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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

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September 2007

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Abstract

Microorganisms and their characteristic features have been investigated as a part of the geochemical characterization of the groundwater in 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 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.

Samples were taken from borehole KLX08 at 197.0–206.7 m, 396.0–400.9 m, and 476.0–485.6 m. The acridine orange direct count (AODC) test indicated that the total number of microorganisms was highest in the KLX08 396 m section. The number found at KLX08 476 m was the lowest so far found in a total of 10 analysed sections in the Oskarshamn area. 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. The KLX08 197 m section had an unusually high ratio, probably due to a problem with the ATP analysis that rendered a very high number. The ATP/AODC ratio for this section should, therefore, be regarded as uncertain. It was also high in KLX08 476 m possibly due to an unusually low total number count. KLX08 396 m had an ATP/AODC ratio that was very close to the overall average of 0.43 determined for deep groundwater. The CHAB determinations were all low. High numbers of CHAB, above 1000 cells mL^{-1}, may indicate surface water contamination. The CHAB numbers found here suggest that there was no surface water contamination, a finding supported by the drill water control results. The percentages of the AODC culturable using MPN during the site investigation in Oskarshamn ranged from 0.12% to 6.4%. The samples from KLX08 were in the 0.84%–5.1% range. The numbers of nitrate-reducing bacteria (NRB) found were somewhat lower in one case and much higher in the second case than the corresponding CHAB numbers were, which suggests that the majority of the CHAB consisted of facultative anaerobes. They are not indicative of drill water contamination, which would have been the case if the CHAB had outnumbered the NRB.

The numbers of sulphate-reducing bacteria (SRB) in groundwater from the two deepest sections of KLX08 were among the highest for any of the studied microbe types at Oskarshamn, while groundwater from the upper section had the third lowest number of SRB found. Acetogens were present in KLX08 groundwater in numbers in the average range detected in the Oskarshamn site investigations. Heterotrophic methanogens were found in relatively high numbers in Oskarshamn, while autotrophic methanogens were more sparsely observed; this finding was upheld in the groundwater investigated here, except in samples from 396 m, which were below the detection limit for both groups.

Sammanfattning

I den geokemiska karakteriseringen av grundvatten i samband med platsundersökning 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 sulfat-reducerande 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 tre sektioner i borrhålet KLX08, 197,0–206,7 m, 396,0–400,9 m och 476,0–485,6 m. Akridinorange räkningar (AODC) visade att totalantalet celler var högst i grundvatten från KLX08 396 m. Antalet i grundvatten från KLX08 476 m var lägst av alla hittills analyserade prov (10 st) i Oskarshamnsområdet. 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. Grundvatten från sektionen KLX08 197 m hade ett ovanligt högt förhållande som troligen berodde på ett problem med ATP analysen vilket gav ett ovanligt högt värde. Förhållandet i denna sektion ska därför behandlas som ett osäkert värde. Även KLX08 497 m, hade ett högt värde, förmodligen beroende på ett ovanligt låg värde för totalantal. Provet från KLX08 396 m hade ett förhållande som var mycket nära medelvärdet på 0,43 för alla bestämningar i djupa grundvatten. Bestämningarna av CHAB visade alla på låga värden med en viss ökning med djupet. Höga CHAB i antal över 1 000 celler mL^{-1} är sällsynta i djupa grundvatten och kan därför betraktas som en indikation på kontamination med mikroorganismer från ekosystem nära ytan. De antal CHAB som påvisades i proverna från KLX08 tyder således inte på kontamination under borrning eller pumpning, vilket är i överensstämmelse med den spolvattenkontroll som utfördes under borrning av KLX08.

Procentsatsen av totalantalet bakterier (AODC) som har odlas med MPN i prover från platsundersökningarna i Oskarshamn (10 prov) varierar från 0,12 % upp till 6,4 %. De prov som rapporteras här låg i området mellan 0,84 % och 5,1 %. Antalet påvisade nitrat-reducerande-bakterier (NRB) var något lägre än antalet CHAB i provet från KLX08 197 m och mycket högre i KLX08 396 m. Detta tyder på att huvuddelen av de påvisade CHAB var fakultativa anerober. De är inte indikatororganismer för ytvattenkontamination, vilket skulle ha varit fallet om CHAB hade påvisats i betydligt större antal än NRB. Procentsatsen av AODC som har kunnat odlas med MPN-metoden under platsundersökningarna i Oskarshamn varierade från 0,008 % upp till 8,7 %. Proverna som rapporteras här låg i området mellan 0,008 % och 0,44 %. Antalet nitrat-reducerande bakterier (NRB) var lika högt eller betydligt högre än antalet CHAB, vilket tyder på att majoriteten av de påviade CHAB var fakultativa anaerober. Denna grupp bakterier är inte typisk för ytvatten vilket skulle ha varit fallet om antalet CHAB varit mycket högre än NRB.

Antalet sulfatreducerande bakterier (SRB) var bland de högsta som uppmätts under platsundersökningarna i Oskarshamn (10 prov) i grundvatten från de två djupaste sektionerna av KLX08. Grundvatten från den översta sektionen hade det tredje lägsta antalet SRB. Acetogener påträffades i antal som låg i mitten av det intervallet i datamängden från Oskarshamn.Heterotrofa metanogener har tidigare påträffats i förhållandevis höga antal i Oskarshamn medan autotrofa metanogener har påträffats i mer begränsade antal. Denna trend bibehölls i proverna från KLX08.

Contents

1 Introduction

This document reports the performance and results of microbe investigations in borehole KLX08 as part of the site investigation programme in Oskarshamn /1/. Microbiological data from the following three borehole sections are presented:

- KLX08, 197.00–206.65 m.
- KLX08, 369.00–400.87 m.
- KLX08, 476.00–485.62 m.

The sampling was carried out in December 2005 and in March and June 2006, as part of the hydrochemical characterisation activities in KLX08, according to the AP PS 400-05-047 activity plan (SKB internal control document; see Table 1-1). The sampling process and the down-hole sampling equipment are described elsewhere /2/. 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 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, only determined once for the borehole reported here. Duplicate sampling and analysis according to standard procedures was not performed. The data set for assessing possible drill water contamination of the samples obtained from KLX08 is thus very limited /3/.

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

Activity Plan	Number	Version
Fullständig kemikaraktärisering med mobilt fältlaboratorium i KLX08.	AP PS 400-05-047	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 /4/. Active microorganisms influence the groundwater geochemistry /5/ and redox potential /6/. 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 /4/. 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 /5/. 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 /7, 8/. These protocols cover the determination of the total number of cells in groundwater (AODC), 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 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/ 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 plans. 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 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 Ziess, 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 that 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 /9/.

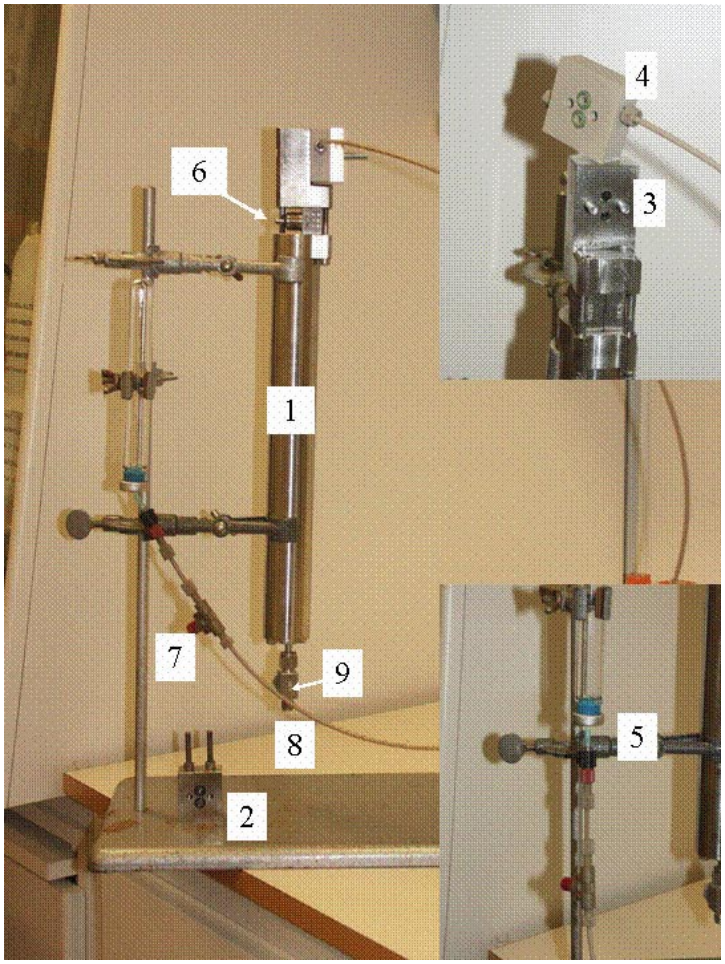

Figure 3-1. *This setup was designed for the oxygen-free transfer of samples from the PVB sampler (1) to nitrogen-flushed, anaerobic tubes stoppered with butyl rubber stoppers (5). (1) PVB sampler, (2) transportation seal, (3) inlet/outlet of the PVB, (4) PEEK sampling device, (5) transfer of sample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

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 /10/. 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}.

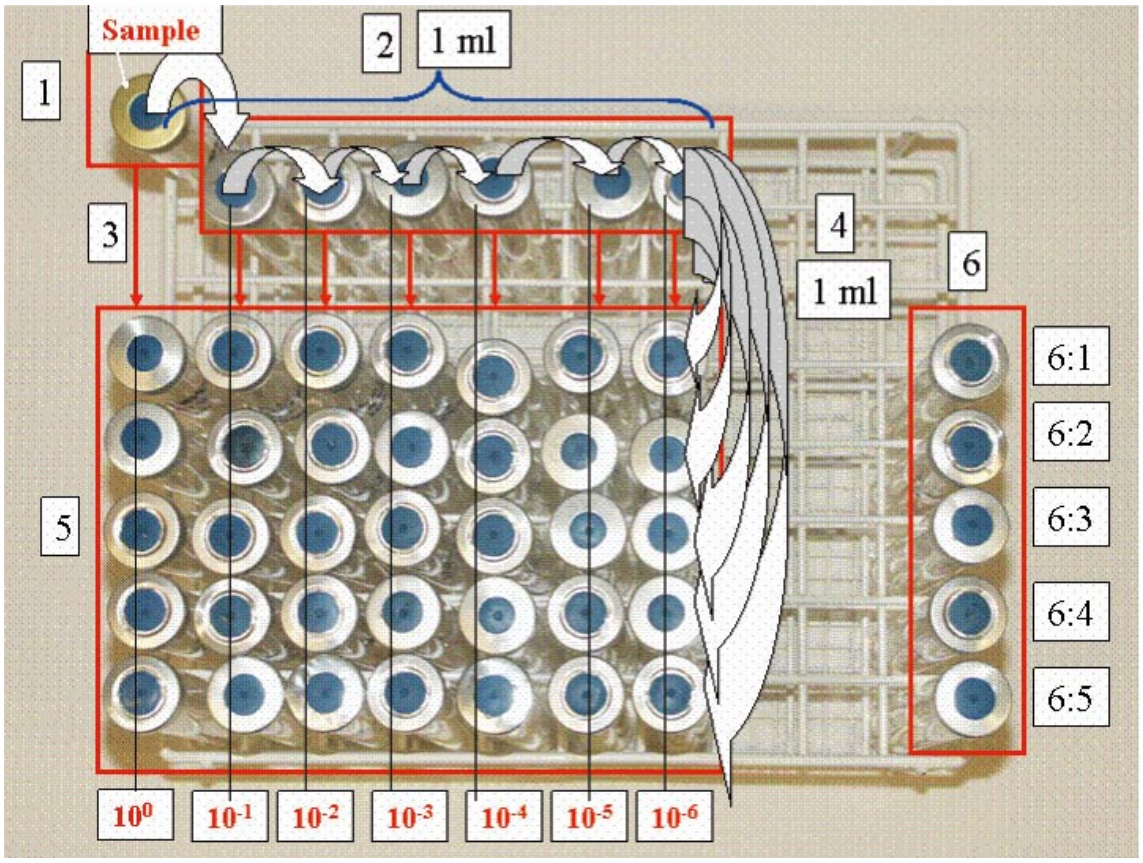

Figure 3-2. *The procedure for most probable number determination. The tube containing the sample is used as the inoculation source (1). Serial dilution is performed first (2); thereafter, subsamples are transferred (3–4) to the growth tubes (5) and control tubes (6).*

3.6.5 Acetate from acetogens

A model 10-148-261-035 kit (Boehringer Mannheim/R-Biopharm Enzyme BioAnalyis, Food diagnosticsm 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 3,400 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 /7, 8/. Quality control procedures have continuously been applied to the analyses of MPN, 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 /11/. 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-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 characterisations 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 on the day of arrival of the samples. ATP was measured on the arrival day and the results were obtained directly. The samples for determination of the total number of cells 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 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 /9/.

4.5 Nonconformities

The MPN of NRB for groundwater from KLX08 476 m was not obtained due to technical problems encountered during the analysis. Likewise, there was a problem with the ATP analysis of groundwater from KLX08197 m and that data should be taken as very uncertain.

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 days 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. The ATP measurements were performed three times for each sample from the different depths; the mean of the three 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 most probable number for each microbial group, as described elsewhere /9/.

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 number of cells was highest in the groundwater sample from KLX08 396 m (Figure 6-1). The number found in the sample from KLX08 476 m was the lowest so far found in a total of 10 analysed samples from the Oskarshamn area (Figure 6-2) /11, 12/. In contrast, the KLX08 197 m sample had the second highest number.

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 count, and to obtain an indication of the activity and viability of the detected microbes, a new 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 AODC and ATP of microbes 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 TNC, revealed that there was a large range of values, for the total dataset, distributed over the averages. The results strongly suggest that ATP to AODC ratios 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 above this average indicate populations that

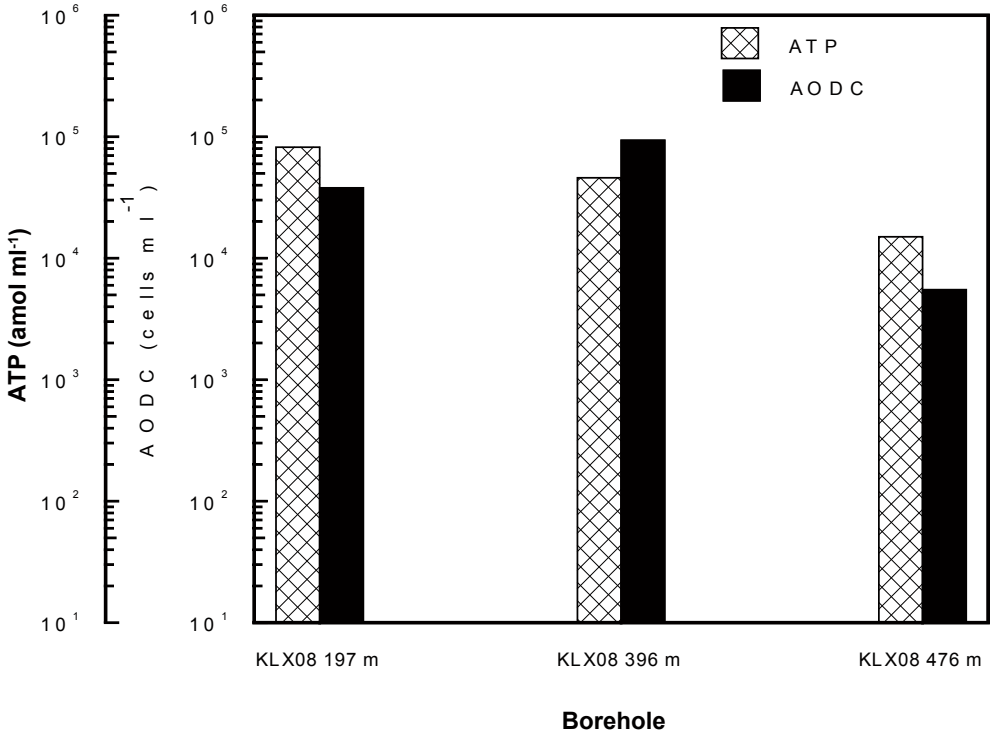

Figure 6-1. *The total numbers of cells (AODC) and the ATP concentrations in the analysed samples from borehole KLX08.*

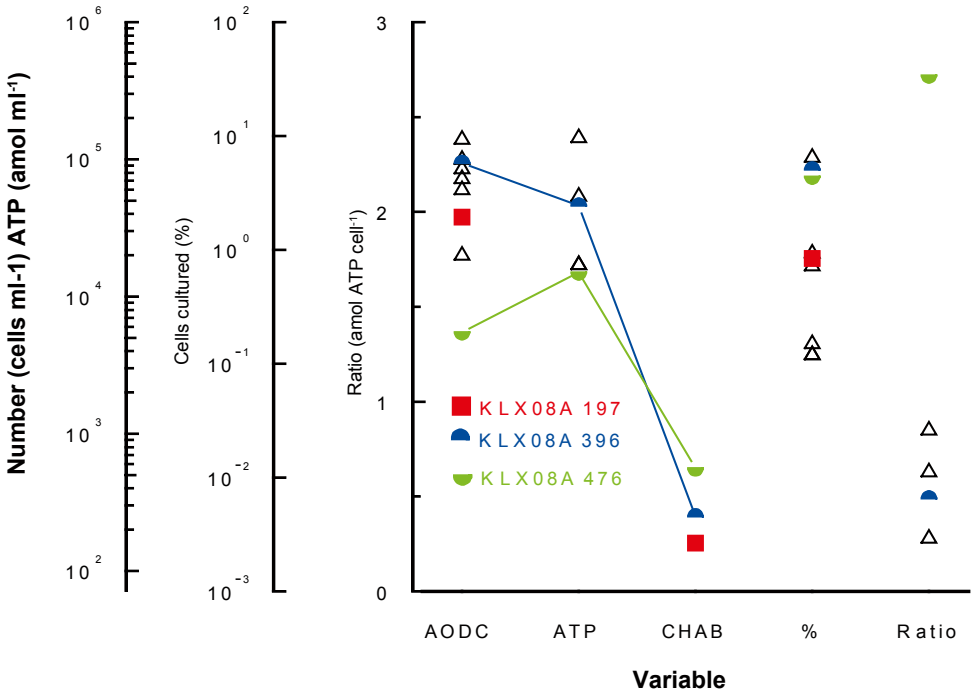

Figure 6-2. *A compilation of all data from the Oskarshamn site investigation /11, 12/ regarding the total number of cells (AODC), ATP, culturable heterotrophic bacteria (CHAB), the % of AODC that could be cultured using the MPN method, and the amount of ATP per total number of cells. At most, 10 samples from 10 sections in three boreholes were analysed. The coloured symbols show the respective borehole sections reported here.*

are more active than are those with ratios below the average. The sample from KLX08 197 m had an unusually high ATP/AODC ratio. This is probably due to a problem with the AODC that rendered a very high value for this sample. The ATP/AODC ratio for this section should, therefore, be regarded as uncertain. The ATP/AODC ratio for the KLX08 197 m sample ws also unusually high, possibly due to a unusually low AODC determined for the groundwater from this section. Groundwater from KLX08 396 m has an ATP/AODC ratio very close to the overall average of 0.43 determined for deep groundwater /13/.

6.2 Numbers of culturable microorganisms

The CHAB determinations were all low, with an increasing range of numbers with depth (Figure 6-2). High numbers of CHAB, above 1,000 cells mL^{-1}, may indicate surface water contamination. The CHAB numbers found here suggest that there was no surface water contamination, a finding supported by the drill water control results /3/.

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 Oskarshamn ranged from 0.12% to 6.4%, i.e. a 50 times range (Figure 6-2). The groundwater samples from the borehole sections reported here were in the 0.84% to 6.4% 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 and A-4) were somewhat lower in one case and much higher in another case than those of CHAB (Table A-1), which suggests that the majority of 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 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 Oskarshamn (10 data points) generally indicate low numbers of IRB and MRB (Figure 6-4). Groundwater from the shallowest borehole section analysed here (KLX08 197 m) contained far fewer IRB that did groundwater from the two other borehole sections analysed (Figure 6-3).

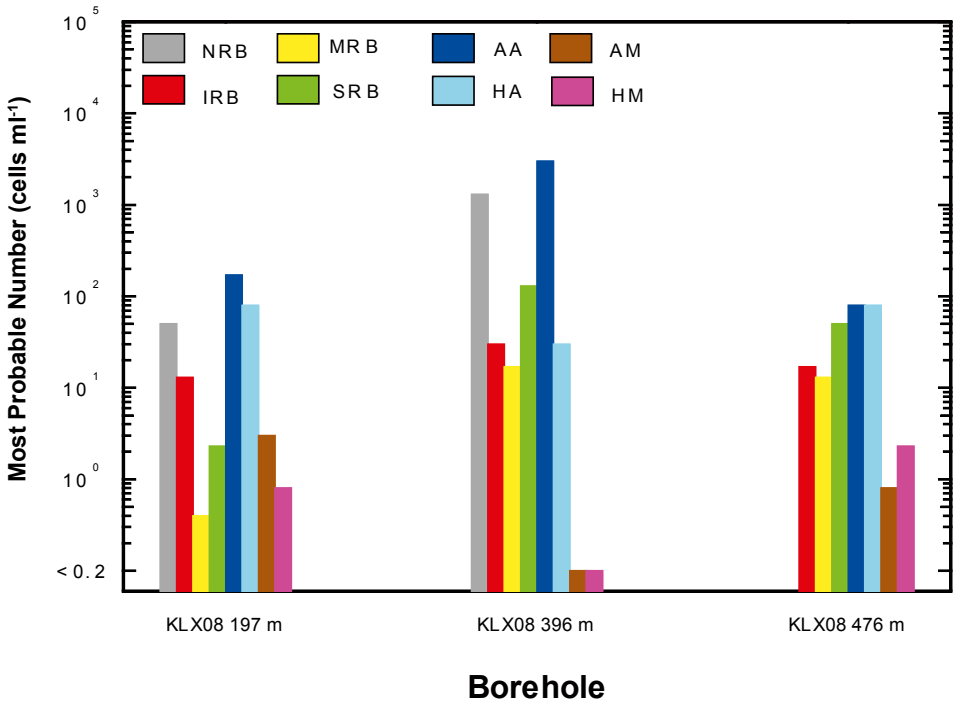

Figure 6-3. *Most probable numbers (MPN) of analysed physiological groups in groundwater samples from KLX08. Abbreviations: NRB (nitrate-reducing bacteria), MRB (manganese-reducing bacteria), AA (autotrophic acetogens), AM (autotrophic methanogens), IRB (iron-reducing bacteria), SRB (sulphate-reducing bacteria), HA (heterotrophic acetogens), and HM (heterotrophic methanogens).*

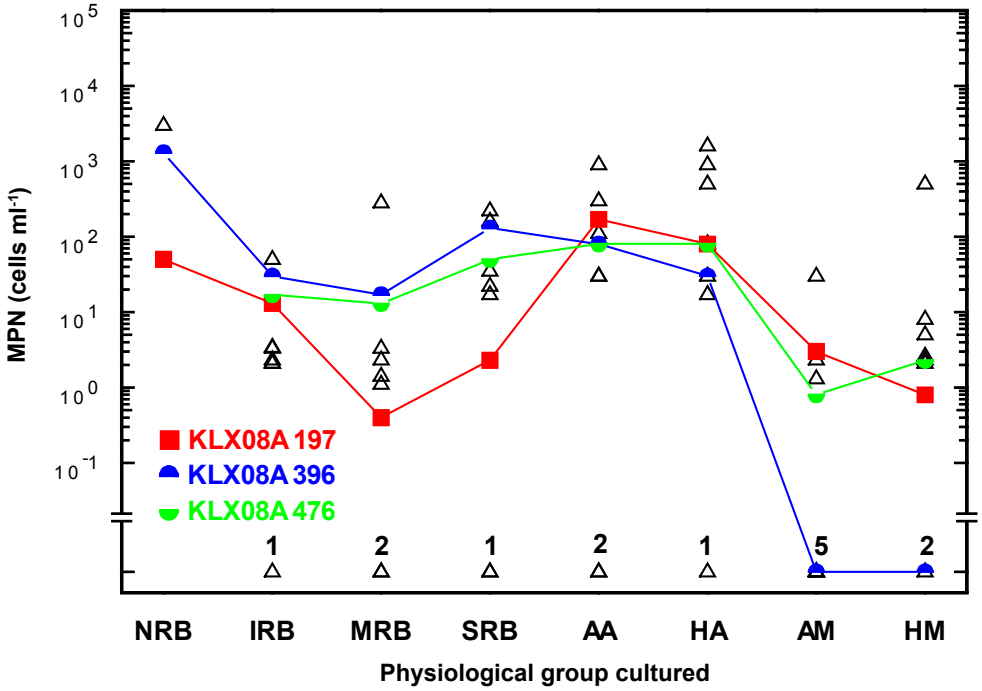

Figure 6-4. *A compilation of all MPN data from the Oskarshamn site investigation /11, 12/. A total of 10 samples from 10 sections in three boreholes have been analysed. The lines connect the individual borehole sections reported here. The number of negative observations for each analysed group is indicated above each lowest symbol.*

6.2.3 Sulphate-reducing bacteria

The numbers of SRB were among the highest of all microbe numbers from Oskarshamn in the samples from the two deepest sections of KLX08 (Figure 6-4), while the sample from the upper section had the third lowest number of SRB found.

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 groundwater investigated here in numbers that were average for the microbes detected in Oskarshamn (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 Oskarshamn, while autotrophic methanogens have been more sparsely observed /11, 12/ (Figure 6-4). This finding was upheld in the samples investigated here, except in groundwater from KLX08 396 m, in which both groups were below the detection limit (Figures 6-3 and 6-4).

7 Conclusions

- The total number of cells was highest in the groundwater sample from KLX08 396 m. The number found in groundwater from KLX08 476 m was the lowest so far found in a total of 10 analysed sections in the Oskarshamn area.
- The groundwater from KLX08 197 m and KLX08 476 both had unusually high ATP/AODC ratios, while KLX08 396 m groundwater had an ATP/AODC ratio very close to the overall average of 0.43 determined for deep groundwater.
- All the CHAB numbers determined were low. High numbers of CHAB, above 1,000 cells mL^{-1}, may indicate surface water contamination. The CHAB numbers found here suggest that there was no surface water contamination, a finding supported by the drill water control results.
- The percentages of the AODC cultivatable using MPN were in the 0.84% to 6.4% range.
- The numbers of SRB in samples from the two deepest sections of KLX08 were among the highest detected in Oskarshamn for any of the investigated microbe types.
- Acetogens are a very versatile and common group, present in KLX08 groundwater in numbers that were average for the microbes detected in Oskarshamn.
- Heterotrophic methanogens were found in relatively high numbers in Oskarshamn, while autotrophic methanogens were more sparsely observed. That finding was upheld in the groundwater samples investigated here, except in KLX08 396 m groundwater in which both groups were below the detection limit.

8 References

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Appendix – Data

Table A-1. Total number of cells and concentration of ATP in groundwater from the analysed sections of KLX08.

Borehole (section m)	Total counts (cells mL^{-1})			ATP (amol mL^{-1})		
	AODC	Standard deviation	Number of observations	ATP	Standard deviation	Number of observations
KLX08 (197.00–206.65)	3.8×10^{4}	$\pm 1.6\times10^{4}$	6	8.2×10^{5}	$\pm 6.1\times10^{4}$	3
KLX08 (396.00–400.87)	9.4×10^{4}	$\pm 1.2\times10^{4}$	6	4.6×10^{4}	$\pm 4.3\times10^{3}$	3
KLX08 (476.00–485.62)	5.5×10^{3}	$\pm 1.5\times10^{3}$	3	1.5×10^{4}	$\pm 0.28\times10^{3}$	3

Table A-2. Number of culturable, heterotrophic aerobic bacteria (CHAB) in groundwater from the analysed sections of KLX08.

Borehole (section m)	CHAB	Standard deviation	Number of observations
KLX08 (197.00–206.65)	0.16×10^{3}	$\pm 0.02\times10^{3}$	3
KLX08 (396.00–400.87)	0.25×10^{3}	$\pm 0.03\times10^{3}$	3
KLX08 (476.00–485.62)	0.56×10^{3}	$\pm 0.32\times10^{3}$	3

Table A-3. Most probable number (MPN) of metabolic groups of microorganisms in groundwater from KLX08, section 197.00–400.87 m.

Metabolic groups	Cells mL^{-1}	
	MPN	Lower–upper 95% confidence limits
Nitrate-reducing bacteria	50	20–170
Iron-reducing bacteria	13	5–39
Manganese-reducing bacteria	0.4	0.1–1.7
Sulphate-reducing bacteria	2.3	0.9–8.6
Autotrophic acetogens	170	70–480
Heterotrophic acetogens	80	30–250
Autotrophic methanogens	3.0	1.0–11
Heterotrophic methanogens	0.8	0.3–2.4

Table A-4. Most probable number (MPN) of metabolic groups of microorganisms in groundwater from KLX08, section 396.00–400.87 m.

Metabolic groups	Cells mL^{-1}	
	MPN	Lower–upper 95% confidence limits
Nitrate-reducing bacteria	1,300	500–3,900
Iron-reducing bacteria	30	10–120
Manganese-reducing bacteria	17	7–40
Sulphate-reducing bacteria	130	50–390
Autotrophic acetogens	3,000	1,000–13,000
Heterotrophic acetogens	300	100–1,300
Autotrophic methanogens	< 0.2	–
Heterotrophic methanogens	< 0.2	–

Table A-5. Most probable number (MPN) of metabolic groups of microorganisms in groundwater from KLX08, section 476.00–485.62 m.

Metabolic groups	Cells mL^{-1}	
	MPN	Lower–upper 95% confidence limits
Nitrate-reducing bacteria	–*	–
Iron-reducing bacteria	17	7–48
Manganese-reducing bacteria	13	5–39
Sulphate-reducing bacteria	50	20–170
Autotrophic acetogens	80	30–250
Heterotrophic acetogens	80	30–250
Autotrophic methanogens	0.8	0.3–2.4
Heterotrophic methanogens	2.3	0.9–8.6

* Not analysed due to technical problems

Table A-6. Ratios of the cells cultured using MPN (Tables A-3 to A-5) and ATP (Table A-1) versus total number of cells (AODC) (Table A-1) in groundwater from KLX08.

Borehole (section, m)	% cultured MPN/AODC	CHAB/AODC	Ratio ATP/AODC
KLX08 (197.00–206.65)	0.84	0.42	21.6
KLX08 (396.00–400.87)	5.1	0.26	0.49
KLX08 (476.00–485.62)	4.4	10.2	2.72