 Decontamination

The PVB system was earlier decontaminated with 70% ethanol, a procedure that worked relatively well but was not optimal. After cleaning, bacteria could still be cultivated in fairly large numbers in the performed decontamination tests. It was instead recommended that the system be decontaminated with a 10 ppm (or more) solution of chlorine dioxide – XiniX FreeBact-20 (DTI Sweden, Märsta, Sweden) yields 22.5 L at 10 ppm. The FreeBact disinfectant should be prepared fresh and pumped through the PVB system. This procedure is used for the Posiva Oy ONKALO investigations and gives very good results; it minimises the risk of contamination of the microbiology samples, compared to the use of 70% ethanol. In addition, ethanol remnants may compromise the organic carbon concentration of the sample.

3.7.2 Reproducibility of the analytical procedures

The reproducibility of the analytical procedures has been extensively tested, and the main finding was that the methods are extremely reproducible from sample to sample. Repeating the sampling and analytical procedures for a specific borehole level gave two datasets that were very nearly identical, and the MPN analyses never differed from one tube to another. Reproducibility over time was demonstrated to be good as well. Two boreholes were each analysed twice at approximately a 3.5-months interval; the two boreholes displayed very different signatures, but the results were reproduced very well within each borehole.

In conclusion, the analytical procedures reported here are reliable, reproducible, and distinguish between different boreholes and borehole sections. The obtained results can be regarded providing borehole- and section-specific signatures that give the required information as to what microbial processes may have been dominant at the time of sampling.

4 Performance

The microbial characterisations were performed according to activity plan AP-PF-400-05-066. Details can be obtained from the appendices attached to this plans.

4.1 Sample transport

Samples were rapidly transported to the laboratory by car and air cargo, reaching the laboratory the day of sampling.

4.2 Preparation of media

The media were prepared less than two weeks before each sampling date. The media incorporated a redox indicator that turned pink if the redox potential went above –40 mV (relative to an H_2 electrode). Tubes in which this happened were not used or analysed, guaranteeing anoxic cultivation conditions. Controls were used for the media and the inoculation procedure.

4.3 Start of analyses

All analyses started on the day of arrival of the samples. ATP was measured on the arrival day and the results were obtained directly. The subsamples were preserved and counted in the following weeks. The CHAB analysis started when the samples arrived, and the plates were counted after approximately 5−7 d. The MPN analyses were inoculated according to specific instructions and cultivated for up to 12 weeks.

4.4 End of analyses

After the specific growth periods required for them, various analyses were started to measure the number of positive and negative MPN tubes in terms of growth. To be regarded as positive, the value of a reading had to be at least twice that of a sterile filtered control, a control with medium only, or adjacent, negative MPN tubes /10/.

4.5 Nonconformities

All the microbial analyses for the investigated boreholes were conducted according to the activity plan.

5 Data handling

5.1 Analyses and interpretation

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

Petri dishes containing agar with nutrients were prepared for determining the number of CHAB. The plates were incubated for between 5 and 7 d at 20°C, after which the number of colony-forming units (CFU) was counted. Plates with between 10 and 300 colonies were counted and the average was reported, along with the standard deviation (SD) and number of observations (n).

The ATP Biomass Kit HS (no. 266-311; BioThema AB, Handen, Sweden) was used to determine total ATP in living cells /13/. The ATP measurements were performed three times for each sample; the mean of the three samples was calculated and reported, along with the standard deviation (SD) and number of observations (n).

The MPN method produced results according to a scheme in which tubes scored positive or negative for growth when analysed. Combinations of three dilutions (15 tubes) were used to calculate the most probable number for each microbial group, as described elsewhere /10/.

6 Results

The detailed results are given in the Appendix.

6.1 Total numbers of microorganisms and ATP concentration

The acridine orange direct count (AODC) indicated the total numbers of microorganisms in the samples (Table A-1). The total numbers of cells were higher in the groundwater samples from KFM01D than in that from KFM08A (Figure 6-1). The numbers found in the samples from KFM01D were the highest so far found in a total of 14 analysed samples from the Forsmark area (Figure 6-2) /12, 14−16/. In contrast, the samples from KFM08A had the fourth lowest numbers and a relatively low ATP content.

AODC numbers, by definition, include active, inactive, and sometimes even dead cells. An inactive microbe can still appear in the AODC analysis, even if it has been inactive for a long time. Because of the uncertainty of the AODC analysis, and to obtain an indication of the activity and viability of the detected microbes, a new ATP analysis was introduced in 2004. The measurement of ATP reflects the living bio-volume, because all living cells contain a relatively constant concentration of ATP. A detailed analysis of the relationship between the total number and ATP of microorganisms has previously been performed /13/. Pure culture experiments have demonstrated that cell volume is nested in metabolic activity, which is reflected by the amount of ATP cell^{-1}. A high amount of ATP cell^{-1} should indicate high activity and large cells. Inspection of the ratio of ATP to AODC in over 100 samples from deep groundwater, plotted versus the total number of cells, revealed that there was a large range of ATP/AODC ratios, distributed over the total number of cells. The results strongly suggest that ratios of ATP to AODC indicate the metabolic state and viability of a groundwater population. The average of all ATP/AODC ratios in deep groundwater was determined to be 0.43 /13/. ATP/AODC ratios

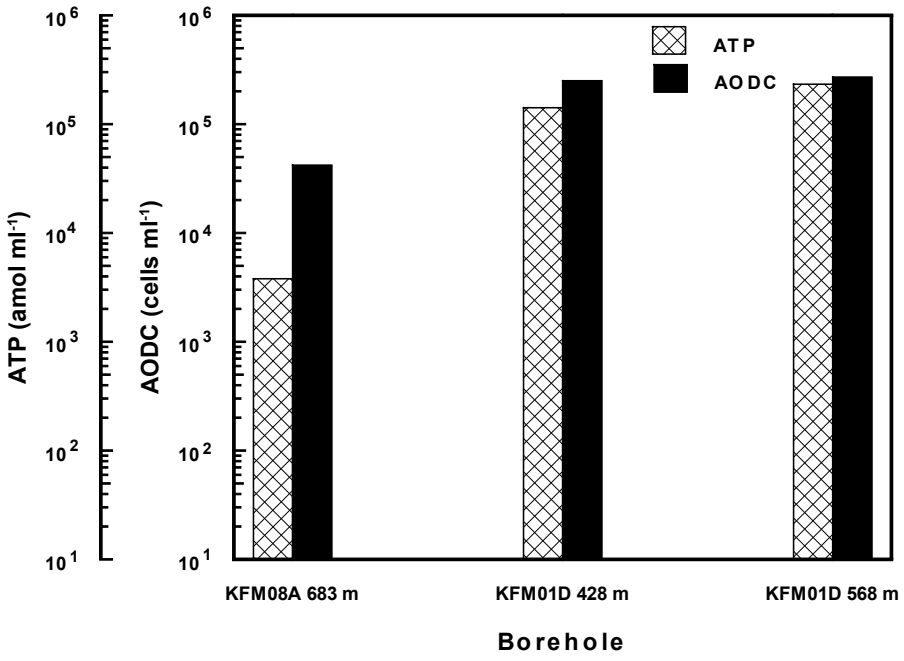

Figure 6-1. *The total numbers of cells (AODC) and the ATP concentrations in the analysed samples from KFM01D and KFM08A.*

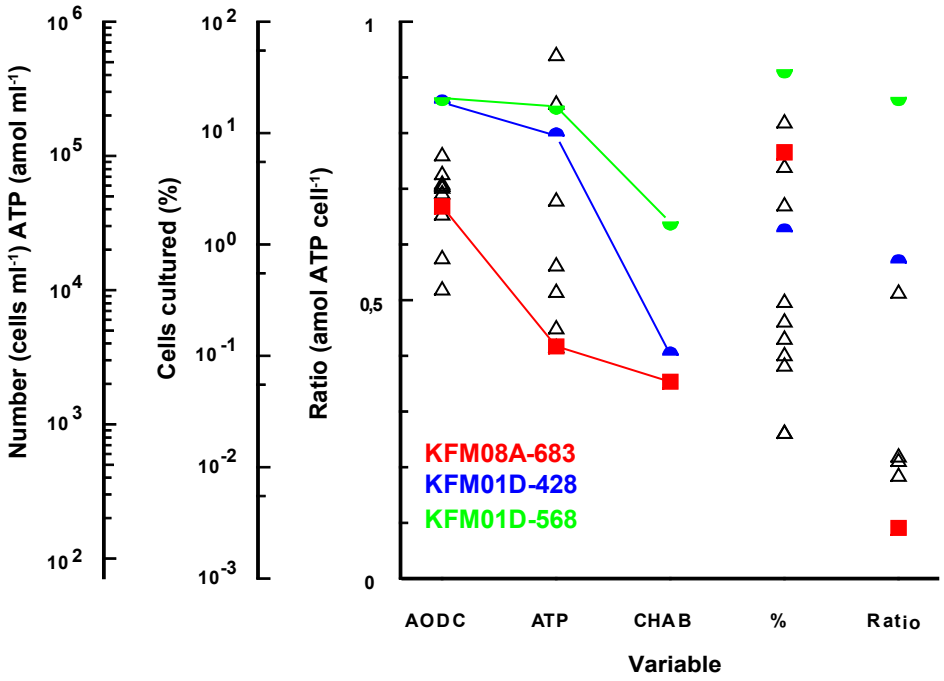

Figure 6-2. *A compilation of all data from the Forsmark site investigation /12, 14−16/ regarding the total number of cells (AODC), ATP, culturable heterotrophic bacteria (CHAB), the percent of AODC that could be cultured using the MPN method, and the amount of ATP per total number of cells. At most, 14 samples from 14 sections in six boreholes were analysed. The lines connect the respective borehole sections reported here.*

above this average indicate populations that are more active than those with ratios below the average. Groundwater from both sections in KFM01D had ATP/AODC ratios above this average displaying the highest ATP/AODC ratio found in Forsmark. In opposite, KFM08A showed the lowest ratio found in Forsmark. The section KFM01D-568 m had the highest values for all the microbiological parameters presented in Figure 6-2, suggesting that the microorganisms in KFM01D-568 m were more viable and active than those in groundwater from the other two sections studied.

6.2 Numbers of culturable microorganisms

The CHAB determinations indicated an increasing range of numbers in the samples consistent with increasing ATP and AODC values: 2,100 cells mL^{-1} in KFM08A-683 m, 3,300 cells mL^{-1} in KFM01D-428 m, and 32,000 cells mL^{-1} in KFM01D-568 m. High numbers of CHAB, above 1,000 cells mL^{-1}, are rare in pristine deep groundwater, and may thus indicate a contamination problem /17/. Such contamination can be caused by 1) a drawdown of shallow water, 2) drill water containing high CHAB numbers, 3) drill water containing a high concentration of organic carbon (> 5 mg L^{-1}), which may support CHAB growth, or 4) a combination of the preceding three. The groundwater in borehole KFM01D had CHAB readings higher than that of KFM08A, with a very high number in the sample from the deepest section. It is not possible to properly evaluate the reasons for these high CHAB numbers, as the control for contaminating bacteria in the drill water was abandoned in Forsmark after drilling KFM06A. However, indications of a drawdown to the deepest section in KFM01D have been noted, and the drill water used to drill this borehole contained a high amount of TOC (i.e. approximately 7 mg L^{-1}). It is thus probable that there was a contamination problem that generated the high numbers of CHAB in KFM01D.

The MPN determinations provided signatures of the metabolic types of microorganisms present in the examined sections. Far from all viable cells can be cultivated, as judged from comparison of the obtained AODC numbers and the numbers of microorganisms cultivated (Table A-6).

This result is common, well-known, and accepted throughout the scientific literature – many microorganisms simply cannot be cultivated. This is not as surprising as it first may seem. There are many animals – fish, birds, and insects – and plants on Earth than can only be studied in their natural environments. 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 to increase the numbers that can be cultivated is to develop our cultivation skills.

The percentages of the AODC culturable using MPN during the site investigation in Forsmark ranged from 0.0015% to 36.6%, i.e. a 10,000 times range (Figure 6-2). The groundwater samples from the borehole sections reported here were in the 1.31–36.6% range (Table A-6).

Each MPN analysis (Figure 6-3) is briefly commented on below. Detailed examination and modelling of the relationships between the MPN data and depth, hydrology, geology, and geochemistry were performed as part of ChemNet.

6.2.1 Nitrate-reducing bacteria

Next to oxygen, nitrate is the most favourable electron acceptor for bacteria. Facultative anaerobic bacteria can generally switch from oxygen to nitrogen when oxygen disappears. NRB can thus survive in deep anaerobic groundwater. The numbers of NRB found (Tables A-3, A-4, and A-5) were significantly lower than those of CHAB in KFM08A and KFM01D-428 m but this number correlates with the high number of CHAB in KFM01D-568 m (Table A-1). This provides an additional indication of surface-related contamination of the groundwater in this section.

6.2.2 Iron- and manganese-reducing bacteria

Iron- and manganese-reducing bacteria are generally observed in larger numbers at shallower than at deeper depths, at which SRB tend to increase in number. The data obtained from Forsmark (14 data points) generally indicate relatively high numbers of IRB and MRB (Figure 6-4). Groundwater from the deepest borehole section analysed here (KFM08A) contained fewer IRB than did groundwater from the two other boreholes (Figure 6-3).

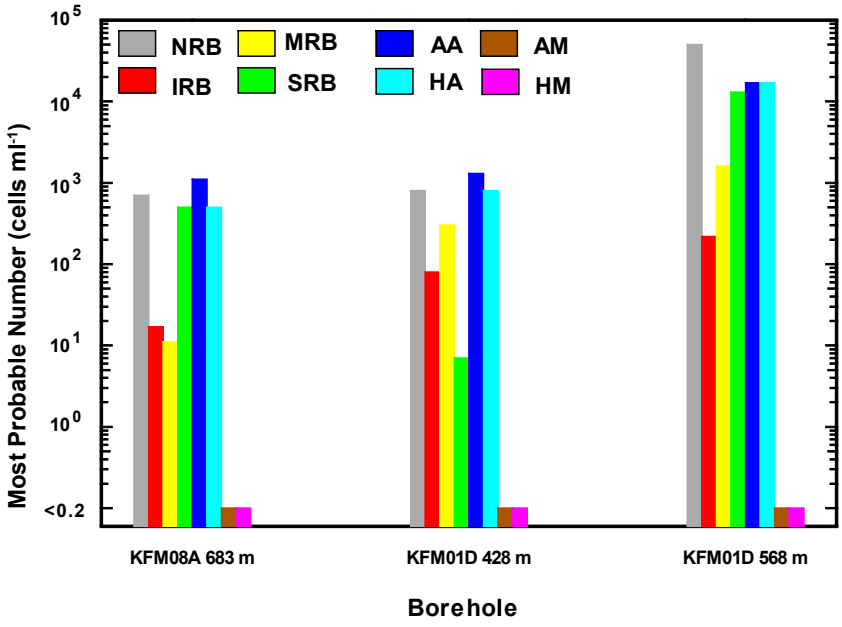

Figure 6-3. *Most probable numbers (MPN) of analysed physiological groups in groundwater samples from KFM08A and KMF01D. Abbreviations: NRB (nitrate-reducing bacteria), MRB (manganese-reducing bacteria), AA (autotrophic acetogens), AM (autotrophic methanogens), IRB (iron-reducing bacteria), SRB (sulphate-reducing bacteria), HA (heterotrophic acetogens), and HM (heterotrophic methanogens).*

6.2.3 Sulphate-reducing bacteria

The numbers of SRB in groundwater from KFM08A were the second highest of all SRB numbers from Forsmark and the highest of all SRB numbers were found in KFM01D-568 m (Figure 6-4).

6.2.4 Acetogens

Acetogens produce acetate from one-carbon organic compounds or from hydrogen and carbon dioxide. They were detected in groundwater from all boreholes and sections, with just a few exceptions, during the site investigations in Forsmark and Oskarshamn, in the Äspö Hard Rock Laboratory, and in shallow and deep groundwater from Olkiluoto. It is thus a very versatile and common group, present in the boreholes investigated here in the highest numbers detected for any microorganism in Forsmark (Figure 6-4).

6.2.5 Methanogens

Methanogens produce methane from small organic compounds (one carbon) and acetate or from hydrogen and carbon dioxide. They were commonly present above the detection limit during the various site investigations. However, they have only been sparsely detected in groundwater from the Forsmark area, in the earlier-investigated boreholes KFM01A, 02A, 03A, and 06A /12, 14−16/ (Figure 6-4), except in groundwater from KFM03A 448−453 m. This finding was upheld in the sections investigated here, none of which contained detectable methanogens (Figures 6-3 and 6-4).

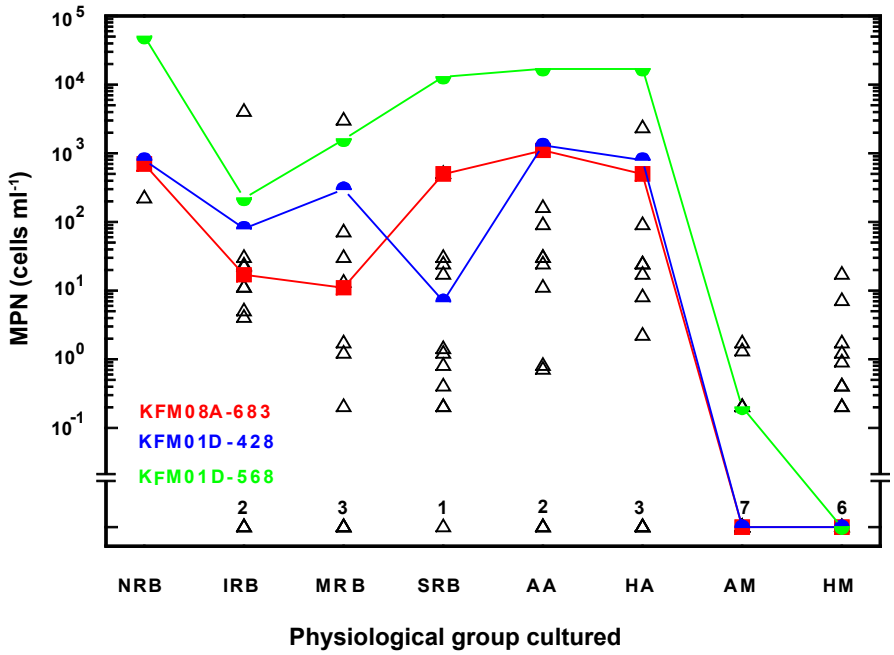

Figure 6-4. *A compilation of all MPN data from the Forsmark site investigation /12, 14−16/. A total of 14 sections in six boreholes have been analysed. The lines connect the individual borehole sections reported here. The number of negative observations for each analysed group is indicated above each lowest symbol.*

7 Conclusions

- The total numbers of cells were higher in KFM1D than in KFM8A. The numbers found in KFM01D were the highest so far found in a total of 14 analysed sections in the Forsmark area. In contrast, KFM08A had the fourth lowest numbers of cells.
- Groundwater samples analysed from KFM01D had ratios of ATP/AODC above the overall average of 0.43 for deep groundwater (n = 100), groundwater from KFM01D-568 m displaying the highest ATP/AODC ratio so far detected in Forsmark. This suggests that the microorganisms in the groundwater from the sampled KFM01D borehole possessed viability and activity above the average for deep groundwater microorganisms.
- The high CHAB numbers that were determined, above 1,000 cells mL^{-1}, may indicate surface water contamination. The numbers of NRB found were significantly lower than those of CHAB, except for in KFM01D-568 m. It was not possible to properly evaluate the possibility of drill water contamination here, as no drill water control was used when drilling KFM08A or KFM01D.
- The percentages of the AODC culturable using MPN were in the 1.31–36.6% range.
- Acetogens are a very versatile and common group, present in the groundwater analysed here in numbers that were the highest detected in Forsmark for any of the investigated microbe types.
- Methanogens were only sparsely detected in the Forsmark area in groundwater from the earlier-investigated boreholes KFM01A, 02A, 03A, and 06A. That finding was upheld in the samples analysed here, all of which contained no detectable methanogens.

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Appendix

Data

Table A-1. Total numbers of cells and concentrations of ATP in groundwater from the analysed sections of KMF01D and KMF08A.

Borehole (section m)	Total counts (cells mL^{-1}) AODC	Standard deviation	Number of observations	ATP (amol mL^{-1}) ATP	Standard deviation	Number of observations
KFM08A (683.5–690.6)	4.2×10^{4}	$\pm1.9\times10^{4}$	6	0.38×10^{4}	$\pm3.8\times10^{2}$	3
KFM01D (428.5−435.6)	2.5×10^{5}	$\pm2.0\times10^{4}$	3	1.4×10^{5}	$\pm9.4\times10^{2}$	3
KFM01D (568.0−575.0)	2.7×10^{5}	$\pm1.2\times10^{5}$	3	2.3×10^{5}	$\pm4.2\times10^{3}$	3

Table A-2. Numbers of culturable, heterotrophic aerobic bacteria (CHAB) in groundwater from the analysed sections of KMF01D and KMF08A.

Borehole (section m)	CHAB	Standard deviation	Number of observations
KFM08A (683.5–690.6)	2.1×10^{3}	$\pm1.1\times10^{2}$	3
KFM01D (428.5−435.6)	3.3×10^{3}	$\pm3.6\times10^{2}$	3
KFM01D (568.0−575.0)	3.2×10^{4}	$\pm4.8\times10^{3}$	3

Table A-3. Most probable number (MPN) of metabolic groups of microorganisms in groundwater from KMF08A, section 683.5–690.6 m.

Metabolic groups	Cells mL^{-1} MPN	Lower−upper 95% confidence limits
Nitrate-reducing bacteria	700	300−2,100
Iron-reducing bacteria	17	7−48
Manganese-reducing bacteria	11	4−29
Sulphate-reducing bacteria	500	200−1,700
Autotrophic acetogens	1,100	400−3,000
Heterotrophic acetogens	500	200−1,700
Autotrophic methanogens	< 0.2	–
Heterotrophic methanogens	< 0.2	–

Table A-4. Most probable number (MPN) of metabolic groups of microorganisms in groundwater from KMF01D, section 428.5–435.6 m.

Metabolic groups	Cells mL^{-1} MPN	Lower−upper 95% confidence limits
Nitrate-reducing bacteria	800	300−2,500
Iron-reducing bacteria	80	30−250
Manganese-reducing bacteria	300	100−1,300
Sulphate-reducing bacteria	7	2−21
Autotrophic acetogens	1,300	500−3,900
Heterotrophic acetogens	800	300−2,500
Autotrophic methanogens	< 0.2	–
Heterotrophic methanogens	< 0.2	–

Table A-5. Most probable number (MPN) of metabolic groups of microorganisms in groundwater from KMF01D, section 568.0–575.0 m.

Metabolic groups	Cells mL^{-1} MPN	Lower−upper 95% confidence limits
Nitrate-reducing bacteria	50,000	20,000−200,000
Iron-reducing bacteria	220	100−580
Manganese-reducing bacteria	≥ 1,600	600−5,300
Sulphate-reducing bacteria	13,000	5,000–39,000
Autotrophic acetogens	17,000	7,000−48,000
Heterotrophic acetogens	17,000	7,000−48,000
Autotrophic methanogens	< 0.2	–
Heterotrophic methanogens	< 0.2	–

Table A-6. Percentage of the cells cultured using MPN (Tables A-3 to A-5) and CHAB (Table A-2) versus total number of cells (AODC) (Table A-1) and the ratio of ATP over AODC in groundwater from KMF08A and KFM01D.

Borehole (section, m)	% cultured MPN/AODC	CHAB/AODC	Ratio ATP/AODC
KFM08A (683.5–690.6)	6.73	4.95	0.64
KFM01D (428.5−435.6)	1,31	1.32	0.57
KFM01D (568.0−575.0)	36.6	11.9	0.86