P-08-08

Oskarshamn site investigation

Microorganisms in groundwater from boreholes KLX13A and KLX17A: numbers, viability, and metabolic diversity

Results from three sections: 432.0–439.2 m in KLX13A and 416.0–437.5 m and 642.0–701.1 m in KLX17A

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ISSN 1651-4416
SKB P-08-08

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Keywords: Microorganisms, ATP, Groundwater, Nitrate, Iron, Manganese, Sulphate, Reduction, Acetogen, Methanogen, Total number, Oskarshamn, AP PS 400-06-075, AP PS 400-06-138.

Abstract

Microorganisms and their characteristic features were investigated during complete chemical characterization as part of the site investigation programme at Oskarshamn. The 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 for determining the numbers of organisms belonging to different physiological groups, the most probable number (MPN) method. This investigation covered 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 was tested using groundwater from a depth of 450 m at the Äspö Hard Rock Laboratory and was found to be excellent.

Samples were taken from boreholes KLX13A at 432.0−439.2 m, KLX17A at 416.0−437.5 m, and KLX17A at 642.0−701.1 m; the sampling dates were 2007-01-18, 2007-04-19, and 2007-03-01, respectively. The total number of cells (TNC) found in groundwater from KLX17A (416.0−437.5 m) was the highest found so far found in a total of 13 analysed sections in the Oskarshamn site investigation programme, while the number found in groundwater from KLX13A (432.0−439.2 m) was the second lowest. A large amount of ATP per cell indicates large, active cells. The average of all previous ATP/TNC ratios ($n \cong 100$) in deep groundwater was determined to be 0.43. The analysed groundwater samples from KLX17A (642.0−701.1 m) had high ATP/TNC ratios, while KLX17A (416.0−437.5 m) groundwater had an ATP/TNC ratio very close to the overall average of 0.43 determined for deep groundwater. The CHAB and NRB numbers found here suggest that there was no surface water contamination, a finding supported by the drill-water control results. The percentages of TNC culturable using the MPN method during the site investigation programme in Oskarshamn were in the 0.12−9.15% range, while the percentages of TNC cultivatable using MPN in groundwater from KLX13A and KLX17A were in the 1.31−9.15% range. The numbers of SRB in samples from the KLX17A sections were the highest so far detected in the site investigation programme in Oskarshamn. Acetogens are a very versatile and common group, present in KLX13A and KLX17A groundwater in numbers in line with the overall average for acetogens detected in the site investigation programme in Oskarshamn. Heterotrophic methanogens have previously been found in relatively high numbers in the site investigation programme in Oskarshamn, while autotrophic methanogens were more sparsely observed. This finding was not upheld in the groundwater samples investigated here, in which all heterotrophic methanogen data were below the detection limit (0.2 cells mL^{-1}).

Sammanfattning

Under fullständig kemikaraktärisering i samband med platsundersökningarna i Oskarshamn ingår undersökning av mikrober. Denna del omfattar bestämning av totalantalet mikroorganismer, mängd adenosin-tri-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 undersökningen ingick de åtta olika grupperna nitrat-, järn-, mangan- och sulfatreducerande bakterier, auto- och heterotrofa acetogener och auto- och heterotrofa metanogener. Metodens reproducerbarhet har befunnits utmärkt vid tester på grundvatten från 450 m djup vid Äspölaboratoriet.

Provtagningarna gjordes i totalt en sektion i borrhålet KLX13A, 432.0−439.2 m, samt i två sektioner i borrhålet KLX17A: 416.0−437.5 m och 642.0−701.1 m. Provtagningarna utfördes 2007-01-18, 2007-04-19 och 2007-03-01 i respektive sektion. Totalantalet celler (TNC) i grundvattenprovet från KLX17A (416.0−437.5 m) var det högsta som hittills uppmätts i totalt 13 grundvattenprover under platsundersökningarna i Oskarshamn, medan TNC i grundvatten från KLX13A (432.0−439.2 m) var det näst lägsta. En stor mängd ATP per cell tyder på att cellerna i provet är aktiva och stora. Medelvärdet för ATP/TNC ($n \cong 100$) i djupa grundvatten har bestämts till 0.43. Grundvattenproven från KLX17A (642.0−701.1 m) hade högt ATP/TNC förhållande, medan grundvatten från KLX17A (416.0−437.5 m) hade ett ATP/TNC förhållande som låg mycket nära medelvärdet för alla djupa grundvatten (0.43). Antalen av CHAB och nitratreducerande bakterier (NRB) in de undersökta proverna från KLX13A och KLX17A tyder starkt på att proverna inte var kontaminerade av ytvatten, vilket också stöds av tidigare erhållna resultat från analyser av det spolvatten som användes vid borrningarna. Andelen av TNC som kunnat odlas med MPN under platsundersökningarna i Oskarshamn varierar från 0.12% upp till 9.15%. I den här undersökningen av grundvatten från KLX13A och KLX17A låg procentsatserna mellan 1.31% och 9.15%. Antalet sulfatreducerande bakterier (SRB) i KLX17A var de högsta som hittills uppmätts under platsundersökningarna i Oskarshamn. Acetogena bakterier är en mycket varierad och vanlig grupp och dessa uppmättes i KLX13A och KLX17A i ungefär samma antal som uppmätts i 10 andra grundvattenprov från platsundersökningarna i Oskarshamn. Heterotrofa metanogener (HM) har tidigare uppmätts i relativt höga antal i grundvattenprover från platsundersökningarna i Oskarshamn, medan autotrofa metanogener endast förekommit sparsamt. I grundvattnen som analyserats och rapporteras här kunde dock HM inte påvisas över detektionsgränsen (0.2 cells mL^{-1}).

Contents

1 Introduction

This document reports the performance and results of microbe investigations of boreholes KLX13A and KLX17A as part of the site investigation programme in Oskarshamn /1/. Microbiological data from the following two borehole sections are presented:

- KLX13A, 432.0−439.2 m, sampling date 2007-01-18.
- KLX17A, 416.0−437.5 m, sampling date 2007-04-19.
- KLX17A, 642.0−701.1 m, sampling date 2007-03-01.

The sampling was done as part of the complete chemical characterization activities for boreholes KLX13A and KLX17A, according to the AP PS 400-06-075 and AP PS 400-06-138 activity plans, respectively (SKB internal control document; see Table 1-1). The sampling process and down-hole sampling equipment are described elsewhere /2, 3/. Subsequent laboratory work was performed over the 12 weeks after the samples had reached the laboratory.

The flushing water used when core drilling the borehole 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 determined several times for the boreholes reported here. Triplicate sampling and analysis according to standard procedures was performed in KLX13A and duplicate control was preformed in KLX17A. The dataset for assessing possible drill water contamination of the samples obtained from KLX13A and KLX17A has been reported elsewhere /4, 5/.

Original data regarding the reported activities are stored in the primary SICADA database. Only data in the database are acceptable 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. Control documents for performance of research activities.

Activity plan	Number	Version
Fullständig kemikaraktärisering i KLX13A.	AP PS 400-06-075	1.0
Fullständig kemikaraktärisering i KLX17A.	AP PS 400-06-138	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 /6/. Active microorganisms influence the groundwater geochemistry /7/ and redox potential /8/. Therefore, a full understanding of geochemical conditions affecting deep groundwater requires knowledge of the presence, diversity, and activity of microorganisms. In their metabolisms, microorganisms oxidize electron- and energy-rich compounds by using a variety of electron acceptors (Figure 2-1). The preferred electron acceptor of many, but far from all, microorganisms is oxygen. This is why the oxygen concentration in 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 belong, is the sulphate-reducing bacteria, which reduce sulphate to sulphide in their metabolisms. The energy and electron donors in the metabolisms of all the above microorganisms are organic material that eventually becomes oxidized 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 oxidized species. The oxidized 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 oxidize hydrogen gas and reduce carbon dioxide to produce methane; acetogens convert the same compounds to acetate. In addition, heterotrophic methanogens and acetogens can utilize organic one-carbon compounds, such as methanol and methylamine, as well as the two-carbon compound acetate.

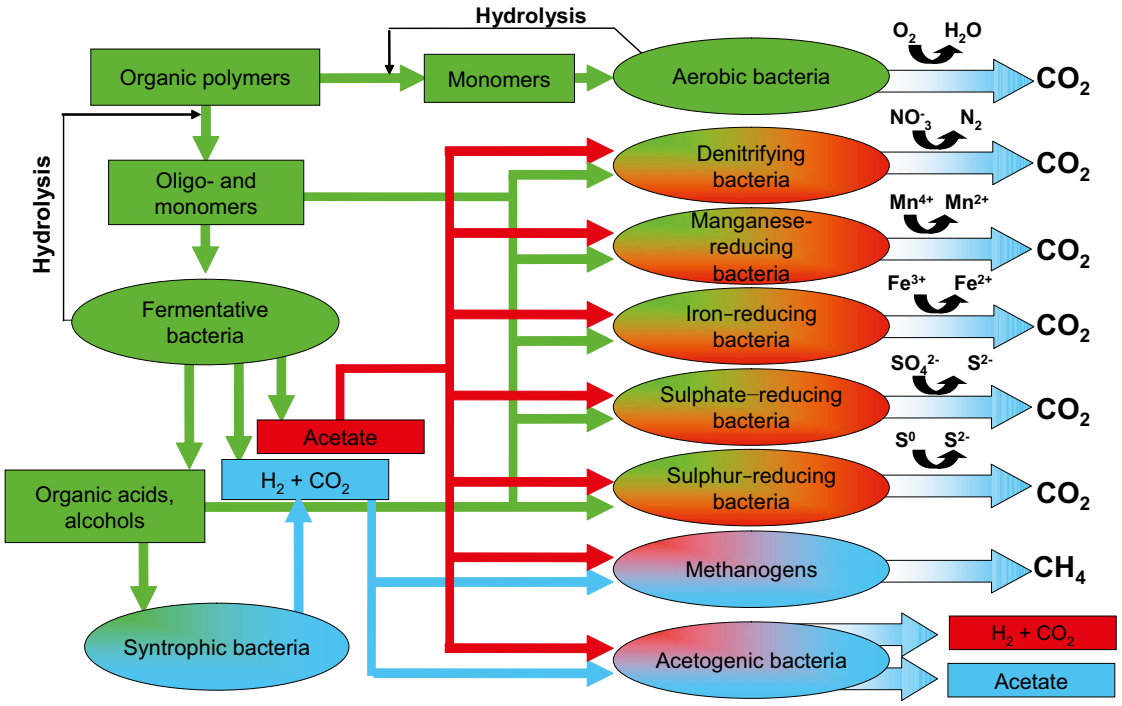

Figure 2-1. *Possible pathways for the flow of carbon in the subterranean environment. Organic carbon is respired with oxygen, if present, or else fermentation and anaerobic respiration occur with an array of different electron acceptors.*

2.1 Objectives

The microbial communities occurring in granitic rock from the surface to a depth of 1,700 m have been studied for two decades /7/. 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 utilize all the electron acceptors mentioned above /7/. These results have been used to formulate a conceptual model of microbially catalysed geochemical reactions in granitic groundwater in the Fennoscandian Shield.

- The major objective here was to enumerate all physiological groups of microorganisms that, through their growth and metabolizing activities, may influence groundwater geochemistry.
- Another important objective of this investigation was to quantify microbial biomass in groundwater from the analysed boreholes.

2.2 Scope

The microbiological analysis programme reported here was carried out according to protocols developed in previous investigations of Finnish groundwater /9, 10/. These protocols cover the determination of the total number of cells (TNC) in groundwater, number of culturable, heterotrophic aerobic bacteria (CHAB), concentration of adenosine-tri-phosphate (ATP), and a statistical cultivation method for estimating the most probable number (MPN) of culturable metabolic groups of microorganisms. These metabolic groups 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 hours; subsampling for analysis was performed immediately on arrival of the PVB sampler.

3 Equipment and methods

3.1 Equipment for transferring samples from the PVB sampler

Transferring 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 pressurized 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 (MPN) 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 elsewhere /11/. 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-

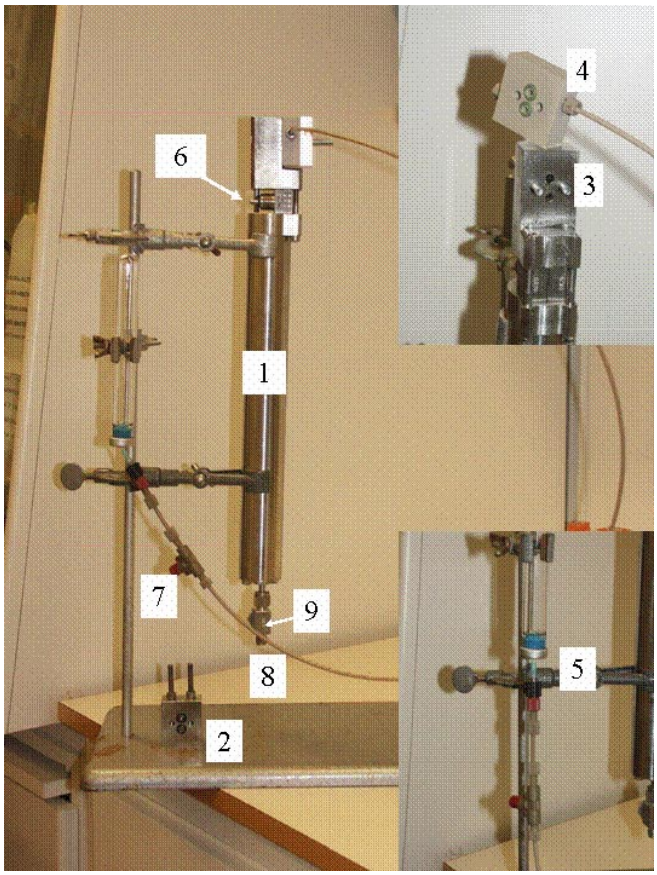

Figure 3-1. *This setup was designed for the oxygen-free transfer of samples from the PVB sampler (1) to nitrogen-flushed, anaerobic tubes stoppered with butyl rubber stoppers (5). (1) PVB sampler, (2) transportation seal, (3) inlet/outlet of the PVB, (4) PEEK sampling device, (5) transfer of sample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

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 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 TNC was determined using the acridine orange direct count (AODC) method. All solutions used were filtered through sterilized 32-mm diameter, 0.2-µm pore size Filtropur S syringe filters (Sartorius, GTF, Göteborg, Sweden). Prior to filtration, 13-mm stainless steel analytical filter holders (no. XX3001240; Millipore, Solna, Sweden), were rinsed with sterile filtered, analytical grade water (AGW) (Millipore Elix 3, Millipore). Samples of 1 mL were suction filtered (−20 kPa) onto 0.22-µm pore size Sudan black-stained polycarbonate isopore filters, 13 mm in diameter (Millipore). 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 100 mL 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, Stockholm, Sweden). 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 (Nikon DIPHOT 300, Tekno-Optik, Göteborg, Sweden). Between 400 and 600 cells, or a minimum of 30 microscopic fields (1 field = 0.01 mm^2), were counted on each filter.

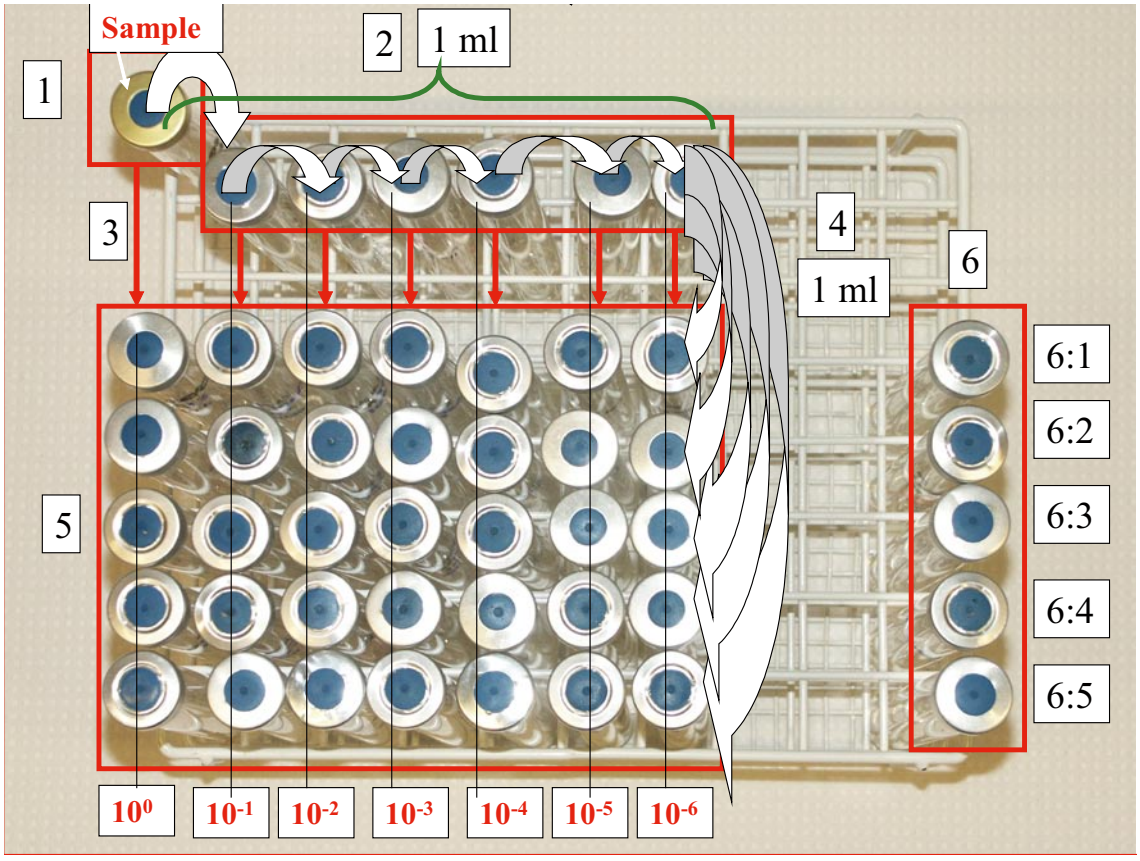

Figure 3-2. *The procedure for most probable number determination. The tube containing the sample is used as the inoculation source (1). Serial dilution is performed first (2); thereafter, subsamples are transferred (3–4) to the growth tubes (5) and control tubes (6).*

3.4 Method for cultivating aerobic, heterotrophic bacteria

Petri dishes containing agar with nutrients were prepared for determining the CHAB. This agar contained 0.5 g L^{-1} of peptone (Merck/VWR, Stockholm, Sweden), 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 /12/, and 15 g L^{-1} of agar (Merck). The medium was sterilized in 1-L batches by autoclaving at 121°C for 20 min; after this the batches were cooled to approximately 60°C in a water bath, and finally distributed in 20-mL portions in 9-cm-diameter plastic Petri dishes (GTF, Göteborg, Sweden). Ten-times dilution series of culture samples were made in AGW with 0.9 g L^{-1} of NaCl; 0.1-mL portions of each dilution were spread with a sterile glass rod on the plates in triplicate. The plates were incubated for between 5 hours and 7 days at 20°C, after which the numbers of colony forming units (CFU) were 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 AB) 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 seconds intervals with a 5 seconds 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 hour of collection, by mixing for 5 seconds 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 1,600 ± 500 amol ATP mL^{-1} (n = 10) were obtained using clean solutions, while solutions displaying values above 1,600 amol ATP mL^{-1} were disposed of. The ATP concentration of the analysed samples was calculated as follows:

$$\text{amol ATP mL}^{-1} = (I_{smp} - I_{bkg}) / ((I_{smp+std} - I_{bkg}) - (I_{smp} - I_{bkg})) \times 10^6 / \text{sample volume} \quad (1)$$

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 nine times each for the samples from the different depths; the mean reading for the nine samples was calculated and reported along with the standard deviation (SD).

3.6 Method for most probable number (MPN) 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 that very closely resembled in situ groundwater in terms of chemistry /11, 12/. Media for the metabolic groups of 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), 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 /11/. 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 NRB, IRB, MRB, SRB, AA, HA, AM, and HM were performed in the laboratory within 6 hours of sample collection from all boreholes. After inoculation, the headspace of only the AA and AM tubes was supplied with H_2 to an overpressure of 2 bars. All MPN tubes were incubated in the dark at 17°C for 8−13 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 whereby tubes score positive or negative for growth, when analysed (see sections 3.6.1–3.6.6). Combinations of three dilutions (15 tubes) were used to calculate the MPNs of all microbial groups, as described elsewhere /11/.

3.6.1 Nitrate consumed by nitrate-reducing bacteria

A chromotropic method (0.2–30 mg L^{-1} NO_3^- − N), i.e. method 10020 for water and wastewater for the HACH DR/2500 spectrophotometer (HACH, Loveland, CO, USA), was used to determine the nitrate consumed by nitrate-reducing bacteria.

3.6.2 Ferrous iron from iron-reducing bacteria

A phenanthroline method (0.02–3 mg L^{-1} Fe^{2+}) method 8146 for water and wastewater for the HACH DR/2500 spectrophotometer (HACH, Loveland, CO, USA), was used to determine the ferrous iron produced by iron-reducing bacteria.

3.6.3 Manganese(II) from manganese-reducing bacteria

A periodate oxidation method (0.2–20 mg L^{-1} Mn^{2+}) method 8034 for water and wastewater for the HACH DR/2500 spectrophotometer (HACH, Loveland, CO, USA), was used to determine the manganese(II) produced by manganese-reducing bacteria.

3.6.4 Sulphide from sulphate-reducing bacteria

Sulphide was measured as copper sulphide, using a spectrophotometer, and compared with a standard curve /12/. The main reagent comprised 1.25 g of $CuSO_4 \cdot 5H_2O$ and 4.14 mL of concentrated HCl dissolved in (AGW) 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 produced by acetogens; the detection limit of this method was approximately 0.15 mg L^{-1}.

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 ionization detector 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 /9, 10, 11/. Ongoing quality control procedures have 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 elsewhere /13/. The main conclusions regarding the stability and reproducibility of the methods are given below.

3.7.1 Decontamination

The PVB system had previously been 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 should be decontaminated with a 10 ppm (or more) solution of chlorine dioxide (XiniX FreeBact-20; DTI Sweden, Märsta, Sweden); one bottle of FreeBact yields 22.5 L at 10 ppm. The FreeBact disinfectant should be prepared fresh and pumped through the PVB system. This procedure was used for the Posiva OY ONKALO investigations, producing very good results; compared to the use of 70% ethanol, it better minimizes the risk of contamination of the microbiology samples. In addition, ethanol remnants may compromize 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 /13/. 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-month 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 as providing borehole- and section-specific signatures that give the required information as to what microbial processes were dominant at the time of sampling.

4 Performance

The microbial characterizations were performed according to the methods described in chapter 3 (with references).

4.1 Sample transport

Samples were rapidly transported to the laboratory by car, reaching the laboratory before 15.00 on 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 the day the samples arrived at the lab. ATP was measured on the arrival day and the results were obtained directly. The samples for determining THC 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 days. 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, 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 containing medium only, or adjacent, negative MPN tubes /11/.

5 Data handling

5.1 Analyses and interpretation

The total numbers of microorganisms were counted on two filtration filter from each of three sample tubes. Each filter was regarded as one independent observation. The mean value for the three filters from the 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 5−7 days at 20°C, after which the numbers of colony-forming units (CFU) were 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) was used to determine total ATP in living cells. The ATP measurements were performed three times for each sample from the different depths; the mean of the nine samples was calculated and reported, along with the standard deviation (SD).

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 MPN for each microbial group, as described elsewhere /11/.

6 Results

The detailed results are given in the Appendix.

6.1 Total numbers of microorganisms and ATP concentration

The AODC indicated the TNC in the samples (Table A-1). The TNC was highest in the groundwater sample from KLX17A (416.0−437.5 m) (Figure 6-1). The number found in the sample from KLX13A (432.0−439.2 m) was among the lowest so far found in a total of 13 analysed samples from the site investigation programme in Oskarshamn (Figure 6-1) /13−15/. In contrast, the KLX17A (416.0−437.5 m) sample had the highest TNC value found so far. The ATP concentrations correlated well with the TNC. The CHAB numbers were also the highest obtained this far, since the onset of this analysis in December 2005.

The TNC values by definition include active, inactive, and sometimes even dead cells; an inactive microbe can still appear in the TNC analysis, even if it has been inactive for a long time. Because of the uncertainty of the TNC count, and to obtain an indication of the activity and viability of the detected microbes, a new type of analysis was introduced in December 2004. The measurement of ATP reflects the living bio-volume, because all living cells contain a relatively

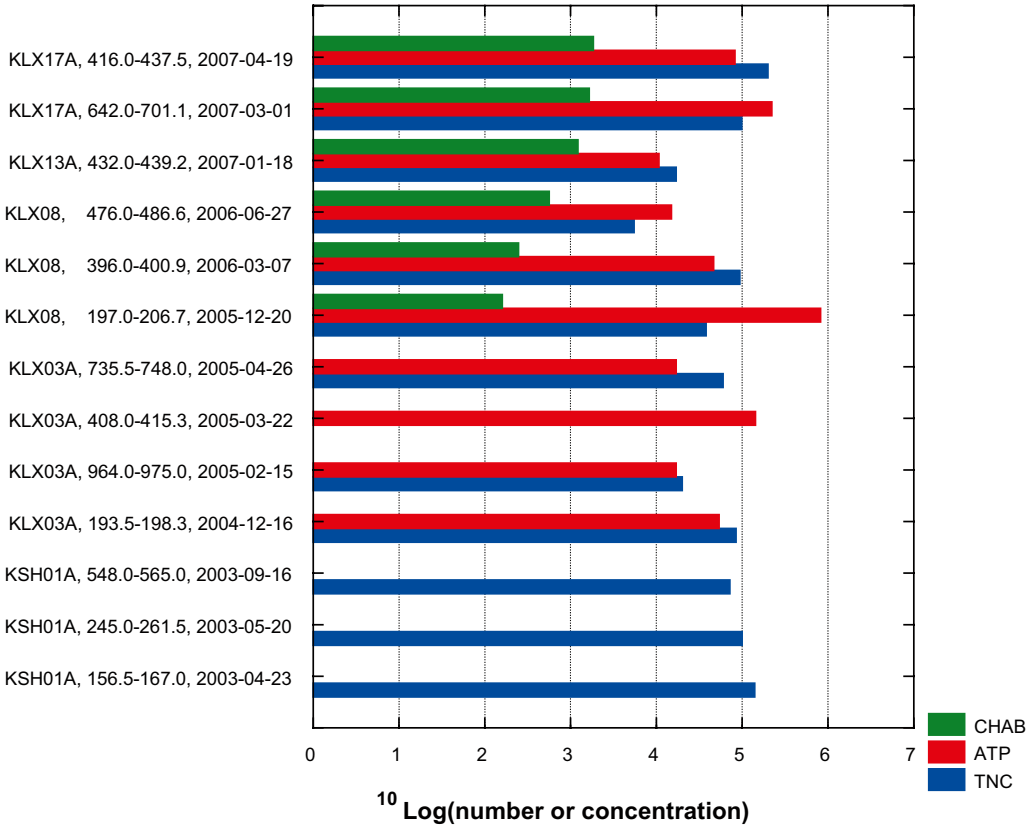

Figure 6-1. *The numbers of culturable heterotrophic, aerobic bacteria (CHAB, cells mL^{-1}), ATP concentrations (amol mL^{-1}), and total numbers of cells (TNC, cells mL^{-1}) in the analysed groundwater samples from boreholes KLX13A and KLX17A (Tables A-1 and A-2), in comparison with all previously obtained data /13−15/.*

constant concentration of ATP. The relationship between the TNC and ATP of microbes has previously been analysed in detail /16/. 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 TNC in over 100 samples from deep groundwater, plotted versus TNC, revealed that there was a large range of values, for the total dataset, distributed over the averages. These results strongly suggest that ATP/TNC ratios indicate the metabolic state and viability of a groundwater population. The average of all ATP/TNC ratios in deep groundwater was determined to be 0.43 /16/. An ATP/TNC ratio above this average indicates populations that are more active than are those with ratios below the average. The groundwater sample from KLX17A (642.0−701.1 m) had a high ATP/TNC ratio, which correlated well with the high percentage of the TNC that could be cultivated using the MPN method (Figure 6-1, Table A-6); this suggests that the microbial populations analysed were very active in this groundwater. Groundwater from KLX17A (416.0−437.5 m) had an ATP/TNC ratio very close to the overall average of 0.43 determined for deep groundwater /16/, while the ratio for KLX13A (432,0–439,2 m) was moderately above average (Figure 6-2). In the KLX13A groundwater, the percentages of TNC cultivated using the MPN and CHAB methods were also high, relative to the other analysed groundwater samples from the site investigation programme in Oskarshamn.

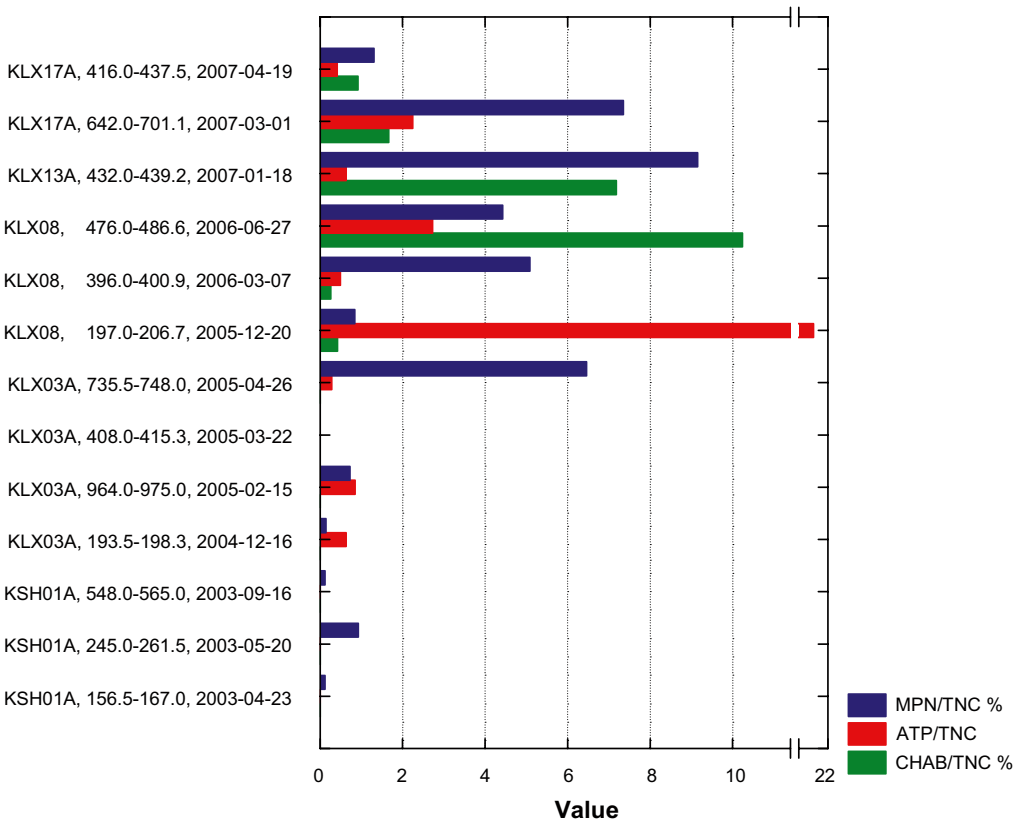

Figure 6-2. *A compilation of the ratios of ATP to TNC (amol cell^{-1}) and the percentages of TNC culturable using the most probable number (MPN) and the culturable heterotrophic aerobic bacteria (CHAB) methods (Table A-6). All previous data from the Oskarshamn site investigation programme /13−15/ are shown as well for comparison. Missing bars reflect lack of data (the CHAB and ATP methods were added consecutively after start of site investigation program).*

6.2 Numbers of culturable microorganisms

The CHAB determinations were all somewhat higher than those found previously in groundwater from the Oskarshamn site investigation programme (Figure 6-1). The CHAB analysis was done under aerobic conditions, unlike all other cultivation methods used, which were done under anaerobic conditions. Many bacteria are known to be facultative anaerobes. These can switch from aerobic respiration using oxygen, to anaerobic respiration using nitrate and commonly also ferric iron and manganese(IV) as alternative electron acceptors (Figure 2-1). Microorganisms in groundwater must be adapted to anoxic conditions, but if oxygen appears for some reason, it is advantageous for the microbe to be able to switch to oxygen respiration. Indigenous groundwater microorganisms should consequently be detectable as both CHAB and NRB, while contaminants from the surface should have a smaller tendency to be detectable in this way. Comparison of the CHAB data with the NRB data reveals reasonably good correlation between them (Tables A-2, A-3, A-4, and A-5), suggesting that the microorganisms analysed as CHAB generally were indigenous. The CHAB and NRB numbers found here, therefore, suggest that there was no surface water contamination, a finding supported by the drill water control results /4, 5/.

The percentages of TNC culturable using the MPN method during the site investigation programme in Oskarshamn ranged from 0.12% to 9.15%, i.e. a 75-times range (Figure 6-2). The groundwater samples from the borehole sections reported here were in the 1.31−9.15%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 will be performed elsewhere.

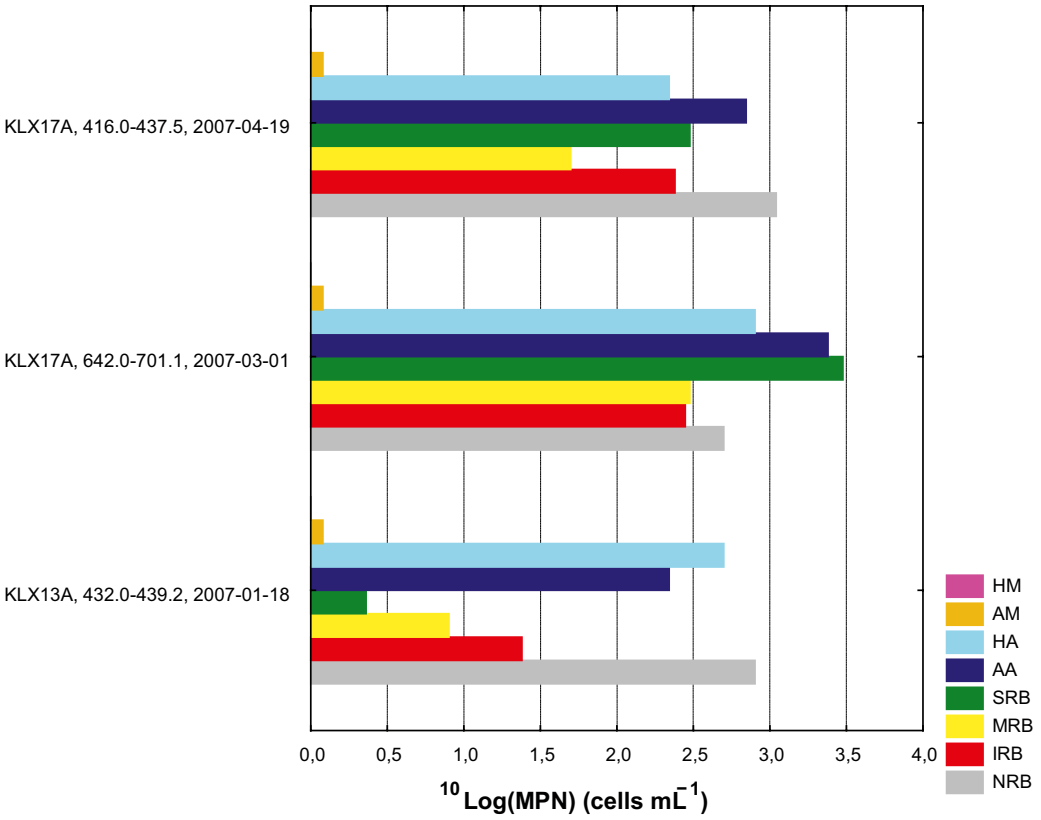

Figure 6-3. *Most probable numbers (MPN) of analysed physiological groups in groundwater samples from KLX13A and KLX17A. NRB = nitrate-reducing bacteria, MRB = manganese-reducing bacteria, AA = autotrophic acetogens, AM = autotrophic methanogens, IRB = iron-reducing bacteria, SRB = sulphate-reducing bacteria, HA = heterotrophic acetogens, and HM = heterotrophic methanogens.*

6.2.1 Nitrate-reducing bacteria

Next to oxygen, nitrate is the most favourable electron acceptor for bacteria. Facultative anaerobic bacteria can generally switch from oxygen to nitrate respiration when oxygen disappears. NRB can thus survive in deep anaerobic groundwater. The numbers of CHAB (Table A-1) found were all within the 95% confidence intervals of the NRB values (Tables A-3, A-4, and A-5), which suggests that most CHAB were facultative anaerobes. This group of microorganisms is able to grow and survive in deep groundwater. They are not indicative of surface water contamination, which would have been the case if the CHAB had significantly outnumbered the NRB.

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 the Oskarshamn site investigation programme (13 data points) generally indicate low numbers of IRB and MRB (Figure 6-4). Groundwater from KLX17A displayed among the highest IRB and MRB values found in the Oskarshamn site investigation programme, while the KLX13A water displayed values closer to those found previously (Figure 6-3).

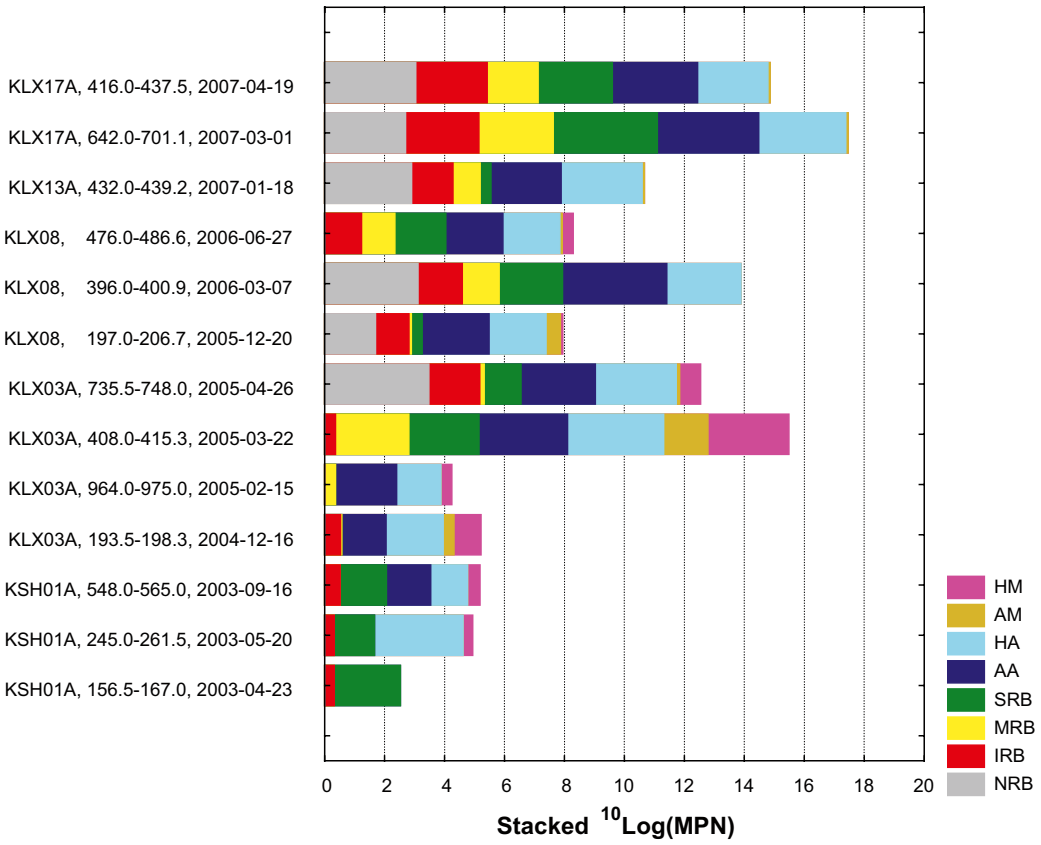

Figure 6-4. *Stacked values of most probable numbers of physiological groups of microorganisms in groundwater from KLX13A and KLX17A. All previous data from the site investigation programme in Oskarshamn /13−15/ are shown as well for comparison. NRB = nitrate-reducing bacteria, IRB = iron-reducing bacteria, MRB = manganese-reducing bacteria, SRB = sulphate-reducing bacteria, AA = autotrophic acetogens, HA = heterotrophic acetogens, AM = autotrophic methanogens, HM = heterotrophic methanogens. Note that analysis of NRB was introduced in April 2005.*

6.2.3 Sulphate-reducing bacteria

The numbers of SRB in KLX17A were the highest of all SRB numbers from the Oskarshamn site investigation programme (Figure 6-4), while the groundwater sample from KLX13A had a low value, just ten times above the detection limit (0.2 cells mL^{-1}).

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 investigation programmes 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 groundwater investigated here in numbers that were average for the microbes detected in the Oskarshamn site investigation programme (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. Heterotrophic methanogens have been found in relatively high numbers in the site investigation programme in Oskarshamn, while autotrophic methanogens have been more sparsely observed /13−15/ (Figure 6-4). This finding was not upheld in the samples investigated here, in which all HM analyses returned below detection data. Some AM could be detected, but only in very low numbers, just above the detection limit (0.2 cells mL^{-1}) (Figures 6-3 and 6-4).

7 Conclusions

- The total number of cells (TNC) was highest in the groundwater sample from KLX17A (642.0−701.1 m). The number found in groundwater from KLX13A (432.0−439.2 m) was the second lowest so far found in a total of 13 analysed sections in the Oskarshamn site investigation programme.
- The groundwater samples from KLX17A had high ATP/TNC ratios, while KLX13A (432.0−439.2 m) groundwater had an ATP/TNC ratio very close to the overall average of 0.43 determined for deep groundwater (n = 100).
- The CHAB and NRB numbers found here suggest that there was no surface water contamination, a finding supported by the drill water control results.
- The percentages of TNC cultivatable using the MPN method were in the 1.31−9.15% range.
- The numbers of SRB in samples from the KLX17A sections were the highest detected in the Oskarshamn site investigation programme this far.
- Acetogens are a very versatile and common group, present in KLX13A and KLX17A groundwater in numbers that were average for the microbes detected in the Oskarshamn site investigation programme.
- Heterotrophic methanogens (HM) have previously been found in relatively high numbers in the site investigation programme in Oskarshamn, while autotrophic methanogens were more sparsely observed. That finding was not upheld in the groundwater samples investigated here, in which all HM data were below the detection limit (0.2 cells mL^{-1}).

8 References

/1/ **SKB, 2001.** Site investigations. Investigation methods and general execution programme. SKB TR-01-29, Svensk Kärnbränslehantering AB.

/2/ **Bergelin A, Nilsson K, Lindquist A, Wacker P, 2007.** Complete chemical characterisation in KLX13A. Results from two investigated borehole sections, 432.0−439.2 m, 499.5−506.7 m. SKB P-07-149, Svensk Kärnbränslehantering AB.

/3/ **Bergelin A, Nilsson K, Lindquist A, Wacker P, 2007.** Complete chemical characterisation in KLX17A. Results from two investigated borehole sections, 416.0−437.5, 642.0−701.1 m. SKB P-07-164, Svensk Kärnbränslehantering AB.

/4/ **Pedersen K, 2006.** Oskarshamn site investigation. Triple control of microorganism content in flushing water used for drilling of KLX13A. SKB P-06-200, Svensk Kärnbränslehantering AB.

/5/ **Pedersen K, 2006.** Oskarshamn site investigation. Double control of microorganism content in flushing water used for drilling of KLX17A. SKB P-06-309, Svensk Kärnbränslehantering AB.

/6/ **Pedersen K, 2002.** Microbial processes in the disposal of high-level radioactive waste 500 m underground in Fennoscandian shield rocks. In: Interactions of Microorganisms with Radionuclides. Edited by Keith-Roach MJ, Livens FR. Elsevier, Amsterdam. pp 279–311.

/7/ **Pedersen K, 2001.** Diversity and activity of microorganisms in deep igneous rock aquifers of the Fennoscandian Shield. In: Subsurface Microbiology and Biogeochemistry. Edited by Fredrickson JK, Fletcher M. Wiley-Liss, New York. pp 97–139.

/8/ **Haveman SA, Pedersen K, 2002.** Microbially mediated redox processes in natural analogues for radioactive waste. *J Contam Hydrol* 55, 161–174.

/9/ **Haveman SH, Pedersen K, Routsalainen P, 1999.** Distribution and metabolic diversity of microorganisms in deep igneous rock aquifers of Finland. *Geomicrobiol J* 16, 277–294.

/10/ **Haveman SA, Pedersen K, 2002.** Distribution of culturable anaerobic microorganisms in Fennoscandian Shield groundwater. *FEMS Microbiol Ecol* 39, 129–137.

/11/ **Hallbeck L, Pedersen K. 2008**. Characterization of microbial processes in deep aquifers of the Fennoscandian Shield *Applied Geochemistry*, 23, 1796–1819.

/12/ **Widdel F, Bak F, 1992.** Gram-negative mesophilic sulfate-reducing bacteria. In: The Prokaryotes, vol. 4, 2nd ed. Edited by Balows A, Trüper HG, Dworkin M, Harder W, Schleifer K-H. Springer-Verlag, New York. pp 3352–3378.

/13/ **Pedersen K, 2005.** Oskarshamn site investigation. Numbers and metabolic diversity of microorganisms in borehole KLX03. Results from sections 193.0–198.0 m, 408.0–415.3 m, 735.5–748.0 m, and 964.5–975.2 m. SKB P-05-182, Svensk Kärnbränslehantering AB.

/14/ **Pedersen K, 2005.** Oskarshamn site investigation. Microorganisms in groundwater from borehole KLX08: numbers, viability, and metabolic diversity. Results from sections 197.0−206.7 m, 396.0−400.9 m, and 476.0−485.6 m. SKB P-07-59, Svensk Kärnbränslehantering AB.

/15/ **Pedersen K, Kalmus A, 2004.** Oskarshamn site investigations. Total numbers and metabolic diversity of microorganisms in borehole KSH01A. Results from three investigated sections, 158.5–167 m, 245–261.6 m, and 548–565 m. SKB P-04-276, Svensk Kärnbränslehantering AB.

/16/ **Eydal HSC, Pedersen P, 2007.** Use of an ATP assay to determine viable microbial biomass in Fennoscandian Shield groundwater from depths of 3−1000 m. *J. Microbiol Meth* 70, 363−373.

Appendix

Data

Table A-1. Total number of cells (TNC) and ATP concentration in groundwater from the analysed sections of KLX13A and KLX17A.

Borehole (section m)	Total counts (cells mL^{-1})			ATP (amol mL^{-1})		
	TNC	Standard deviation	Number of observations	ATP	Standard deviation	Number of observations
KLX13A (432.0–439.2)	1.7×10^4	$\pm 2.7 \times 10^3$	3	1.07×10^4	$\pm 2.86 \times 10^3$	9
KLX17A (416.0–437.5)	2.0×10^5	$\pm 3.8 \times 10^3$	3	8.25×10^4	$\pm 8.88 \times 10^3$	9
KLX17A (642.0–701.1)	9.9×10^4	$\pm 1.1 \times 10^4$	3	2.22×10^5	$\pm 5.71 \times 10^4$	9

Table A-2. Number of culturable, heterotrophic aerobic bacteria (CHAB) in groundwater from the analysed sections of KLX13A and KLX17A.

Borehole (section m)	CHAB	Standard deviation	Number of observations
KLX13A (432.0–439.2)	1.22×10^3	$\pm 0.006 \times 10^3$	3
KLX17A (416.0–437.5)	1.84×10^3	$\pm 0.17 \times 10^3$	3
KLX17A (642.0–701.1)	1.65×10^3	$\pm 0.07 \times 10^3$	3

Table A-3. Most probable number (MPN) of metabolic groups of microorganisms in groundwater from KLX13A, section 432.0–439.2 m.

Metabolic groups	Cells mL^{-1}	
	MPN	Lower–upper 95% confidence limits
Nitrate-reducing bacteria	800	300–2,500
Iron-reducing bacteria	24	10–94
Manganese-reducing bacteria	8	3–25
Sulphate-reducing bacteria	2.3	0.9–8.6
Autotrophic acetogens	220	100–580
Heterotrophic acetogens	500	200–1,700
Autotrophic methanogens	0.4	0.1–1.7
Heterotrophic methanogens	<0.2	–

Table A-4. Most probable number (MPN) of metabolic groups of microorganisms in groundwater from KLX17A, section 416.0–437.5 m.

Metabolic groups	Cells mL^{-1}	
	MPN	Lower–upper 95% confidence limits
Nitrate-reducing bacteria	1,100	400–3,000
Iron-reducing bacteria	240	100–940
Manganese-reducing bacteria	50	20–170
Sulphate-reducing bacteria	300	100–1,200
Autotrophic acetogens	700	300–2,100
Heterotrophic acetogens	220	100–580
Autotrophic methanogens	0.2	0.1–1.1
Heterotrophic methanogens	<0.2	–

Table A-5. Most probable number (MPN) of metabolic groups of microorganisms in groundwater from KLX17A, section (642.0–701.1 m).

Metabolic groups	Cells mL^{-1}	
	MPN	Lower–upper 95% confidence limits
Nitrate-reducing bacteria	500	200–1,700
Iron-reducing bacteria	280	120–690
Manganese-reducing bacteria	300	100–1,300
Sulphate-reducing bacteria	3,000	1000–12,000
Autotrophic acetogens	2,400	1,000–9,400
Heterotrophic acetogens	800	300–2,500
Autotrophic methanogens	0.2	0.1–1.1
Heterotrophic methanogens	<0.2	–

Table A-6. Ratios of the cells cultured using the most probable number (MPN) method (Tables A-3 to A-5), CHAB (Table A-2), and ATP (Table A-1) versus total number of cells (TNC) (Table A-1) in groundwater from KLX13A and KLX17A.

Borehole (section m)	% cultured		Ratio
	MPN/TNC	CHAB/TNC	ATP/TNC
KLX13A (432.0–439.2)	9.15	7.18	0.63
KLX17A (416.0–437.5)	1.31	0.92	0.41
KLX17A (642.0–701.1)	7.35	1.67	2.24