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Oskarshamn site investigation

Microorganisms in groundwater from borehole KLX15A – numbers, viability, and metabolic diversity

Results from one section: 623.00–634.51 m in KLX15A

Karsten Pedersen, Microbial Analytics Sweden AB

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Svensk Kärnbränslehantering AB
Swedish Nuclear Fuel
and Waste Management Co
Box 250, SE-101 24 Stockholm
Tel +46 8 459 84 00

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Abstract

Microorganisms and their characteristic features have been investigated as a part of the complete chemical characterization programme in the site investigation programme at Oskarshamn. The investigation consists of determining the total numbers of microorganisms (TNC), 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 KLX15A, 623.00–634.51 m. The sample date was 2007-07-16. The TNC found in groundwater from KLX15A (623.00–634.51 m) were among the average of the number range found in a total of 14 analysed sections in the Oskarshamn site investigation programme. The groundwater sample from KLX15A had a low ATP/TNC ratio. The CHAB and NRB numbers found here suggest that there was no surface water contamination. The percentages of the TNC cultivatable using MPN were the highest, 13.2, observed in a total of 14 analysed sections in the Oskarshamn site investigation programme. The numbers of SRB in samples from the KLX15A were among the average of the number range found in a total of 14 analysed sections in the Oskarshamn site investigation programme. Acetogens are a very versatile and common group, present in KLX15A 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 Oskarshamn site investigation programme, while autotrophic methanogens (AM) were more sparsely observed. That finding was not upheld in the groundwater samples investigated here, where all AM and HM 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.

Provtagning gjordes i en sektion i borrhålet KLX15A, 623,00–634,51 m. Provtagningen utfördes 2007-07-16. Totalantalet celler (TNC) i grundvattenprovet från KLX15A (623,00–634,51 m) var i medelområdet av vad som uppmätts i totalt 14 grundvattenprover från platsundersökningarna i Oskarshamn. 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 KLX15A (623,00–634,51 m) hade ett ovanligt lågt ATP/TNC-förhållande. Antalen av CHAB och nitratreducerande bakterier (NRB) i det undersökta provet från KLX15A tyder starkt på att provet inte var kontaminerat av ytvatten, vilket också stöds av tidigare erhållna resultat från analyser av det spolvatten som användes vid borrningar av borrhålen KLX13A och KLX17A. Andelen av TNC som kunnat odlas med MPN i platsundersökningarna i Oskarshamn varierar från 0,12 % upp till 9,15 %. I den här undersökningen av grundvatten från KLX15A låg procentsatsen på 13,1 %. Antalet sulfatreducerande bakterier (SRB) i KLX17A var i medelområdet av vad som uppmätt i totalt 14 grundvattenprover från platsundersökningarna i Oskarshamn. Acetogena bakterier är en mycket varierad och vanlig grupp och dessa uppmättes i KLX15A i ungefär samma antal som uppmätts i 11 andra grundvattenprov från platsundersökningarna i Oskarshamn. Heterotrofa methanogener (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 AM och HM inte påvisas över detektionsgränsen (0,2 cells mL^{-1}).

Contents

1 Introduction

This document reports the performance and results of microbe investigations in borehole KLX15A as part of the site investigation programme in Oskarshamn /1/. Microbiological data from the following borehole section is presented:

- KLX15A, 623.00–634.51 m, the sampling date was 2007-07-16.

The sampling was carried out as a part of the complete chemical characterization in KLX15A, according to the activity plan AP PS 400-07-053 (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 sample 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 not determined for the boreholes reported here. Triplicate sampling and analysis was performed for the earlier sampled boreholes in KLX13A and duplicate control was performed in KLX17A according to standard procedures. The data set for assessing possible drill water contamination of the samples obtained from KLX13A and KLX17A has been reported /3, 4/.

Original data from the reported activity are stored in the primary data base SICADA. 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. Control documents for performance of the activity.

Activity plan	Number	Version
Fullständig kemikaraktärisering i KLX15A.	AP PS 400-07-053	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 (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 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.

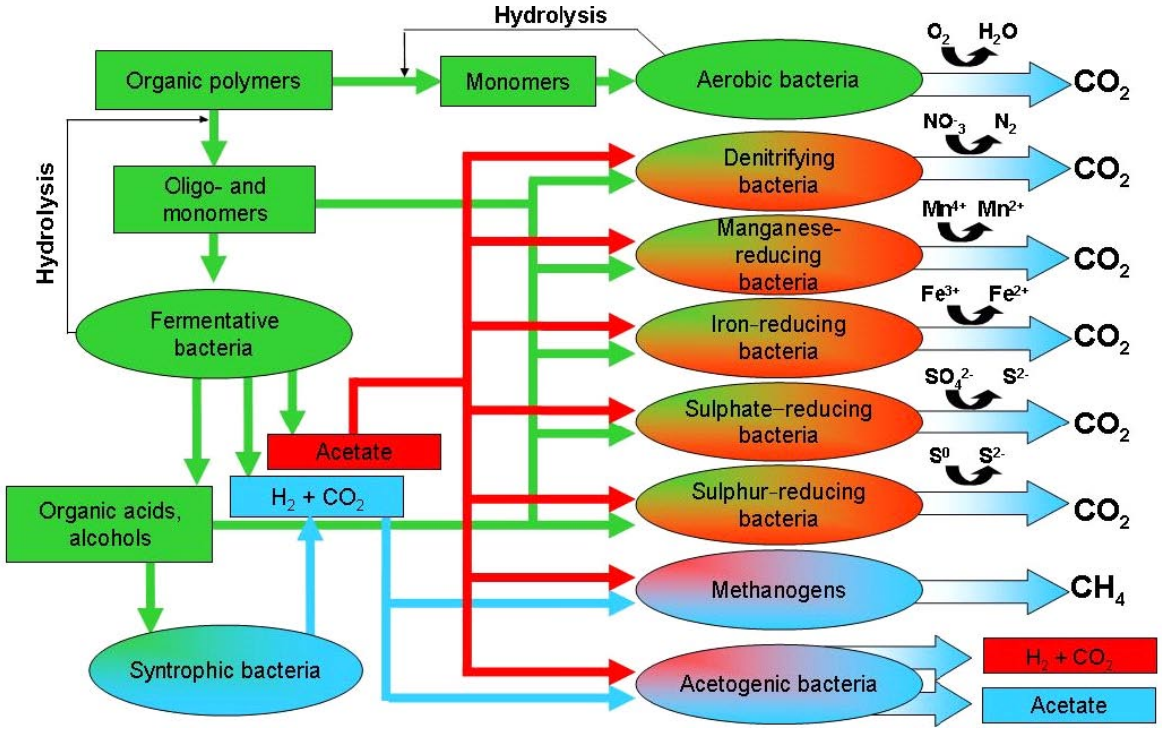

Figure 2-1. *Possible pathways for the flow of carbon in the subterranean environment. Organic carbon is respired with oxygen, if present, or else fermentation and anaerobic respiration occur with an array of different electron acceptors.*

2.1 Objectives

The microbial communities occurring in granitic rock from the surface to a depth of at most 1,700 m have been studied for two decades /6/. 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.

- The major objective here was to enumerate all physiological groups of microorganisms that, through their growth and metabolising 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 /8, 9/ These protocols cover the determination of the total number of cells in groundwater (TNC), 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 the borehole section /2/ and sent to the laboratory in Göteborg within 4–6 hours; 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 sample from the PVB sampler to the culturing tubes required a procedure that did not expose the sample 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 elsewhere /10/. 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

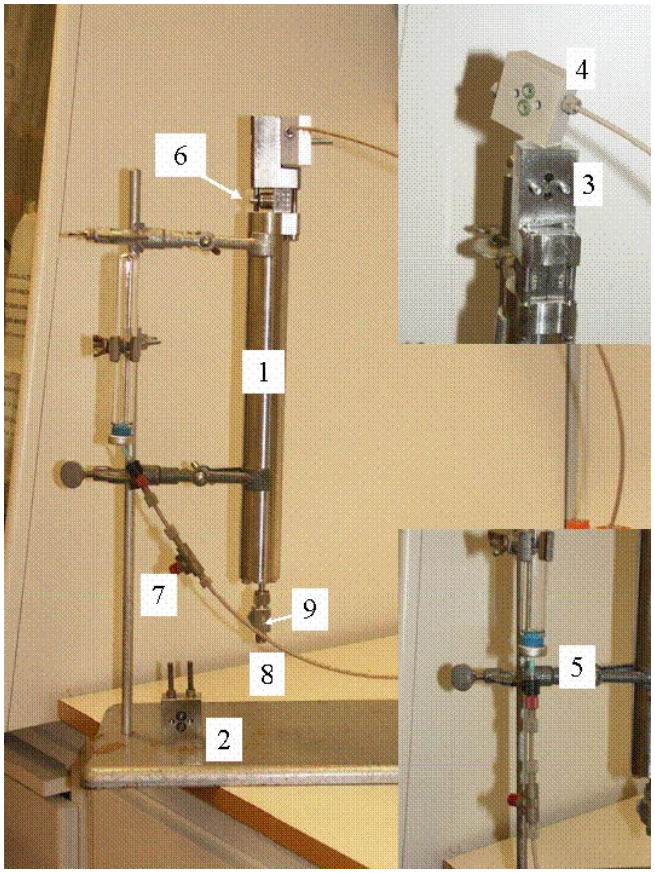

Figure 3-1. *This setup was designed for the oxygen-free transfer of samples from the PVB sampler (1) to nitrogen-flushed, anaerobic tubes stoppered with butyl rubber stoppers (5). (1) PVB sampler, (2) transportation seal, (3) inlet/outlet of the PVB, (4) PEEK sampling device, (5) transfer of sample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

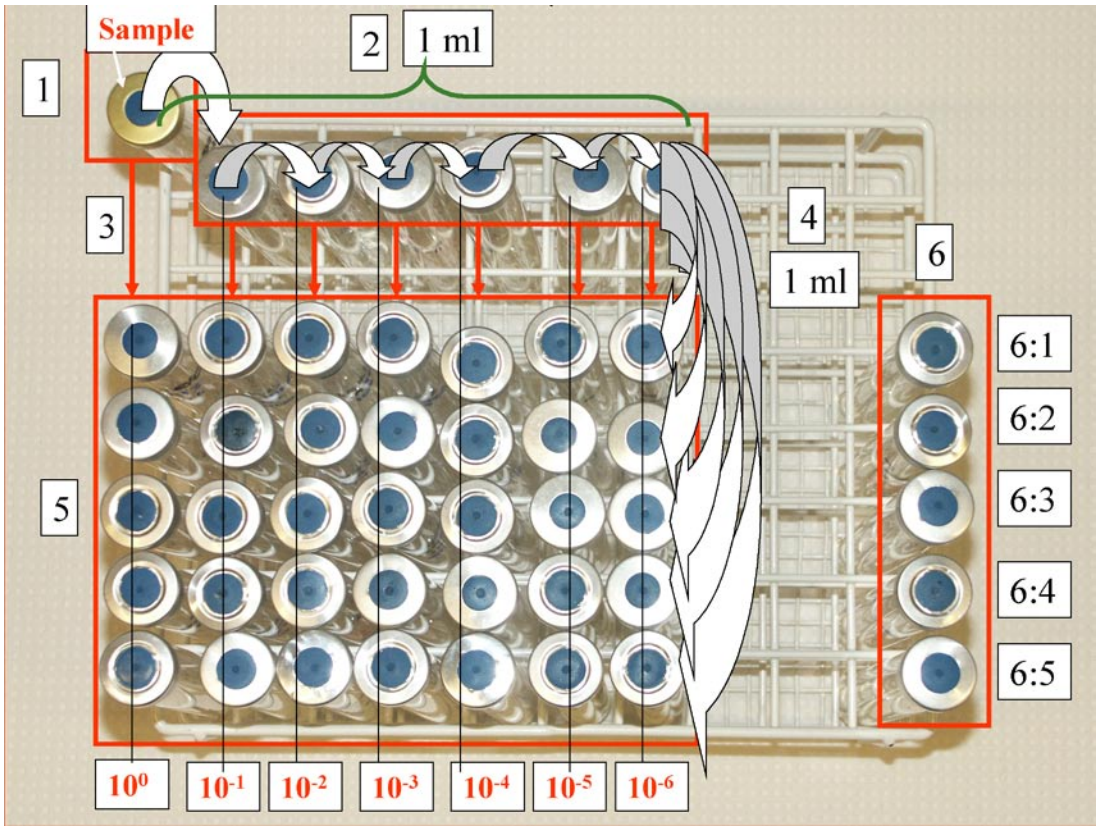

Figure 3-2. *The procedure for most probable number determination. The tube containing the sample is used as the inoculation source (1). Serial dilution is performed first (2); thereafter, subsamples are transferred (3–4) to the growth tubes (5) and control tubes (6).*

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 (TNC) was determined using an acridine orange direct count procedure. All solutions used were filtered through sterilised 32 mm diameter, 0.2 µm pore size Filtropur S syringe filters (Sartorius, GTF, Göteborg, Sweden). Prior to filtration, stainless steel analytical filter holders, 13 mm (no. XX3001240; Millipore, Solna, Sweden), were rinsed with sterile filtered, analytical grade water (AGW) (Millipore Elix 3, Millipore, Solna, Sweden). 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, Solna, Sweden). The filtered cells were stained for 5 minutes 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). 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.

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, 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 /10/, and 15 g L^{-1} of agar (Merck). The medium was sterilised in 1-L batches by autoclaving at 121°C for 20 minutes; 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 (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 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 materials, 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-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 off. 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 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 /11/. 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 /10/. 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 with tubes that 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 most probable numbers of all microbial groups, as described elsewhere /10/.

3.6.1 Nitrate consumed by nitrate-reducing bacteria

A chromotropic method (0.2–30 mg L^{-1} NO_3^- - N) was used, according to HACH DR/2500, Method 10020 for water and wastewater.

3.6.2 Ferrous iron from iron-reducing bacteria

A phenanthroline method (0.02–3 mg L^{-1} 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^{-1} 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 (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 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^{-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 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 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 /10/. 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 should be decontaminated with a 10 ppm (or more) solution of chlorine dioxide – XiniX FreeBact-20 (DTI Sweden, Märsta, Sweden) which 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 /10, 12, 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 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 /10/.

5 Data handling

5.1 Analyses and interpretation

The total numbers of microorganisms were counted on one filtration filters from three sample tubes. Each filter was regarded as one independent observation. The mean of three 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 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 most probable number for each microbial group, as described elsewhere /10/.

6 Results

The detailed results are given in the Appendix.

6.1 Total number of microorganisms and ATP concentration

The AODC indicated the TNC in the samples (Table A-1). The number found in the sample from KLX15A (623.00–634.51 m) was in the average number range so far found in a total of 14 analysed samples from the site investigation programme in Oskarshamn area (Figure 6-1) /12, 13, 14, 15/. The ATP concentration correlated well with the TNC. The CHAB were the highest obtained this far, since the onset of this analysis in December 2005.

The TNC numbers, 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 analysis was introduced in December 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 TNC and ATP of microbes has previously been performed /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. The 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 KLX15A (623.00–634.51 m) had a low ATP/TNC ratio which did not correlate well with a high percentage of the TNC that could be cultivated with MPN (Figure 6-1, Table A-4). The percent of TNC cultured with MPN suggests that the microbial populations analysed were very active in this groundwater. The percentage of TNC cultivated with CHAB was also high, relative to the other groundwater samples analysed from the site investigation programme in Oskarshamn. It seems clear that the microbial population in KLX15A (623.00–634.51 m) was diversified and active as judged from the cultivation data, although contradicted by the low ATP/TNC quotient.

6.2 Numbers of culturable microorganisms

The CHAB determination was higher than found previously in groundwater from the Oskarshamn site investigations (Figure 6-1). The analysis of CHAB was done under aerobic conditions in opposite to all other cultivation methods that were performed under anaerobic conditions. Many bacteria are known to be facultative anaerobes. These can switch from an aerobic respiration with oxygen to anaerobic respiration with nitrate and commonly also with ferric iron and manganese(IV) as alternative electron acceptors (Figure 2-1). Microorganisms in groundwater must be adapted to anoxic conditions, but if oxygen appear for some reason, it is advantageous for the microbe to switch to oxygen respiration. Indigenous groundwater microorganisms should consequently be detectable both as CHAB and NRB, while contaminants from the surface should have a smaller tendency to do so. A comparison of the CHAB data with the NRB data shows a reasonable good correlation (Tables A-2 and A-3), 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 that unfortunaltely can not be confirmed with drill water data, as such an investigation was not done this time.

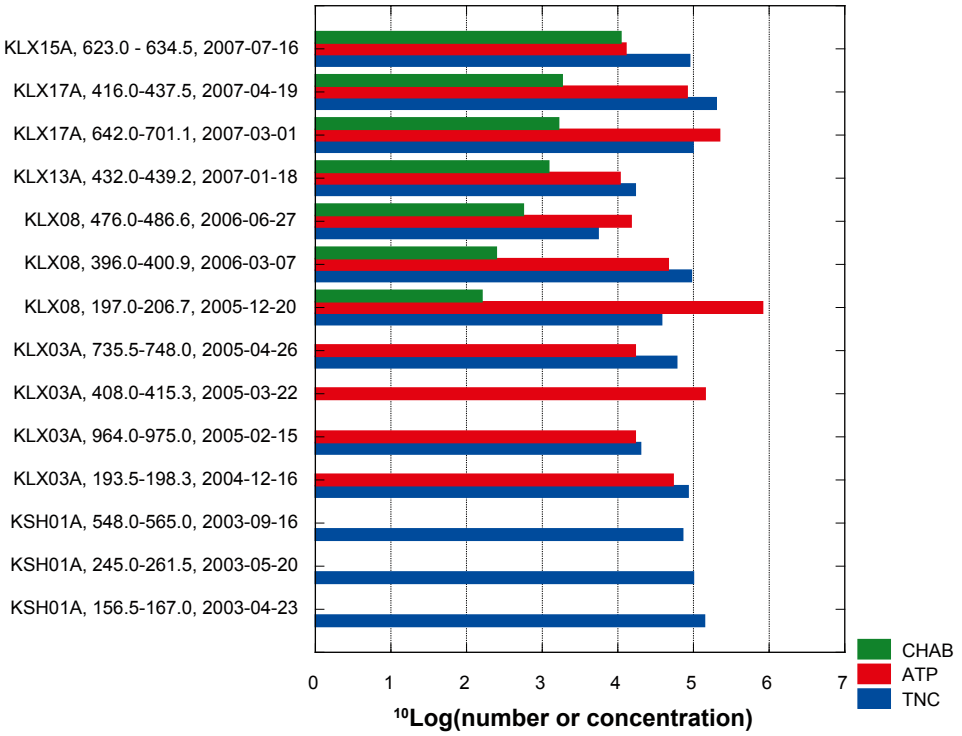

Figure 6-1. *The numbers of culturable heterotrophic, aerobic bacteria (CHAB, cells mL^{-1}), the ATP concentrations (amol mL^{-1}) and the total numbers of cells (TNC, cells mL^{-1}) in the analysed groundwater samples from boreholes KLX15A (Tables A-1 and A-2), in comparison with all previously obtained data /12, 13, 14, 15/.*

The percentages of the TNC that was 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 12.4%, which is the highest quotient observed (Table A-4).

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 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 CHAB (Table A-1) found were within the 95% confidence intervals of the NRB values (Table A-3), 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 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 site investigation programme in Oskarshamn (14 data points) generally indicate low numbers of IRB and MRB (Figure 6-4). Groundwater from KLX15A, together with the previously analysed borehole KLX17A showed among the highest values found in the Oskarshamn site investigations of IRB and MRB.

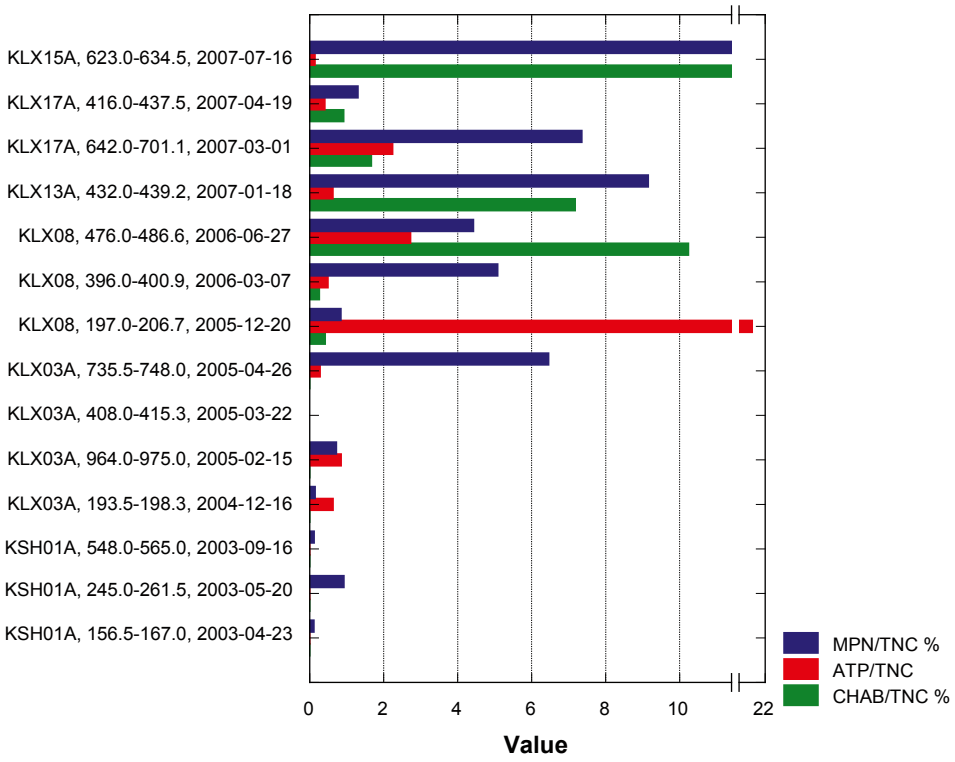

Figure 6-2. *A compilation of the ratios of ATP to the total number of cells (TNC) (amol cell^{-1}) and the percentages of the TNC that could be cultured using the most probable number (MPN) and the culturable heterotrophic aerobic bacteria (CHAB) methods (Table A-4). All previous data from the Oskarshamn site investigation /12, 13, 14, 15/ are shown as well for comparison. Missing bars reflect lack of data due to that the methods for CHAB and ATP has been added consecutively after start of the Oskarshamn site investigation programme.*

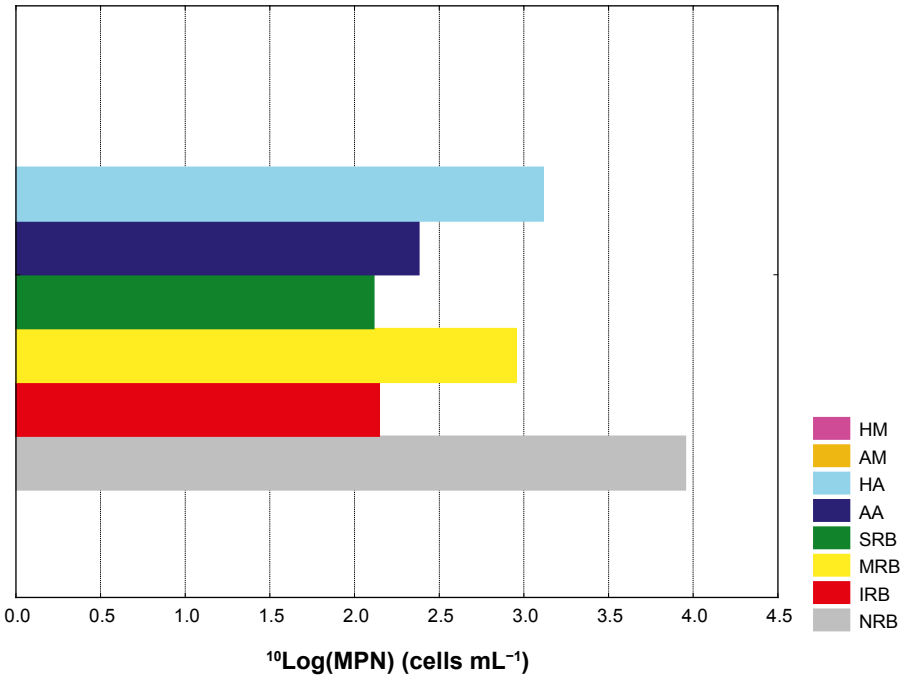

Figure 6-3. *Most probable numbers (MPN) of analysed physiological groups in groundwater samples from KLX15A (623.00–634.51 m). Abbreviations: NRB (nitrate-reducing bacteria), MRB (manganese-reducing bacteria), AA (autotrophic acetogens), AM (autotrophic methanogens), IRB (iron-reducing bacteria), SRB (sulphate-reducing bacteria), HA (heterotrophic acetogens), and HM (heterotrophic methanogens).*

6.2.3 Sulphate-reducing bacteria

The numbers of SRB in KLX15A (623.00–634.51 m) were the average of the SRB number range from Oskarshamn site investigations (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 groundwater investigated here in numbers that were average for the microbes detected in the Oskarshamn site investigation (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 /12, 13, 14, 15/ (Figure 6-4). This finding was not upheld in the samples investigated here where both the AM and the HM analyse returned below detection data (0.2 cells mL^{-1}) (Table A-3).

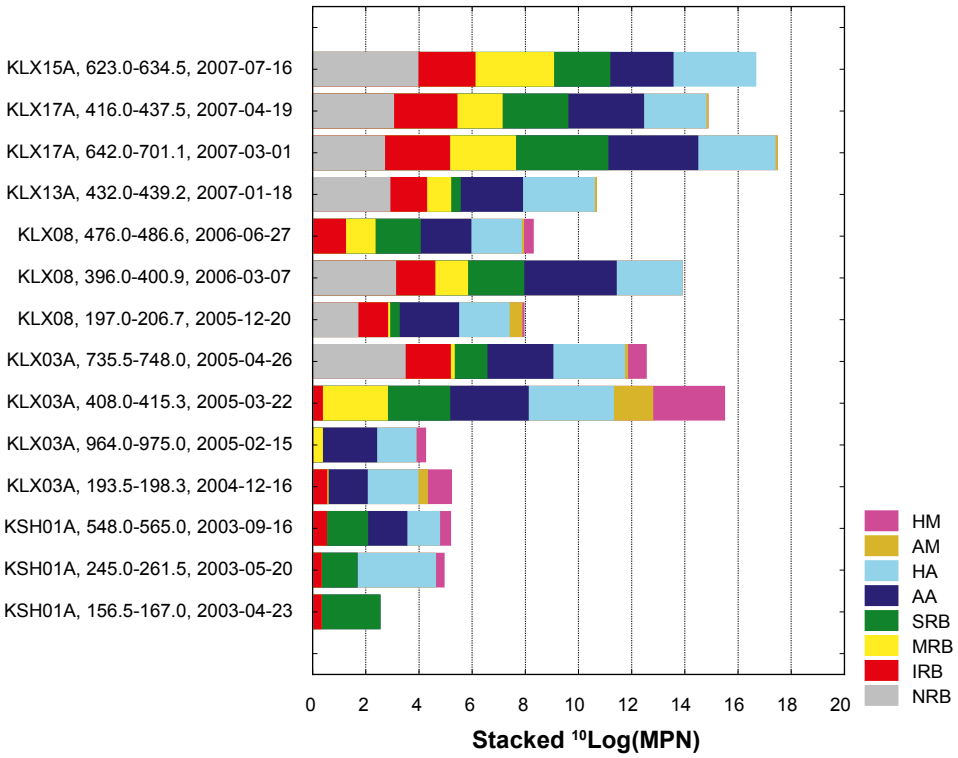

Figure 6-4. *Stacked values of most probable numbers of physiological groups of microorganisms in KLX15A (623.00–634.51 m). All previous data from the Oskarshamn site investigation /12, 13, 14, 15/ are shown as well for comparison. NRB = nitrate-reducing bacteria, IRB = iron-reducing bacteria, MRB = 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.*

7 Conclusions

- The number found in groundwater from KLX15A (623.00–634.51 m) were among the average of the number range found in a total of 14 analysed sections in the site investigation programme in Oskarshamn.
- The groundwater sample from KLX15A (623.00–634.51 m) had a low ATP/TNC ratio.
- 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 percentage of the TNC cultivatable using MPN were the highest, 13.2 observed in a total of 14 analysed sections in the site investigation programme in Oskarshamn.
- The number of SRB in samples from the KLX15A (623.00–634.51 m) were among the average of the number range found in a total of 14 analysed sections in the site investigation programme in Oskarshamn.
- Acetogens are a very versatile and common group, present in KLX15A (623.00–634.51 m) groundwater in numbers that were average for the microbes detected in 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. That finding was not upheld in the groundwater samples investigated here where all AM and HM data were below the detection limit (0.2 cells mL^{-1}).

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9 Appendix

Data

Table A-1. Total number of cells and concentration of ATP in groundwater from the analysed section of KLX15A, section 623.00–634.51.

Borehole (section m)	Total counts (cells mL^{-1})			ATP (amol mL^{-1})		
	TNC	Standard deviation	Number of observations	ATP	Standard deviation	Number of observations
KLX15A (623.00–634.51)	8.9×10^4	$\pm 4.5\times10^3$	3	1.28×10^4	$\pm 1.74\times10^3$	9

Table A-2. Number of culturable, heterotrophic aerobic bacteria (CHAB) in groundwater from the analysed section (623.00–634.51 m) of KLX15A.

Borehole (section m)	CHAB	Standard deviation	Number of observations
KLX15A (623.00–634.51)	11.0×10^3	$\pm 0.44\times10^3$	3

Table A-3. Most probable number (MPN) of metabolic groups of microorganisms in groundwater from KLX15A, section 623.00–634.51.

Metabolic groups	Cells mL^{-1}	
	MPN	Lower–upper 95% confidence limits
Nitrate-reducing bacteria	9,000	4,000–25,000
Iron-reducing bacteria	140	60–360
Manganese-reducing bacteria	900	300–2,900
Sulphate-reducing bacteria	130	50–390
Autotrophic acetogens	240	100–940
Heterotrophic acetogens	1,300	500–3,900
Autotrophic methanogens	< 0.2	–
Heterotrophic methanogens	< 0.2	–

Table A-4. Ratios of the cells cultured using MPN (Tables A-3), CHAB (Table A-2) and ATP (Table A-1) versus total number of cells (TNC) (Table A-1) in groundwater from KLX15A, section 623.00–634.51.

Borehole (section, m)	% cultured		Ratio
	MPN/TNC	CHAB/TNC	ATP/TNC
KLX15A (623.00–634.51)	13.16	12.36	0.144