Working Report 2010-60

Microbiology of Olkiluoto and ONKALO Groundwater

Results and Interpretations, 2008—2009

Karsten Pedersen, Johanna Arlinger,
Johanna Edlund, Lena Eriksson,
Sara Lydmark, Jessica Johansson,
Sara Jägevall and Lisa Rabe

August 2010

POSIVA OY
Olkiluoto
FI-27160 EURAJOKI, FINLAND
Tel +358-2-8372 31
Fax +358-2-8372 3709

Working Report 2010-60

Microbiology of Olkiluoto and ONKALO Groundwater

Results and Interpretations, 2008–2009

Karsten Pedersen, Johanna Arlinger,
Johanna Edlund, Lena Eriksson,
Sara Lydmark, Jessica Johansson,
Sara Jägevall and Lisa Rabe
Microbial Analytics Sweden AB

August 2010

Base maps: ©National Land Survey, permission 41/MML/10

Working Reports contain information on work in progress
or pending completion.

The conclusions and viewpoints presented in the report
are those of author(s) and do not necessarily
coincide with those of Posiva.

ABSTRACT

Microbiology cultivation, DNA, and RNA data were assembled from 18 groundwater samples from Olkiluoto, from deep drillholes ranging in depth from 62 to 708 m, and from groundwater from eight ONKALO drillholes ranging in depth from 7.1 to 318 m. Biomass was determined by counting total numbers of microbial cells (TNC) and determining adenosine triphosphate (ATP) concentrations. The aerobic cultivation method used comprised aerobic plate counts. Anaerobic most probable number (MPN) methods were used to determine counts of nitrate-, iron-, manganese-, and sulphate-reducing bacteria, acetogenic bacteria, and methanogens. Molecular methods for analysing the diversity and abundance of microorganisms have been continuously developed and applied to groundwater samples. These methods included the sampling of DNA and RNA, extraction of nucleic acids, cloning and sequencing of environmental nucleic acids, and real-time quantitative polymerase chain reaction (qPCR) for analysing amounts of DNA and RNA. The results of these analyses have been merged and interpreted, and the outcomes are reported here. The four methods for biomass-related analysis correlated well. These methods focus on different characteristics of microbial cells: TNC analyses whole cells using a microscope, ATP analyses a cell component using a biochemical method, MPN is based on cultivation and qPCR analyses DNA (genes) and RNA (gene expression). The range of analytical focus encompassed by the methods ensures that the biomass-related information in this and previous reports from Olkiluoto and ONKALO is reliable and reflects a diverse range of the biomass-related characteristics of the analysed microorganisms.

The distribution of the MPN data over depth from 2008 to 2009 followed the distribution found earlier. There were generally more cultivable microorganisms between depths of 200 and 400 m than in the shallower 50–200-m depth range. These new results agree with previous results, suggesting that microorganisms are more active in the boundary area between overlying sulphate-rich groundwater and deeper methane-rich groundwater at a depth of approximately 300 m. The MPN analyses have indicated that all the physiological groups analysed for were present in Olkiluoto deep groundwater and in ONKALO groundwater. In 2007, analysis of molecular diversity and numbers started. The molecular results agreed well with the cultivation and biomass results. Sequences belonging to iron-, manganese-, and sulphate-reducing bacteria were found. *Archaea* sequences representative of methane-producing microorganisms were also detected, and sequences identified as belonging to ANME consortia were found. The pumping tests conducted in drillholes OL-KR6_422, OL-KR13 T360, and ONK-PVA3 conclusively demonstrated the importance of understanding the mechanisms that control microbial diversity, activity, and numbers as a function of sampling methodology, including pumping and draining. Flow rates during sampling, volumes let out before the sample was taken, and configuration of drillhole equipment were factors that exerted influence on microbial numbers; they should consequently be further investigated.

Keywords: Olkiluoto, ONKALO, microbiology, archaea, sulphate-reducing bacteria, methanogens, ANME, DNA, RNA

Olkiluodon ja ONKALOn mikrobiologia
Tulokset ja tulkinnat, 2008–2009

TIIVISTELMÄ

Vuosien 2008 ja 2009 aikana suoritettiin 18:sta Olkiluodon syvästä kairareiästä (7-708 metrin syvyydeltä) ja kahdeksasta ONKALOn kairareiästä (7-318 metrin syvyydeltä) mikrobiologisia tutkimuksia perinteisellä viljely- menetelmällä sekä uusilla DNA- ja RNA- menetelmillä. Mikrobien kokonaismassa määritettiin laskemalla mikrobiologisten solujen kokonaismäärä (TNC Total Number of Cells) ja määrittämällä adenosiini trifosfaatti (ATP) konsentraatiot. Aerobinen viljelymenetelmä piti sisällään myös bakteerien luvun määrittämisen. Anaerobista MPN (Most Probable Number) menetelmää käytettiin määritettäessä nitraattia-, rautaa-, mangaania-, ja sulfaattia pelkistävien bakteerien sekä asetogeenisten bakteereiden ja metanogeenien lukumäärä. Diversiteetin ja mikrobien lukumäärän analysointia molekyylimenetelmillä on jatkuvasti kehitetty sekä sovellettu sopimaan paremmin syville pohjavesinäytteille. Nämä menetelmät pitävät sisällään DNA- ja RNA -näytteenoton, nukleiinihapon uuttamisen, kloonauksen, sekä ympäristönukleiinihapon sekvennoinnin, että DNA:n ja RNA:n määrien analysoinnin qPCR -menetelmän (real-time quantitative Polymerase Chain Reaction) avulla. Tässä raportissa on raportoitu edellä mainittuja analyysimenetelmiä käyttämällä saadut mikrobitulokset sekä tulosten tulkinta. Mikrobien kokonaismassan analysoinnissa käytetyt neljä eri menetelmää korreloivat hyvin keskenään. Menetelmät keskittyvät mikrobisolujen eri ominaisuuksiin: TNC analysoidaan mikroskopoimalla koko mikrobisolu, ATP analysoi solukomponentit käyttämällä biokemiallisia menetelmiä, MPN perustuu soluviljelyyn ja qPCR analysoi geenin DNA:n ja RNA:n. Analyysimenetelmien monipuolisuus varmistaa, että mikrobien kokonaismassaan liittyvä informaatio on luotettavaa.

MPN aineisto jakautuu syvyyden mukaan vuoden 2008 ja 2009 tutkimuksissa samaan tapaan kuin aikaisempina vuosina tehdyissä tutkimuksissa. Useimmiten viljelyyn soveltuvia mikrobeja on 200–400 metrin syvyydellä enemmän kuin 50–200 metrin syvyydellä. Nämä uudet tutkimustulokset täsmäävät aikaisempien tulosten kanssa ja viittaavat siihen, että mikrobit ovat aktiivisimpia sulfaatti-rikkaan ja syvän metaani -rikkaan pohjaveden raja-alueella noin 300 metrin syvyydellä. MPN analyysit osoittavat, että kaikki fysiologiset ryhmät, jotka analysoitiin Olkiluodon syvistä pohjavesistä, ovat läsnä. Vuonna 2007 aloitettiin molekyylien diversiteetin ja lukumäärän analysoiminen ja havaittiin, että nämä menetelmät ovat yhdenmukaisia viljely- ja kokonaismääritystulosten kanssa. Sekvenssit raudan-, mangaanin- ja sulfaatin pelkistäjille löydettiin. Tutkimuksessa löydettiin myös Arkkien sekvensseille tyypillinen metaania muodostava mikrobi ja sekvenssi, jonka tunnetaan kuuluvan ANME yhtymään. Kairanrei'issä OL-KR6 (422-425 m), OL-KR13 (361-365 m) ja ONK-PVA3 tehdyt pumppauskokeet osoittavat, että on tärkeää ymmärtää ne mekanismit, jotka kontrolloivat mikrobien diversiteettiä, aktiivisuutta ja lukumäärää näytteenottopumppausten yhteydessä sekä pumppauksen aikana että pumppaustauolla näytteenottovälin ollessa luonnontilassa. Erityisesti on havaittu, että virtaus, ennen näytteenottoa kairareiästä valutettu kokonaisvesimäärä ja näytteenottolaitteiston kokoonpano ovat asioita, jotka vaikuttivat mikrobien kokonaislukumäärään. Näitä näytteenottoon liittyviä tekijöitä tullaan tutkimaan tarkemmin tulevien näytteenottojen yhteydessä.

Avainsanat: Olkiluoto, ONKALO, mikrobiologia, arkit, sulfaatinpelkistäjä, metanogeeni, ANME, DNA, RNA.

TABLE OF CONTENTS

ABSTRACT

TIIVISTELMÄ

1 INTRODUCTION 5
1.1 OLKILUOTO (OL) investigations, 2008–2009 5
1.2 ONKALO (ONK) investigations, 2008–2009 6

2 MATERIALS AND METHODS 7
2.1 Sampling procedures 7
2.1.1 Sampling deep Olkiluoto groundwater for microbiology using PAVE 7
2.1.2 Sampling drillhole OL-KR13, 361–365-m section, using a drillhole pump 7
2.1.3 Sampling ONKALO groundwater, 2008 9
2.1.4 Sampling ONKALO groundwater and fracture surfaces, 2009 10
2.2 Methods for microbiological analysis 11
2.2.1 Determining total number of cells 11
2.2.2 ATP analysis 11
2.2.3 Determining cultivable aerobic bacteria 11
2.2.4 Analysis of most probable number of cultivable anaerobic microorganisms 11
2.3 Molecular Biology: DNA and RNA methods 12
2.3.1 DNA extraction 12
2.3.2 RNA extraction 12
2.3.3 Quantitative PCR (qPCR) 12
2.3.4 16S rRNA gene PCR: cloning and sequencing 13

3 RESULTS 21
3.1 Deep Olkiluoto groundwater microbiology 21
3.1.1 Total number of cells 21
3.1.2 ATP 21
3.1.3 Cultivable heterotrophic aerobic bacteria 21
3.1.4 Most probable number of cultivable anaerobic microorganisms 22
3.1.5 qPCR results for groundwater samples from 2009 29
3.2 Drillhole OL-KR13_T360: effects of pumping on bacterial diversity and chemistry 31
3.2.1 Total number of cells 31
3.2.2 Most probable number of cultivable anaerobic microorganisms 34
3.2.3 qPCR of biomass and SRB 34
3.2.4 Diversity: cloning and sequencing of 16S rRNA genes in drillhole OL-KR13 T360 34
3.3 ONKALO groundwater: drillholes ONK-KR1-3 and ONK-PVA1-2 39
3.3.1 Total number of cells 39
3.3.2 Biomass and activity based on 16S rRNA and apsa RNA qPCR 39
3.4 ONKALO groundwater: drillhole ONK-PVA3 draining 41
3.4.1 Total number of cells 41
3.4.2 Biomass and activity based on 16S rRNA Bacteria gene and apsa mRNA qPCR 41
3.5 Draining of drillhole ONK-PP128 42
3.5.1 Total number of cells 42

3.5.2 Most probable number of SRB, AM, and ANME 42
3.5.3 Biomass and activity based on 16S rRNA Bacteria gene, 16S rRNA ....
Archaea gene, and apsa mRNA qPCR 42
3.5.4 GenBank accession numbers 43
3.5.5 ONKALO 2009 43
3.6 Dissolved gas in groundwater 46

4 DISCUSSION 53

4.1 Evaluation of applied analysis methods 53
4.1.1 Comparison of biomass-related quantitative analytical methods 53
4.1.2 qPCR of phylogenetic groups 55
4.1.3 Cloning and sequencing 56
4.2 Deep Olkiluoto groundwater microbiology 56
4.3 ONKALO groundwater microbiology 59
4.4 The effect of pumping and draining drillholes 60
4.4.1 Drillhole OL-KR13 T360 60
4.4.2 Drillholes ONK-PVA-3 and ONK-PP128 61
4.5 Biofilms on fracture surfaces in groundwater conducting aquifers 62
4.6 DNA signatures of ANME in Olkiluoto and ONKALO groundwater 63
4.7 Dissolved gas 63

REFERENCES 65

A. APPENDIX 69

Key to abbreviations used frequently in the text

Abbreviation	Meaning	Brief description
AA	Autotrophic acetogens	Microbes able to produce acetate from carbon dioxide and hydrogen
AGW	Analytical-grade water	Purified distilled water
AM	Autotrophic methanogens	Microbes able to produce methane from carbon dioxide and hydrogen
ANME	Anaerobic methane-oxidizing consortia	Microbes able to oxidize methane to hydrogen and carbon dioxide, and using hydrogen to reduce sulphate to sulphide
AODC	Acridine orange direct count	Method based on nucleic acid staining for determining cell numbers
apsA	Adenosine-5'-phosphosulphate reductase alpha subunit gene	Part of a key enzyme in the reduction of sulphate by sulphate-reducing bacteria
ATP	Adenosine triphosphate	Energy carrier in living organisms
CFU	Colony-forming unit	A cell that has divided repeatedly, e.g., on an agar plate, forming a dense colony of many identical cells
CHAB	Cultivable heterotrophic aerobic bacteria	Microbes able to live on oxygen and organic carbon and that grow in the laboratory
DNA	Deoxyribonucleic acid	The genetic code, which builds the genome unique to each organism
fthfs	Formyltetrahydrofolate synthetase gene	Part of a key enzyme in the production of acetate by acetogens
HA	Heterotrophic acetogens	Microbes able to produce acetate from organic carbon
HM	Heterotrophic methanogens	Microorganisms able to produce methane from organic carbon
IRB	Iron-reducing bacteria	Microbes able to reduce iron(III) in their respiration
MOB	Methane-oxidizing bacteria	Oxygen-dependent microbes able to use methane as a carbon and energy source
MPN	Most probable number	Method for enumerating microbes
MRB	Manganese-reducing bacteria	Microbes able to reduce manganese(IV) in their respiration
NRB	Nitrate-reducing bacteria	Microbes able to reduce nitrate in their respiration
PCR	Polymerase chain reaction	Technique used to exponentially amplify DNA or RNA
qPCR	Quantitative polymerase chain reaction	Technique used to quantify DNA or RNA using the PCR method
RNA	Ribonucleic acid	Part of the ribosome, which constructs all the proteins in an organism
rDNA	Ribosomal DNA	DNA encoding for the ribosome
SRB	Sulphate-reducing bacteria	Microbes able to reduce sulphate in their respiration
TNC	Total number of cells	The number of cells in a water sample or on a solid phase, usually determined by means of microscopy using the AODC method

1 INTRODUCTION

The microbiology of shallow and deep groundwater in Olkiluoto, Finland and of ONKALO groundwater was previously analysed for almost four years from 2004 to 2007. The extensive sampling and analysis programme produced a substantial database, including 84 analytical datasets on the microbiology of Olkiluoto and ONKALO groundwater, described and interpreted previously in a series of working reports (Pedersen 2006 2007; Pedersen et al. 2008a), a Posiva report (Pedersen 2008), and a scientific publication (Pedersen et al. 2008b). The reasons for the research program can be found in Posiva Report 2008-02 (Pedersen 2008). Shallow groundwater in Olkiluoto contains dissolved oxygen at approximately 10 % or less saturation. The presence of aerobic and anaerobic microorganisms, including methane-oxidizing bacteria (MOB), was documented. The data confirmed earlier findings suggesting that oxygen-reduction processes were ongoing in the shallow part of the bedrock. These microbial processes reduce intruding oxygen in the shallow groundwater using dissolved organic carbon and methane as the main electron donors. Microbiological and geochemical data indicated that anaerobic microbial oxidation of methane (ANME) is active at depths down to approximately 300 m in Olkiluoto, as has been suggested previously, based on interpretations of geochemical data (Andersson et al. 2007 Chapter 7). However, proof of the presence and activity of ANME microorganisms was deemed necessary before the existence of active ANME processes in Olkiluoto groundwater could be accepted. Part of the research on behalf of Posiva Oy in 2007, therefore, focused on developing and testing methods for detecting ANME microorganisms. Groundwater from the ONKALO tunnel was also analysed as part of the ANME research. In 2008 and 2009, these methods were applied and the results are presented here.

1.1 OLKILUOTO (OL) investigations, 2008–2009

Microbiology cultivation, DNA, and RNA data were assembled from 18 groundwater samples from Olkiluoto, from deep drillholes ranging in depth from 62 to 708 m. Sampling and analysis protocols had previously been adapted and tested for quality and reproducibility (Pedersen 2008; Pedersen et al. 2008b), and contamination controls were also performed. Investigation of data variability as a function of drillhole pumping time was executed on drillhole OL-KR13 T360 (361–365-m section). Biomass was determined by counting total numbers of microbial cells (TNC) and determining ATP concentrations. The aerobic cultivation method used comprised aerobic plate counts. Anaerobic most probable number (MPN) methods were used to determine counts of nitrate-, iron-, manganese-, and sulphate-reducing bacteria, acetogenic bacteria, and methanogens. Molecular methods for analysing the diversity and abundance of microorganisms have been continuously developed and applied to groundwater samples. These methods included the sampling of DNA and RNA, extraction of nucleic acids, cloning and sequencing of environmental nucleic acids, and real-time quantitative polymerase chain reaction (qPCR) for analysing of amounts of DNA and RNA. The results of these analyses have been merged and interpreted, and the outcomes are reported here. Methods for detecting ANME microorganisms in deep groundwater that were developed and tested in 2007 were further developed and used in 2008 and 2009.

1.2 ONKALO (ONK) investigations, 2008–2009

Microbiology cultivation, DNA, and RNA data were assembled from groundwater samples collected from eight drillholes in ONKALO ranging in depth from 7.1 to 318 m. Sampling and analysis protocols had previously been adapted and tested for quality and reproducibility (Pedersen 2008; Pedersen et al. 2008b), and contamination controls were also performed. Investigation of data variability as a function of drillhole draining time was executed. The work focused on using molecular methods for analysing the diversity and abundance of microorganisms, including the sampling of DNA and RNA, extraction of these nucleic acids, cloning and sequencing of environmental nucleic acids, and qPCR for analysing amounts of DNA and RNA. Aerobic and anaerobic cultivation methods were used at a small scale for drillhole ONK-PP128. Fracture surfaces in drill cores from two new drillholes (2009), ONK-PVA6 and ONKPVA-7, were analysed using DNA methodology for the presence and amount of microorganisms.

2 MATERIALS AND METHODS

Materials, methods, and analytical procedures were described in detail in Posiva Report 2008-2 (Pedersen 2008) and in Working Report no. 2008-34 (Pedersen et al. 2008a). These reports should be referred to for descriptions of materials, methods, and analytical procedures used previously; descriptions and details of new materials, methods, and analytical procedures will be presented in the present working report. The findings of previous work up to 2007 have been summarized by Pedersen et al. (2008b).

2.1 Sampling procedures

Deep groundwater samples for the analysis of microbiology were taken as described previously (Pedersen 2008). New in 2008 was the sampling of a deep drillhole by pumping groundwater from the sampling depth to the surface (drillhole OL-KR13, 361–365-m section) and repeated samplings in ONKALO. New in 2009 was the DNA analysis of deep groundwater and the analysis of fracture material from ONKALO drill cores.

2.1.1 Sampling deep Olkiluoto groundwater for microbiology using PAVE

Deep groundwater was sampled using the PAVE system. The procedures for microbiological analysis using PAVE have been evaluated using appropriate quality controls (Hallbeck and Pedersen 2008). The sampled drillholes and depths are listed in Table 2-2 and Table A-1. The positions of the sampled surface drillholes (OL-KR) are shown in Figure 2-1.

2.1.2 Sampling drillhole OL-KR13, 361–365-m section, using a drillhole pump

Drillhole OL-KR13 was equipped with a pump and new tubing to the surface before the start of sampling. The tubing between the sampling system at the surface and the sampled section was new and filled with freshwater from the surface. Pumping began on 28 October 2009 at a flow rate of 20 mL min^{-1} and continued for about two days. The first sampling was done approximately 1 h after the start of pumping. Samples for MPN of IRB, SRB, and AM were taken at different pumping time intervals either using sterile syringes or directly into sampling vessels. Three sealable, sterilized anaerobic glass tubes (no. 2048-00150; Bellco Glass, Vineland, NJ, USA), sealed with butyl rubber stoppers (no. 2048-117800; Bellco Glass) and aluminium crimp seals (no. 2048-11020; Bellco Glass), were each filled with approximately 10 mL of sampled groundwater for analysis of the MPN of cultivable microorganisms. On each sampling occasion, sterile 50-mL polypropylene tubes (Sarstedt, Landskrona, Sweden) were filled with groundwater. Three samples were subsequently analysed for TNC. In addition, samples were taken for RNA and DNA analysis, as described for the respective methods in section 2.3, Molecular Biology: DNA and RNA methods.

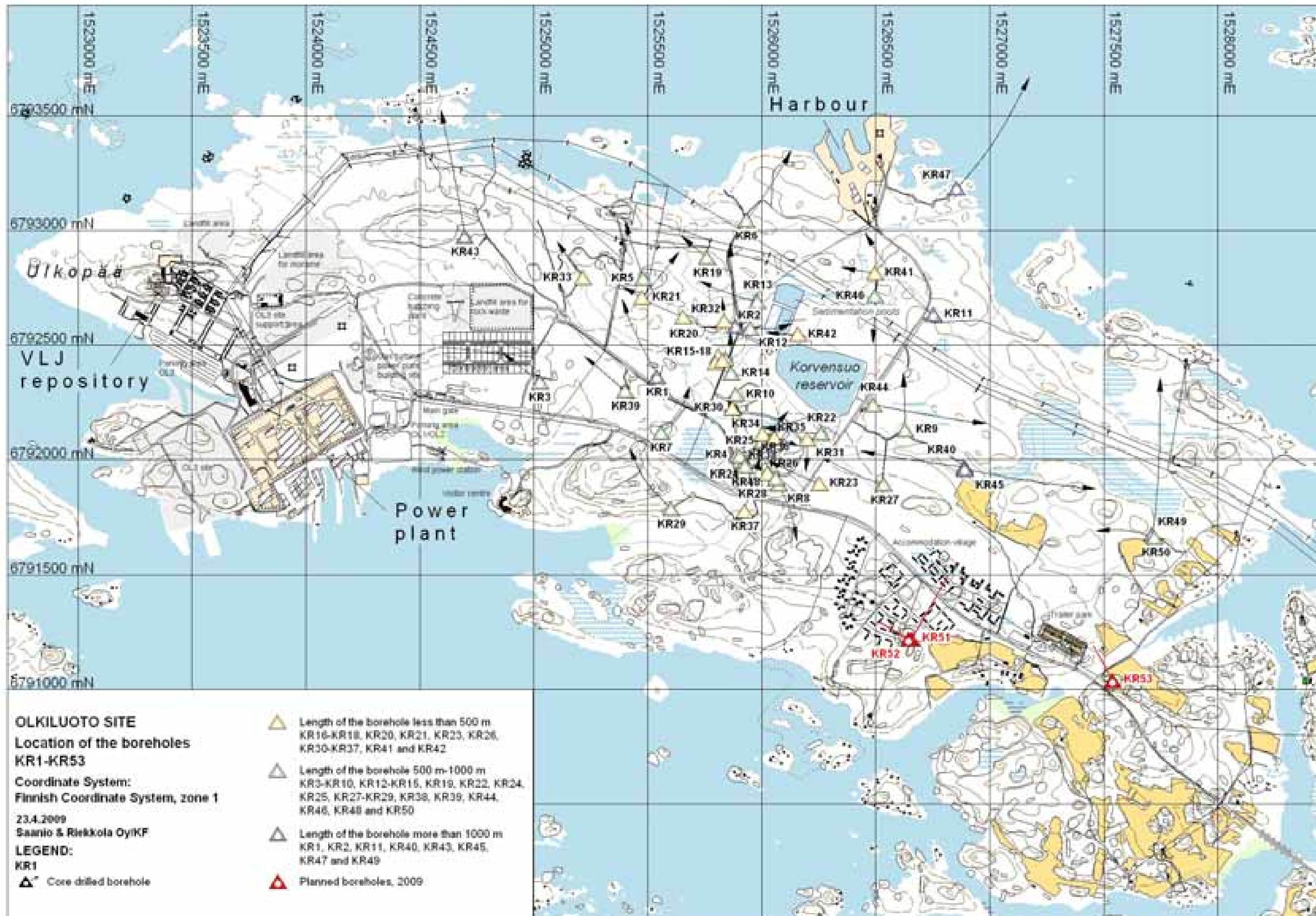

Figure 2-1. *Map showing the positions of KR drillholes in Olkiluoto.*

Table 2-1. *Flow rates in ONKALO drillholes in September 2008. The flow rates were adjusted using valves to particular sampling flow rates.*

Drillhole	**Depth (m)**	**Sampling flow rate (mL min^{-1})**
ONK-KR1	7.1	30
ONK-KR2	30.5	32
ONK-KR3	44.8	6
ONK-PVA1	14.6	740
ONK-PVA2	65.2	5
ONK-PVA3	78.5	200

2.1.3 Sampling ONKALO groundwater, 2008

The sampled ONKALO drillholes and sampling dates are listed in Table A-1. The drillholes stood open before and during sampling, and the flow rates were adjusted according to Table 2-1. The samples from drillholes ONK-KR1–3 and ONK-PVA1–3 were placed into the respective sampling vessels in the tunnel. The sampling vessels were as follows: sealable, sterilized anaerobic glass tubes (no. 2048-00150; Bellco Glass), sealed with butyl rubber stoppers (no. 2048-117800; Bellco Glass), and aluminium crimp seals (no. 2048-11020; Bellco Glass), were filled with approximately 10 mL of sampled groundwater for analysis of the MPN of cultivable microorganisms. At each sampling point, two sterile 15-mL polypropylene tubes (Sarstedt) were filled with groundwater. One sample was subsequently analysed for TNC. In addition, samples were taken for DNA (15 mL) and RNA (500 mL) analysis, as described for the respective methods below. The following samples were collected and analyses as listed above were performed.

Drillholes ONK-KR1-3 and ONK-PVA1-2

These drillholes were sampled as described earlier (Pedersen et al. 2008a). Sterile flasks (1000 mL) were placed in all but ONK-PVA3 drillholes and collected when full.

Draining drillhole ONK-PVA3

Drillhole ONK-PVA3 was chosen to study the effect of draining a drillhole on bacterial numbers and diversity. Groundwater samples of four different volumes, i.e., 1, 3, 14, and 175 L, were collected during draining of this drillhole.

ONK-PP128

Approximately 50 mL of groundwater was sampled in 50-mL sterile plastic tubes on three occasions after the start of pumping. Samples for MPN of SRB, AM, and ANME were first taken; thereafter, two 15-mL samples of groundwater were taken in sterile plastic tubes for DNA and TNC analyses. Finally 500 mL of groundwater was sampled for analysis of RNA. The first sample was taken on 10 September 2008 at 9:30 after

approximately 20 L of groundwater was first discharged. This sample contained large amounts of concrete material from injection activities at the nearby tunnel construction site. The second sample was taken on 10 September 2008 at 14:10 and was free of turbidity from concrete. However, after the first sample, someone closed the valve at an unknown time, so the discharged volume cannot be calculated. The third sample was collected on 11 September 2008 at 9:50; it was calculated that at least 1800 L of groundwater was discharged before this sample was taken.

Previous studies have detected the presence of anaerobic methane oxidation at Olkiluoto (Pedersen et al. 2008a). ANME signatures and diversity were investigated by cloning and sequencing (Table 2-3). Methanotrophs, methylotrophs, methanogens, and SRB were previously found by cloning and sequencing samples from various drillhole locations in the ONKALO tunnel and from ONKALO slime (Pedersen et al. 2008a). Drillhole ONK-PP128 was studied on three occasions after draining was started (ONK-PP128-1-3).

2.1.4 Sampling ONKALO groundwater and fracture surfaces, 2009

ONK-PVA1, ONK-PVA2, ONK-PVA4, and ONK-PVA5 groundwater

A volume corresponding to half the section volume was allowed to drain, after which groundwater was collected. A total of 1000–2500 mL (the final volume depended on the flow rate) of groundwater from each of the four drill holes, i.e., ONK-PVA1, ONK-PVA2, ONK-PVA4, and ONK-PVA5, was vacuum filtered on site within 3 min of sampling in 500-mL sterile plastic 0.2-µm bottle top filters (Filtropur BT50, cat. no. 83.1823.101; Sarstedt) screwed onto 1-L glass bottles (Thermo Fisher Scientific, Waltham, MA, USA) and connected to a vacuum pump (LABPORT, no. N810 FT.18; KNF Lab, Neuberger, Germany). The filter was cut out immediately after filtration and placed into 15 mL of RNAlater (cat. no. AM7022; Ambion, Stockholm); it was washed with the RNAlater solution and then discarded. The samples were subsequently subjected to DNA extraction and qPCR analysis (Table 2-3).

ONK-PVA6 and ONK-PVA7 groundwater and fracture surface material biofilms

Drillholes ONK-PVA6 and ONK-PVA7 were sampled immediately after drilling for fracture surface material biofilms on 4 November 2009 and for groundwater on 19 November 2009. ONK-PVA7 was dry and groundwater could not be sampled; a sample of 300 mL was obtained from ONK-PVA6 and filtered as described above for ONK-PVA1-5.

Immediately after a drill core was retrieved, it was observed for fractures that could be sampled for biofilm analysis. To sample biofilms that may be present in fracture surface material (FSM), one mL of RNAlater (RNAlater, 1.5 mL, cat. no. AM7022; Ambion) was applied by pipette to the sampling point on each fracture surface (Finnpipette, 1000 µL, cat. no. 4641100; Thermo Fisher Scientific); approximately 1 cm^2 of FSM was removed using a sterile scalpel, collected using the pipette, and transferred to a 2.0-mL Eppendorf tube filled with 1.5 mL of RNAlater for subsequent DNA extraction and qPCR analysis (Table 2-3).

2.2 Methods for microbiological analysis

2.2.1 Determining total number of cells

The total number of cells (TNC) was determined using the acridine orange direct count (AODC) method as devised by Hobbie et al. (1977) and modified by Pedersen and Ekendahl (1990) (Table 2-3).

2.2.2 ATP analysis

The ATP Biomass Kit HS (no. 266-311; BioThema, Handen, Sweden) was used to determine total ATP in living cells (Table 2-3). This analysis kit was developed based on the results of Lundin et al. (1986) and Lundin (2000). The ATP biomass method used here has been described, tested in detail, and evaluated for use with Fennoscandian groundwater, including Olkiluoto groundwater (Eydal and Pedersen 2007).

2.2.3 Determining cultivable aerobic bacteria

Petri dishes containing agar with nutrients were prepared as described elsewhere (Pedersen and Ekendahl 1990) for determining the numbers of cultivable heterotrophic aerobic bacteria (CHAB) in groundwater samples (Table 2-3). Ten-times dilution series of culture samples were prepared in sterile analytical-grade water (AGW) (Millipore Elix 3; Millipore, Solna, Sweden) containing 1.0 g L^{-1} of NaCl and 0.1 g L^{-1} of K_2HPO_4; 0.1-mL portions of each dilution were spread with a sterile glass rod on the Petri dishes in triplicate. The plates were incubated for 7–9 days at 20 °C, after which the number of colony-forming units (CFUs) was counted; plates with 10–200 colonies were counted.

2.2.4 Analysis of most probable number of cultivable anaerobic microorganisms

Anaerobic media for determining the MPN of various anaerobic microorganisms in groundwater were prepared according to the procedures described by Widdel and Bak (1992). The specifications of the media were formulated based on previously measured chemical data from Olkiluoto. This allowed the formulation of artificial media that most closely mimicked *in situ* groundwater chemistry for optimal microbial cultivation (Haveman and Pedersen 2002). Media for the 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 autoclaved and anaerobically dispensed according to the formulations described elsewhere (Pedersen 2008). The MPN procedures resulted in protocols with tubes that scored positive or negative for growth. The results of the analyses were rated positive or negative versus control levels. Three dilutions in five replicate tubes each were used to calculate the MPN of each group, according to the calculations found in Greenberg et al. (1992). The lower and upper 95 % confidence intervals for the MPN method applied to the five replicate tubes with approximately one-third and three times the obtained values, respectively; the detection limit was 0.2 cells mL^{-1}.

Medium for ANME was mixed, autoclaved, and anaerobically dispensed according to the formulation for SRB medium described elsewhere (Hallbeck and Pedersen 2008). The composition was similar to that of a SRB medium but with the following modifications: 0.1 mL L^{-1} of 50 g L^{-1} yeast extract was used and lactate was not added. After inoculation, the headspaces of the culture tubes were filled with CH_4 to an overpressure of 2 bars.

2.3 Molecular Biology: DNA and RNA methods

2.3.1 DNA extraction

Total genomic DNA was extracted from groundwater samples filtered at the drillhole site within a few minutes of sampling in 500-mL sterile plastic 0.2-µm bottle top filters (Filtropur BT50, cat. no. 83.1823.101; Sarstedt) screwed onto 1-L glass bottles connected to the vacuum pump. The filter was stored in RNAlater solution (no. AM7021; Ambion). The solutions containing cells were first centrifuged in 1.5-mL portions in Eppendorf tubes at 7440 × g in a micro-centrifuge (MIKRO 200; Hettich Zentrifugen, Tuttlingen, Germany) for 10 min. Supernatants from all centrifuged samples were discarded and DNA was extracted from the water sample pellets using the DNeasy Blood&Tissue kit (no. 69504; QIAGEN, Solna, Sweden) according to the manufacturer's protocol for Gram-positive bacteria. The DNA concentration and purity of the extractions were analyzed on a NanoDrop ND-1000 UV-Vis spectrophotometer (NanoDrop Technologies, Wilmington, DE). The extracted DNA was stored at –20 °C.

2.3.2 RNA extraction

RNA was extracted from 500-mL samples of groundwater filtered at the drillhole site within a few minutes of sampling in 500-mL sterile plastic 0.2-µm bottle top filters (Filtropur BT50, cat. no. 83.1823.101; Sarstedt) screwed onto a 1-L glass bottle and connected to the vacuum pump. The filter was cut out immediately and placed in 15 mL of RNAlater solution. The filter was washed with the RNAlater solution and then discarded. The solutions containing cells were first centrifuged at 7440 × g in a micro-centrifuge (MIKRO 200; Hettich Zentrifugen, Tuttlingen, Germany) for 15 min; the supernatant was then discarded, and the total RNA was extracted from the pellet using the RNeasy Mini kit (no. 74104; QIAGEN) according to the manufacturer's protocol. RNA concentrations were measured on a NanoDrop ND-1000 UV-Vis spectrophotometer (NanoDrop Technologies) and the extracted RNA was stored at –80 °C. The RNA quality was checked on a 1 % formaldehyde agarose gel.

2.3.3 Quantitative PCR (qPCR)

Gene amplification using the polymerase chain reaction requires short DNA strings specific to each target gene. These strings come in pairs and are denoted "primers". The primers used here to detect the biomass of various types of bacteria were as listed in Table 2-4; the primers were designed according to the corresponding literature references in this table. The qPCR method used to quantify the various groups detected with the respective primer pairs is briefly described in Table 2-3.

2.3.4 16S rRNA gene PCR: cloning and sequencing

Cloning and sequencing for *Bacteria* 16S rRNA gene sequences were carried out as described in Pedersen et al. (2008a) and outlined in Table 2-3. *Archaea* 16S rRNA gene sequences were amplified, cloned, and sequenced using the universal primers A21F (5′-TTCCGGTTGATCCYGCCGGA-3′) and U1406R (5′-ACGGGCGGTGTGTRC-3′) (Pedersen et al. 2008a). Each PCR mixture for the *Archaea* 16S rRNA contained 0.5 µL of each primer solution (10 pmol μL^{-1}), 20 ng of DNA, 12.5 µL × 2 of iProof Mastermix (no. 172-5310; Bio-Rad Laboratories, Sundbyberg, Sweden) in a final reaction volume of 25 µL. Amplification was carried out on a thermal PCR cycler (MyCycler; Bio-Rad Laboratories). After initial denaturation for 5 min at 98 °C, 35 cycles were performed, each cycle consisting of 30 s at 98 °C, 60 s at 60.9 °C, and 90 s at 72 °C; a final extension step was carried out for 7 min at 72 °C. PCR products were visualized on a 1 % agarose gel containing ethidium bromide (no. 161-0433; Bio-Rad Laboratories); the bands were then cut, cloned, and sequenced as described in Pedersen et al. (2008a).

__Table 2-2.__ Olkiluoto and ONKALO groundwater sample and analysis scheme.

Sample	Date sampled (Y-M-D)	TNC	ATP	CHAB	Dissolved gas	MPN	qPCR DNA	qPCR RNA	Cloning and sequencing
OL-KR41_213_1	2008-02-12	×	×	×	×	×			
OL-KR41_257_2	2008-03-03	×	×	×	×	×			
OL-KR43_96_1	2008-03-05	×	×	×	×	×			
OL-KR43_214_1	2008-05-06	×	×	×	×	×			
OL-KR6_393_1	2008-05-07	×	×	×	×	×			
OL-KR46_175_1	2008-05-26	×	×	×	×	×			
OL-KR46_131_1	2008-07-01	×	×	×	×	×			
OL-KR46_82_1	2008-09-23	×	×	×	×	×			
OL-KR47_131_1	2008-09-30	×	×	×	×	×			
OL-KR13 T360_1-7	2008-10-28-30	×				×	×	×	×
OL-KR47_708_1	2008-11-17	×	×	×	×	×			
OL-KR45_117_1	2008-12-01	×	×	×	×	×			
OL-KR47_413_1	2009-01-07	×	×	×	×	×			
OL-KR47_145_1	2009-02-17	×	×	×	×	×			
OL-KR45_295_1	2009-04-14	×	×	×	×	×	×		
OL-KR49_614_1	2009-06-29	×	×	×	×	×	×		
OL-KR47_77_1	2009-09-29	×	×	×	×	×	×		
OL-KR47_217_1	2009-11-17	×	×	×	×	×	×		
OL-KR40_786_1	2009-12-08	×	×	×	×	×	×		

Sample	Date sampled (Y-M-D)	TNC	ATP	CHAB	Dissolved gas	MPN	qPCR DNA	qPCR RNA	Cloning and sequencing
ONK-KR1	2008-09-09	×					×		
ONK-KR2	2008-09-09	×					×		
ONK-KR3	2008-09-09	×					×		
ONK-PVA1	2008-09-09	×					×		
ONK-PVA2	2008-09-09	×					×		
ONK-PVA3 :1 L	2008-09-09	×					×		
ONK-PVA3:2 L	2008-09-09	×					×		
ONK-PVA3: 14 L	2008-09-09	×					×		
ONK-PVA3:175 L	2008-09-09	×					×		
ONK-PP128-1	2008-09-10	×				×	×	×	×
ONK-PP128-2	2008-09-10	×				×	×	×	×
ONK-PP128-3	2008-09-11	×				×	×	×	×
ONK-PVA1	2009-11-18				×		×		
ONK-PVA2	2009-11-19				×		×		
ONK-PVA4	2009-11-19				×		×		
ONK-PVA5	2009-11-19				×		×		
ONK-PVA6	2009-11-19				×		×		

Table 2-3. *Summary of analyses performed in this work, with their objectives, brief descriptions of the associated methods, key strengths/weaknesses, and key references.*

Analysis	Name	Objective	Brief description of method	Key strengths/weaknesses	Key references
Total number of cells	TNC	Determination of total number of microorganisms in a sample	The sample is passed through a 0.2-µm filter; the bacteria on the filter are stained and the number of cells on the filter is counted in an epifluorescence microscope at 1000× magnification.	Reliable numbers are obtained. Gives a good overview of the size and morphology of sampled microorganisms. / Fairly time consuming. Difficult to use for turbid samples containing non-living particles and colloids and for cells adhering to mineral surfaces.	Hobbie et al. (1977), Pedersen and Ekendahl (1990)
Adenosine-tri-phosphate	ATP	Determination of the living biomass in a sample	All living cells contain ATP. A kit with enzymes and reagents is used, with a luminometer, to determine ATP. The photon counts are proportional to the amount of ATP in the groundwater sample.	Sensitive method that can detect even a very few cells. / High salt concentrations, like those found in deep groundwater, may disturb the analysis.	Eydal and Pedersen (2007)
Cultivable, aerobic, heterotrophic bacteria	CHAB	Determination of number of bacteria that can be cultivated in air at 20 °C	Ten-times dilution series of culture samples are made in a sterile salt-buffer solution and 0.1-mL portions of each dilution are spread on nutrient agar plates in triplicate. The number of CFUs is counted after 7-9 days.	Very basic and well-known methodology. / This method does not detect obligate anaerobic microorganisms or microorganisms living on inorganic energy sources with carbon dioxide as the source of cell carbon.	Pedersen and Ekendahl (1990)
Dissolved gas	Gas	Determination of total amount of dissolved gas and its constituents	Pressurized samples are collected using the Posiva PAVE or SKB PVB sampler. Dissolved gases are extracted from the groundwater under vacuum to glass vials and analysed using gas chromatography.	The gas chromatography method is very reproducible and stable. / The main difficulty is keeping the sample separated from contaminating air and pressure vessel gas.	Hallbeck and Pedersen (2008)

Analysis	Name	Objective	Brief description of method	Key strengths/weaknesses	Key references
Numbers of physiological groups of micro-organisms	MPN	Determination of microorganisms with different physiologies with respect to their utilized electron acceptor	Ten-times dilution series are made and five anaerobic culture tubes are inoculated from each dilution. After 8–12 weeks of cultivation, the tubes are analysed for growth of physiological groups and the most probable number of each group is obtained from tables or probability calculations.	The method is robust and produces good data. It is subsequently possible to determine the dominant species in a sample using cloning and sequencing (see below). / The results are obtained long after sampling. The samples may contain microorganisms that do not grow under the conditions used.	Hallbeck and Pedersen (2008)
Numbers of phylogenetic groups of micro-organisms	qPCR DNA	Determination of the numbers of various types of microorganism based on known genetic information	Cells are collected and disintegrated using biochemical methods and the genetic material, DNA, is extracted. The DNA is quantitatively PCR amplified using pairs of short DNA sequences (i.e., primers) specific to various microorganisms. Known amounts of DNA from the analysed genes are included as standards and analysed concomitantly with the samples.	The results can be obtained within 24 h. The sensitivity is very high, but related to the available sample volume. All types of microorganisms are detected provided proper primers are available. The selected standard gene sequences may differ from those of the sample microorganisms. Discrepancies in numbers may arise due to differences in gene copies between standard and sampled microorganisms. Genetic analysis is influenced by sample quality, e.g., traces of metals and organic material may disturb the amplification process.	Ginzinger (2002)

Analysis	Name	Objective	Brief description of method	Key strengths/weaknesses	Key references
Microbial activity of micro-organisms	qPCR RNA	Determination of the metabolic activity of various types of microorganisms based on known genetic information	Cells are collected and disintegrated using biochemical methods and the messenger RNA for genes specific to various metabolic processes is extracted. A DNA copy of the messenger RNA is subsequently made. Then the quantification proceeds as described for qPCR DNA above.	The results are obtained within 48 h. This is the only method that can detect metabolic activity in a preserved sample of microorganisms. / The same as for qPCR of DNA above. The method requires immediate access to fresh samples on the sample site that can be filtered within a few minutes. It is impossible to ship groundwater samples for analysis.	Ginzinger (2002)
Microbial diversity	Cloning and sequencing	Determination of the diversity and species of microorganisms	Cells are collected and disintegrated using biochemical methods and the genetic material, DNA, is extracted. The DNA is PCR amplified using pairs of short DNA sequences (i.e., primers) specific to various microorganisms. Amplified DNA is cloned into *Escherichia coli* and the target sequences are sequenced. The obtained DNA sequences are compared against international databases for identity to deposited genes; the most closely related sequence in the database will reveal species-related information about the analysed microorganisms.	The method reveals the diversity of sample microorganisms within a week, including organisms difficult or impossible to cultivate. The alternative diversity and identification analysis methods require cultivation, and the many biochemical analyses required are time-consuming and costly. / The method tends to be biased in favour of dominant microorganisms in the sample. Many more than 100 clones may need to be sequenced for a full diversity assessment, which is expensive and time consuming.	Pedersen (1997), Pedersen et al. (2004)

Table 2-4. *Gene primers used for qPCR analysis of groundwater and biofilms samples in this work.*

Target organisms	Target gene name	Species used as standard in the qPCR analysis	Primer pair names for the qPCR amplification	References
Bacteria	*Bacteria* 16S	*Desulfovibrio aespoeensis*	Q926f – Q1100r	Lane et al. (1985), Lane (1991)
Archaea	*Archaea* 16S	*Methanobacterium subterraneum*	Arch349f – Arch806r	Horz et al. (2001), Zhang and Fang (2006)
Eukarya	*Eukarya* 18S	*Saccharomyces cerevisiae*	18Sf – 18Sr	Nazarenko et al. (2002)
Nitrate-reducing bacteria	*narG1*	*Pseudomonas fluorescens*	narG1f – narG1r	Smith et al. (2007)
Nitrate-reducing bacteria	*narG2*	*Pseudomonas fluorescens*	narG2f – narG2r	Smith et al. (2007)
Nitrate-reducing bacteria	*nirK*	*Pseudomonas fluorescens*	nirK876f – nirK1040r	Henrya et al. (2004)
Pseudomonas fluorescens	*pvsA*	*Pseudomonas fluorescens*	pvsA1f15964 – pvsA2r16315	Mossialos et al. (2002)
Sulphate-reducing bacteria DNA / mRNA	*apsA / apsa*	*Desulfovibrio aespoeensis*	apsAf304 – apsAr416	Ben-Dov et al. (2007)
Autotrophic acetogens	*fthfs*	*Acetobacterium carbinolicum*	Acetofthfs231f – Acetofthfs478r	Pedersen et al. (2008a)
Methanotrophs	*pmoA*	*Methylocystis aldrichii*	189f – 682r	Horz et al. (2001), Zhang and Fang (2006)
Methanotrophs	*mmoX*	*Methylocystis aldrichii*	882f – 1403r	Horz et al. (2001), Zhang and Fang (2006)
Methylotrophs	*mxaF*	*Methylocystis aldrichii*	1003f – 1561r	Horz et al. (2001), Zhang and Fang (2006)
Anaerobic methane-oxidizing communities	ANME-1	Not available	ANME1337 f – ANME1724r	Girguis et al. (2005)
Anaerobic methane-oxidizing communities	ANME-2a	Not available	ANME2a647f – Ee1MS932r	Orphan et al. (2002)
Anaerobic methane-oxidizing communities	ANME-2c	Not available	ANME2c468f – ANME2c736r	Orphan et al. (2002)

3 RESULTS

3.1 Deep Olkiluoto groundwater microbiology

Eleven complete analyses were performed of deep groundwater samples in 2008; the complete dataset, including statistical details, can be found in Table A-1 to Table A-3. An additional seven complete analyses were performed in 2009.

3.1.1 Total number of cells

The total number of cells (Table 3-1) in deep groundwater was within the range, i.e., 2.7 $\times 10^3$ to 1.5×10^5 cells mL^{-1}, found previously in Olkiluoto (Pedersen et al. 2008a). The average of all previous analyses (n = 25) was 5.8×10^4 cells mL^{-1}, and all observations for 2008–2009 were below this average value, except for OL-KR41_213_1. There is no obvious trend with depth for deep TNC when all shallow and deep data below 50 m from 2005–2009 are considered (Figure 3-1).

3.1.2 ATP

The amount of ATP (Table 3-1) in deep groundwater was within the range, i.e., 3.1 $\times$ 10^3 to 2.1×10^5 attomol (amol) mL^{-1}, found previously in Olkiluoto (Pedersen et al. 2008a). The average of all previous analyses (n = 23) was 2.7×10^4 amol mL^{-1}, and all observations for 2008–2009 were below this average value, except for OL-KR49_614_1. This and the OL-KR43_214_1 groundwater sample had the highest ATP/TNC values, suggesting that they had the most active microbial populations of all deep groundwater samples investigated in 2008 and 2009, respectively. There were two peaks in the ATP values over depth: shallow groundwater above 50 m and a zone at approximately 300 m had elevated ATP concentrations in several samples, compared with the overall average (Figure 3-2). The lack of deeper observations, except for two data points from 2008 and 2009, makes it impossible to evaluate ATP trends at depths greater than 350 m.

3.1.3 Cultivable heterotrophic aerobic bacteria

The numbers of CHAB (Table 3-1) in deep groundwater were within the range, i.e., 1.0 $\times 10^1$ to 1.2×10^5 cells mL^{-1}, found previously in Olkiluoto (Pedersen et al. 2008a), except for the deepest sample, OL-KR40_786_1. The average of all (n = 17) previous analyses was 2.8×10^4 cells mL^{-1}, and all observations for 2008–2009 were at or below this average value except for the CHAB value for the OL-KR49_614_1 sample, which was above the previous average. A large proportion of the TNC from this groundwater sample could be cultivated as CHAB, which supports the inference from the ATP/TNC value that the microbial population from this drillhole sample was active. However, the TNC value was low in this sample and did not correlate well with ATP or CHAB. In fact, it appears as the TNC value underestimated the total population when compared with CHAB or ATP, possibly due to an unidentified methodological error during the TNC analysis of this sample. The CHAB values scatter without a clear trend when all shallow and deep data from 2005–2009 are considered (Figure 3-4).

Table 3-1. Cell numbers and biomass (ATP) determinations for deep groundwater from Olkiluoto sampled in 2008 and 2009. TNC = total number of cells, CHAB = cultivable heterotrophic aerobic bacteria. Statistical details can be found in Table A-1.

Sample number	Sampled (Y-M-D)	Section (m)	TNC (cells mL^{-1})	ATP (amol mL^{-1})	CHAB (cells mL^{-1})	CHAB/ TNC (%)	ATP/ TNC
OL-KR41_213_1	2008-02-12	213–222	68,000	10,600	1650	2.43	0.156
OL-KR41_257_2	2008-03-03	257–266	38,000	15,200	5230	13.76	0.400
OL-KR43_96_1	2008-03-05	96–102	25,000	8600	1030	4.12	0.344
OL-KR43_214_1	2008-05-06	214–219	11,000	11,800	4830	43.91	1.073
OL-KR6_393_1	2008-05-07	393–398	15,000	9930	6500	43.33	0.662
OL-KR46_175_1	2008-05-26	175–182.5	6300	3500	20	0.32	0.556
OL-KR46_131_1	2008-07-01	131–137	4800	3600	357	7.44	0.75
OL-KR46_82_1	2008-09-23	82–88	3300	2500	267	8.09	0.76
OL-KR47_131_	2008-09-30	131–137	39,000	14,400	380	0.97	0.37
OL-KR47_708_1	2008-11-17	708–714	20,000	7610	1070	5.35	0.38
OL-KR45_117_1	2008-12-01	708–714	19,000	7540	4170	21.95	0.40
KR47_413_1	2009-01-07	413–419	3200	2350	103	3.22	0.73
KR47_145_1	2009-02-17	145–151	11,000	8960	2500	22.73	0.81
KR45_295_1	2009-04-14	295–300	26,000	7440	5600	21.54	0.29
KR49_614_1	2009-06-29	614–622	24,000	35,300	31,400	130.83	1.47
KR47_77_1	2009-09-29	77–81	15,000	4610	890	5.93	0.31
KR47_217_1	2009-11-17	217–222	41,000	19200	10,900	26.59	0.47
KR40_786_1	2009-12-08	786–790.5	51,000	No data*	3.33	0.01	No data*

**ATP could not be analysed due to high salinity that disturbed the analysis.*

3.1.4 Most probable number of cultivable anaerobic microorganisms

Base-10 logarithms were calculated for the MPN values for each metabolic group and stacked in bar graphs (Figure 3-3). The obtained stacked number then represent both the diversity (i.e., how many metabolic groups could be cultivated) and numbers of cultivable organisms in each metabolic group in a groundwater sample. A large stacked number is obtained if both the diversity and numbers of cultivated metabolic groups are large. The number of each cultivated group can be judged from the bar length for each metabolic group. The stacked numbers of MPN values for all deep groundwater samples analysed from 2005 to 2009 are shown in Figure 3-3. The NRB analysis was introduced into the sampling programme in 2005, so NRB values are missing from some of the oldest stacked MPN bars for deep groundwater samples. This may partly explain why the stacked number bars for drillholes OL-KR2, OL-KR7, OL-KR10, OL-KR19, and OL-KR27 are shorter than the remaining bars, which incorporate NRB data. In addition, IRB and MRB were not analysed for OL-KR13 in 2007. The stacked MPN numbers in deep groundwater remained fairly homogenous over depth for the first 200 m; deeper, however, several samples displayed high stacked numbers. Several samples taken from

below depths of 200–300 m displayed significantly larger stacked numbers than did all other analysed deep groundwater samples.

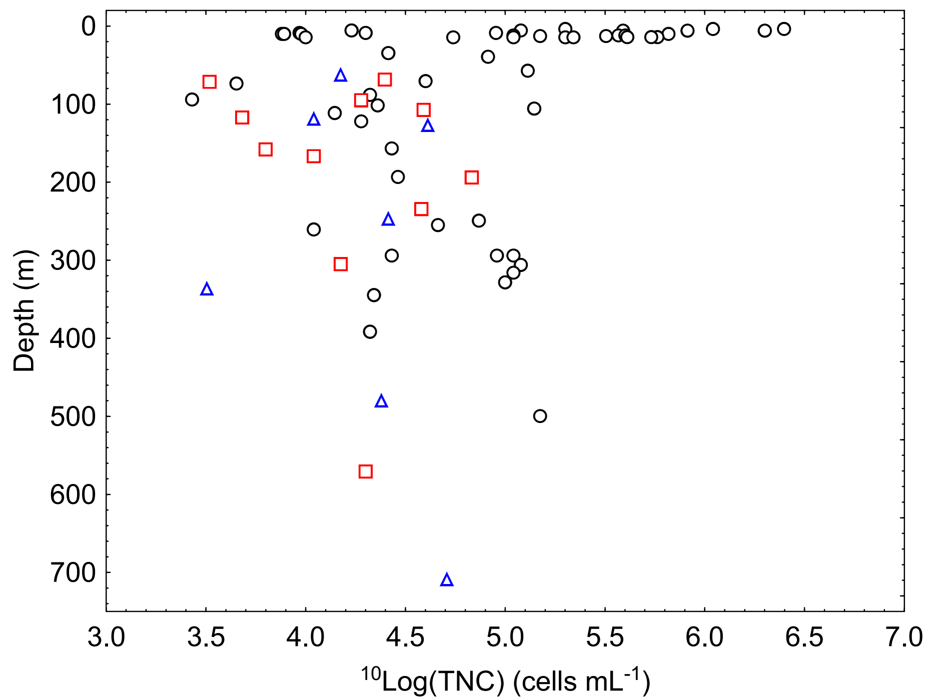

Figure 3-1. *The distribution of total number of cells (TNC) versus depth in Olkiluoto groundwater. Data: 2005–2007, black circles; 2008, red squares; 2009, blue triangles.*

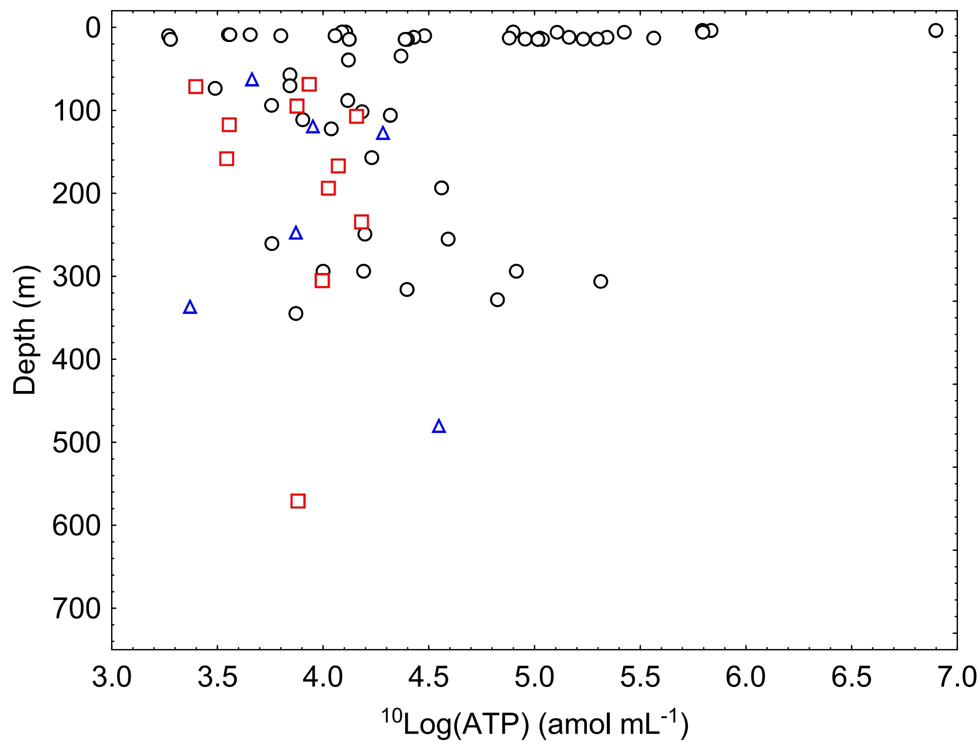

Figure 3-2. *ATP concentration distributed over depth in Olkiluoto groundwater. Data: 2005–2007, black circles; 2008, red squares; 2009, blue triangles.*

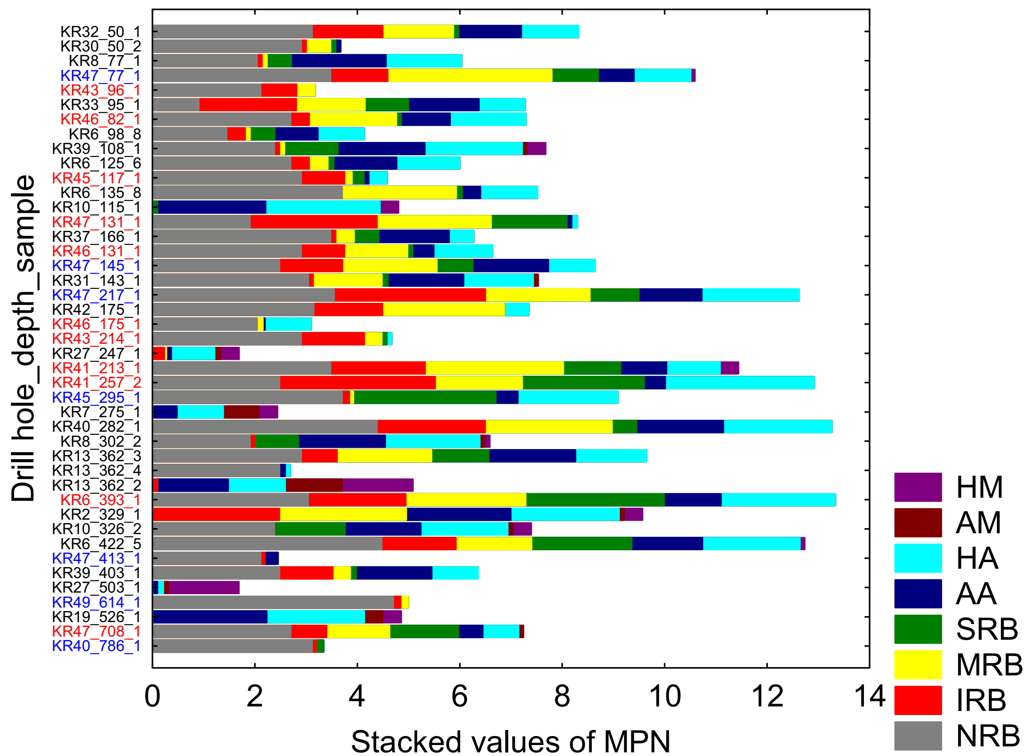

Figure 3-3. *Stacked values of most probable numbers of various physiological groups of microorganisms in deep Olkiluoto groundwater, 2005–2009. The drillholes are listed in order of increasing depth. Data obtained in 2008 are indicated in red text; data obtained in 2009 are indicated in blue text. NRB = nitrate-reducing bacteria, IRB = iron-reducing bacteria, MRB = manganese-reducing bacteria, SRB = sulphate-reducing bacteria, AA = autotrophic acetogens, HA = heterotrophic acetogens, AM = autotrophic methanogens, and HM = heterotrophic methanogens. The obtained stacked values represent both the diversity, i.e., how many metabolic groups could be cultivated, and the number of cultivable organisms in each metabolic group in a drillhole. The values for each cultivated group can be appreciated from the bar length for each metabolic group.*

The distribution of MPN of NRB over depth displayed a range over four orders of magnitude in the groundwater samples (Figure 3-5). The highest NRB value was found at depths of 480 m and 247 m in drillholes OL-KR49 and OL-KR45, respectively. The MPN of IRB was low in many samples, with few values above 10 cells mL^{-1} in the shallow groundwater (Figure 3-6). The deep groundwater samples displayed a peak relative to the other MPN of IRB, with several IRB values significantly above 100 cells mL^{-1} in the 100–300-m depth interval. In the case of MRB, the situation was similar to that of IRB (Figure 3-7). The MPN of SRB followed the trends of IRB and MRB, with scattered values in shallow groundwater up to approximately 1000 cells mL^{-1} and eight values above the detection limit at a depth of approximately 300 m (Figure 3-8). The MPN results for AA and HA displayed similar patterns. The data were scattered over a range of four orders of magnitude (10^0 to 10^3 cells mL^{-1}) (Figure 3-9 and Figure 3-10). At a depth of approximately 300 m, there was a systematic tendency towards higher AA and HA MPN values than the overall average, as was also observed for NRB and SRB.

There were some detectable AM and HM in shallow groundwater and there were very few detectable methanogens at depth (Figure 3-11, Figure 3-12).

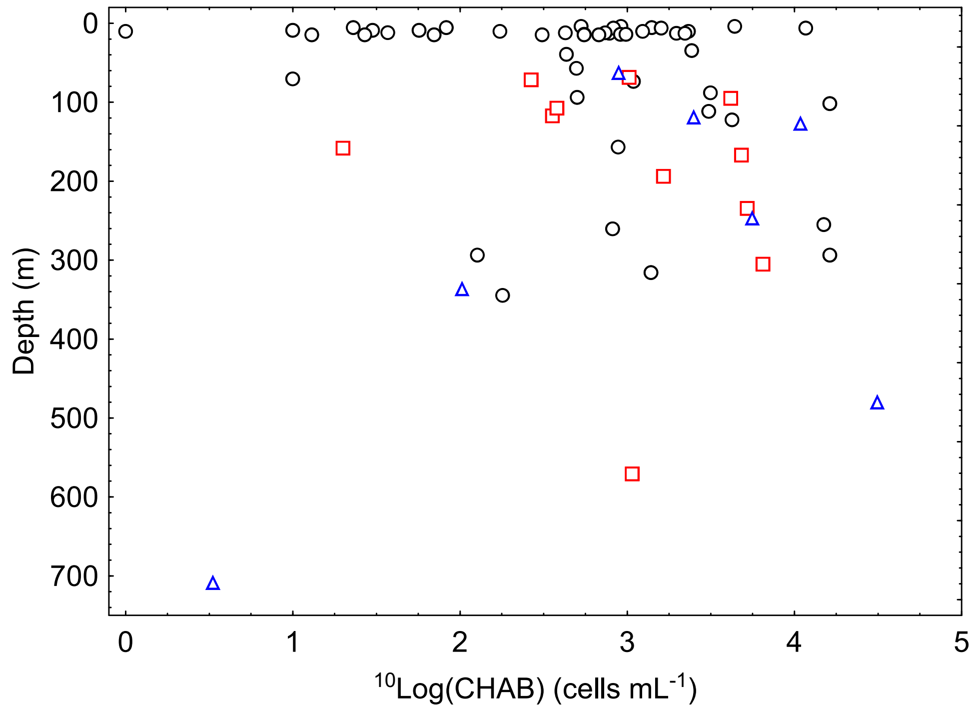

Figure 3-4. *The distribution of cultivable heterotrophic aerobic cells (CHAB) versus depth in Olkiluoto groundwater. Data: 2005–2007, black circles; 2008, red squares; 2009, blue triangles.*

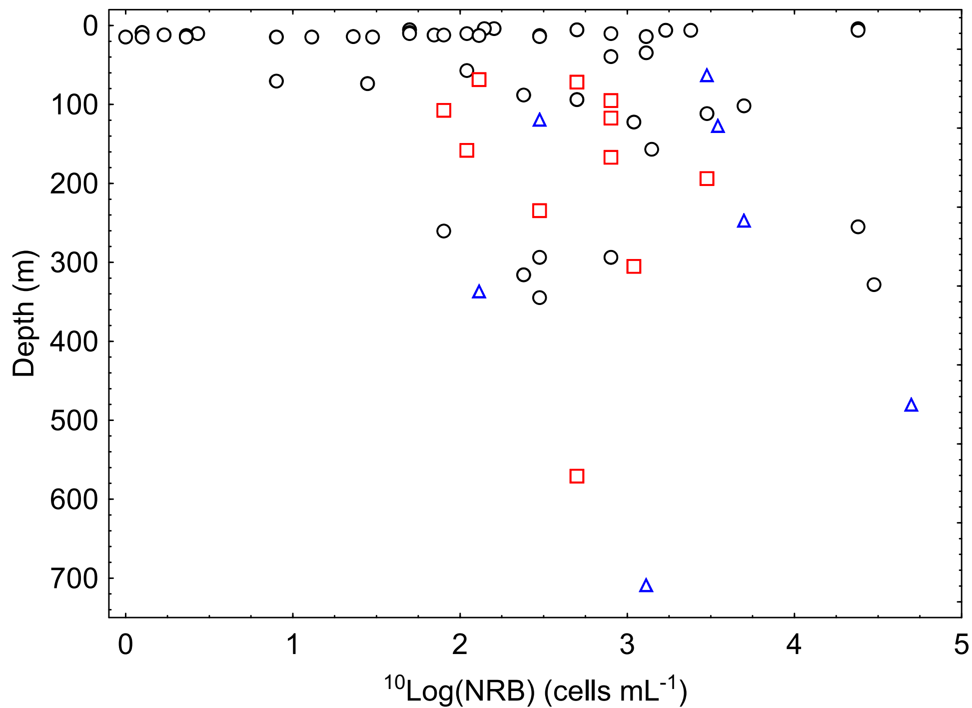

Figure 3-5. *The distribution of nitrate-reducing bacteria (NRB) versus depth in Olkiluoto groundwater. Data: 2005–2007, black circles; 2008, red squares; 2009, blue triangles.*

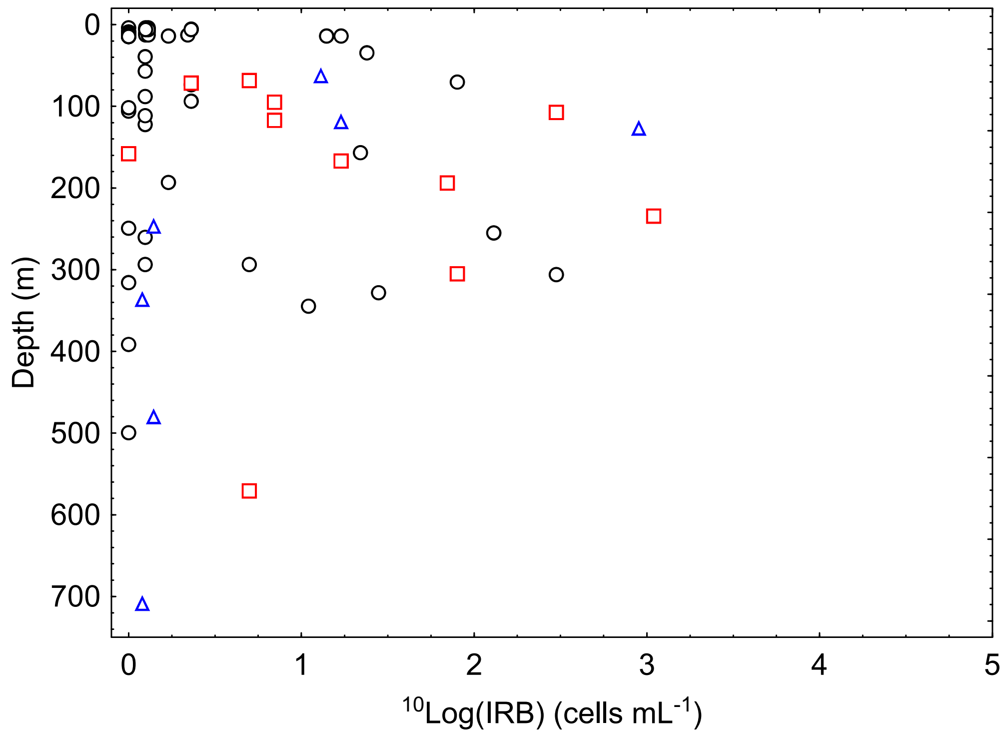

Figure 3-6. *The distribution of iron-reducing bacteria (IRB) versus depth in Olkiluoto groundwater. Data: 2005–2007, black circles; 2008, red squares; 2009, blue triangles.*

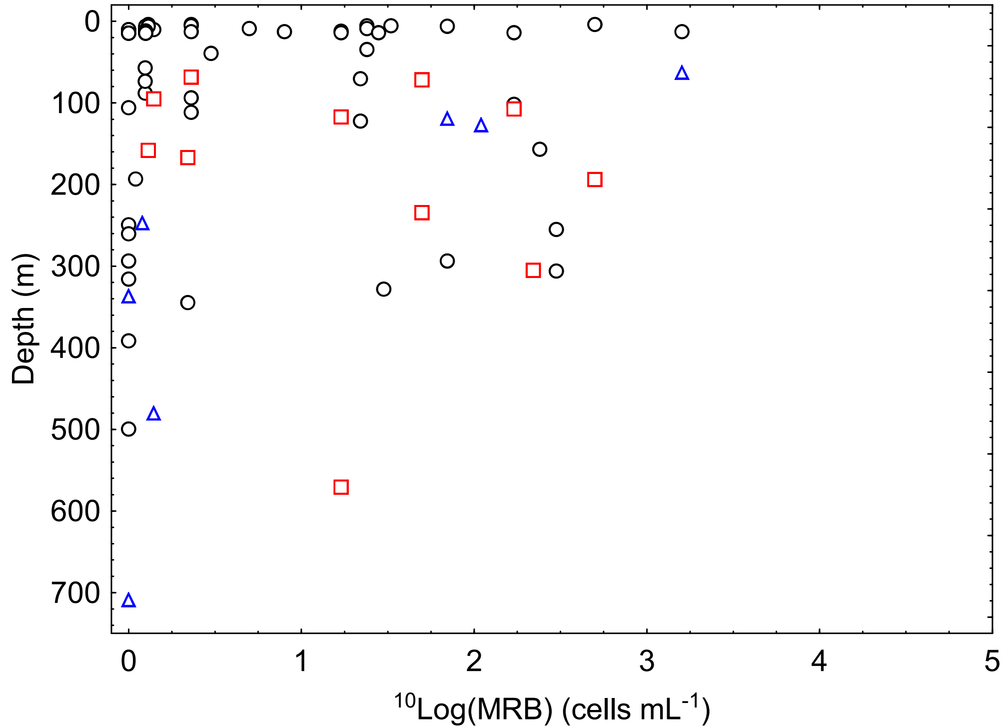

Figure 3-7. *The distribution of manganese-reducing bacteria (MRB) versus depth in Olkiluoto groundwater. Data: 2005–2007, black circles; 2008, red squares; 2009, blue triangles.*

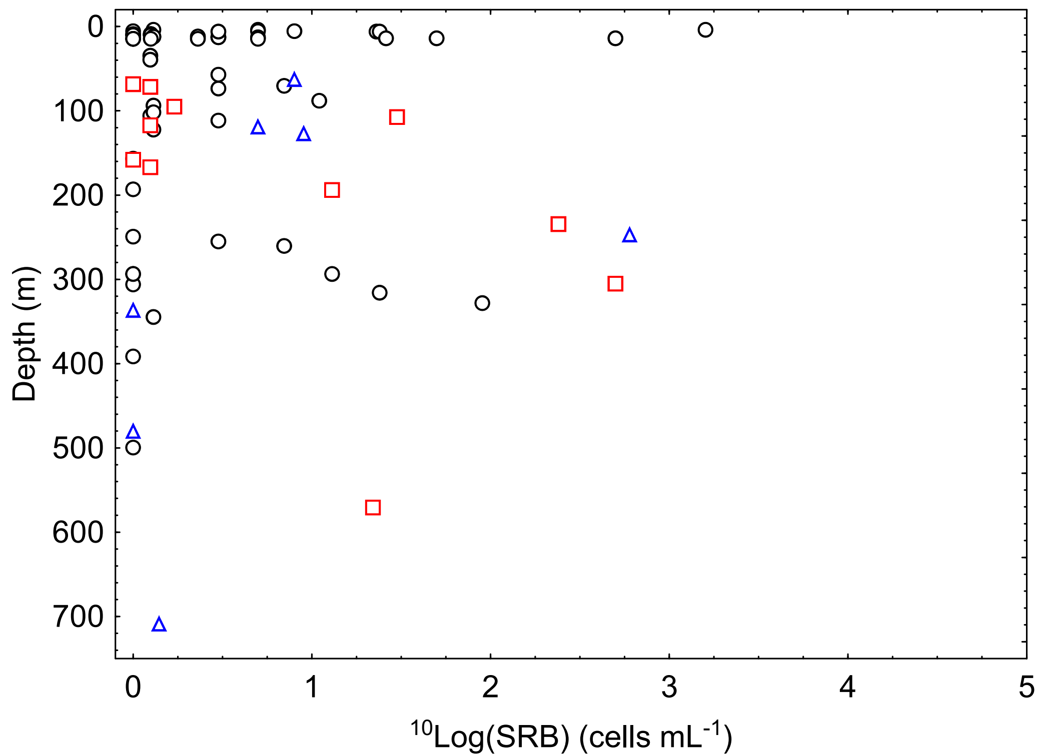

Figure 3-8. *The distribution of sulphate-reducing bacteria (SRB) versus depth in Olkiluoto groundwater. Data: 2005–2007, black circles; 2008, red squares; 2009, blue triangles.*

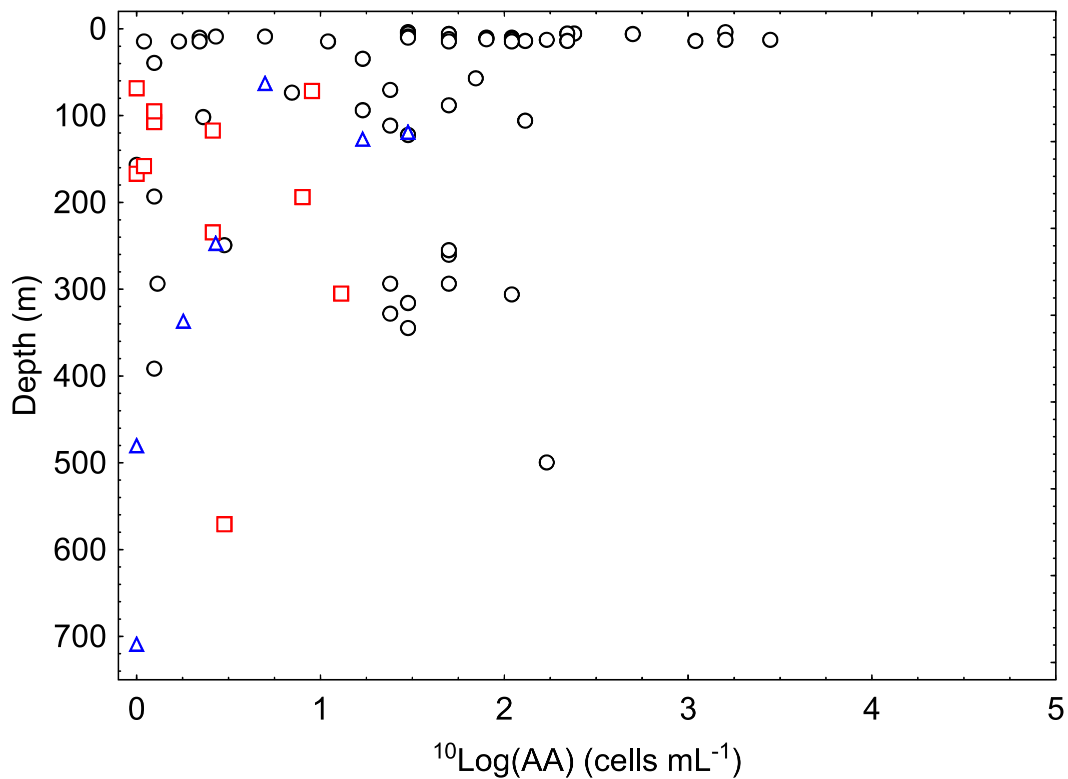

Figure 3-9. *The distribution of autotrophic acetogens (AA) versus depth in Olkiluoto groundwater. Data: 2005–2007, black circles; 2008, red squares; 2009, blue triangles.*

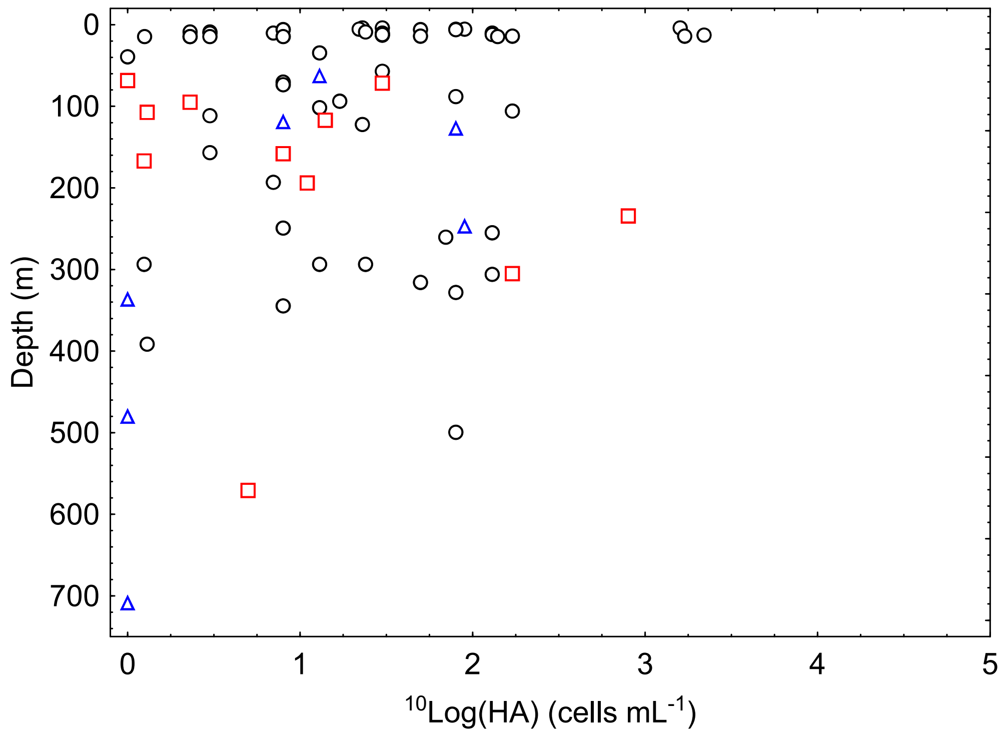

Figure 3-10. *The distribution of heterotrophic acetogens (HA) versus depth in Olkiluoto groundwater. Data: 2005–2007, black circles; 2008, red squares; 2009, blue triangles.*

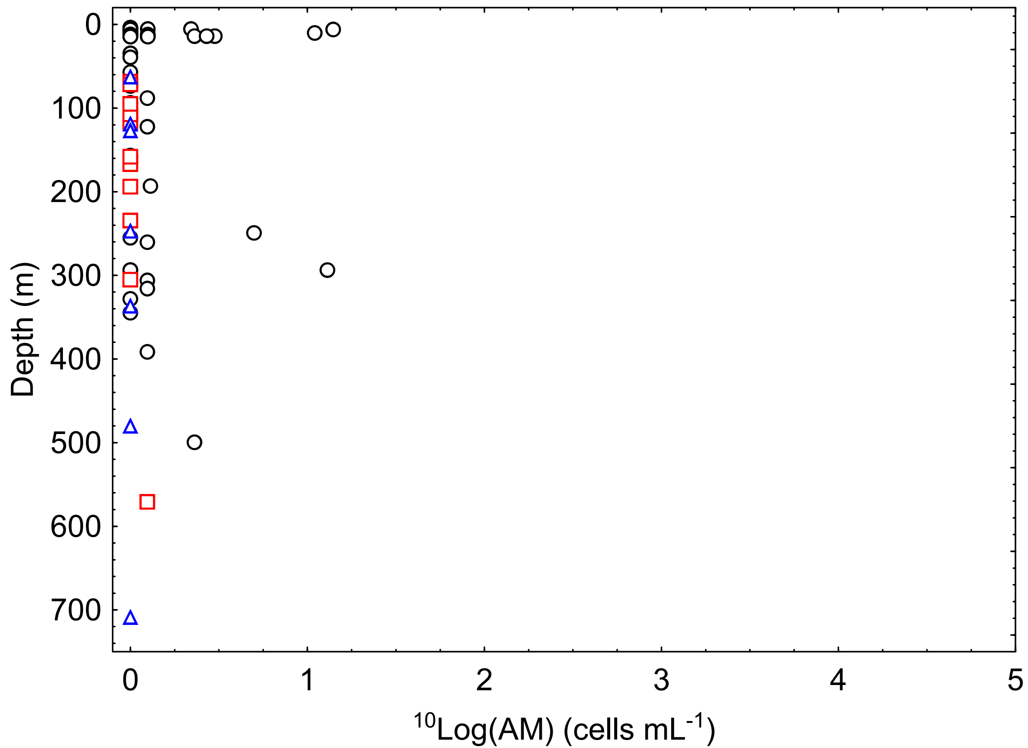

Figure 3-11. *The distribution of autotrophic methanogens (AM) versus depth in Olkiluoto groundwater. Data: 2005–2007, black circles; 2008, red squares; 2009, blue triangles.*

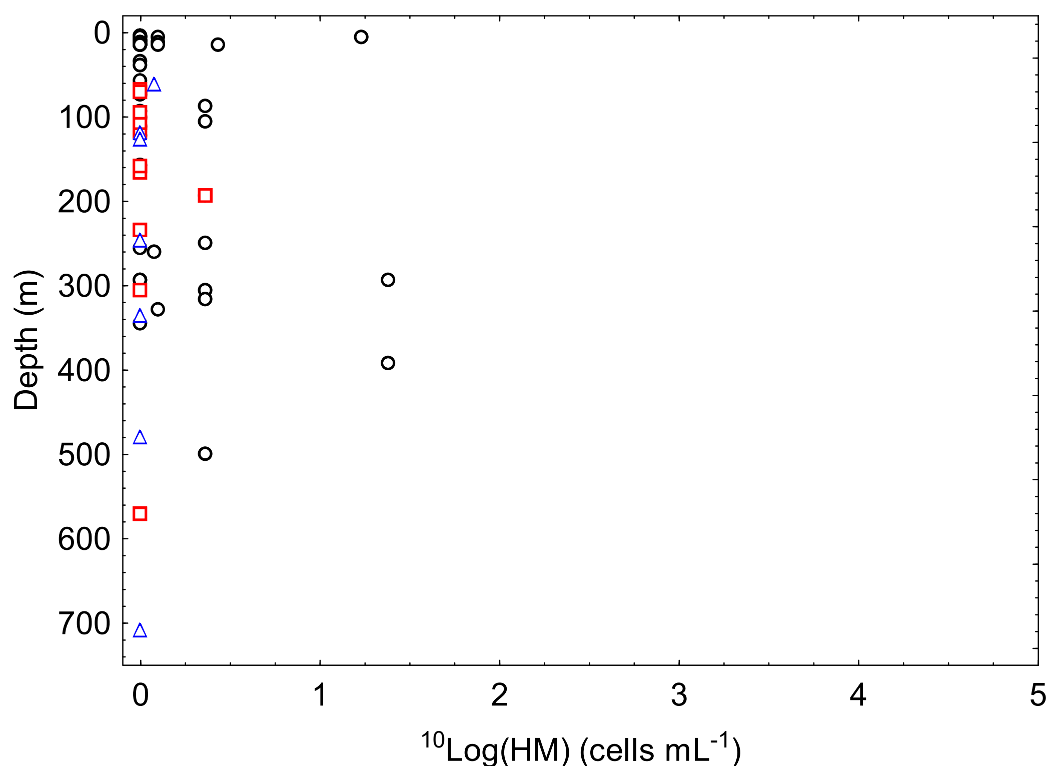

Figure 3-12. *The distribution of heterotrophic methanogens (HM) versus depth in Olkiluoto groundwater. Data: 2005–2007, black circles; 2008, red squares; 2009, blue triangles.*

3.1.5 qPCR results for groundwater samples from 2009

Five groundwater samples from deep drillholes were analysed using qPCR, as outlined in Table 2-2. The method detected *Bacteria*, *Archaea*, *Eukarya*, and some methane-oxidizing, nitrate-reducing, and ANME 2c-related gene signatures (Table 3-2; see also Table 2-4). This was the first set of deep groundwater samples collected using the PAVE system that was analysed using qPCR. The results are evaluated in the Discussion section.

Table 3-2. *qPCR results for five Olkiluoto groundwater samples collected using the PAVE sampler in 2009. The table shows standard units mL^{-1} for each analysis and standard deviation for double qPCR. See Table 2-4 for an explanation of the primers used.*

Sample	***Bacteria***	**SD**	***Archaea***	**SD**	***Eukarya***	**SD**	***apsA***	**SD**	***fthfs***	**SD**	***pmoA***	**SD**
OL-KR45_295_1	167 000	742	939	52.9	<1000	-	<100	-	<100	-	144	46.7
OL-KR49_614_1	9030	146	429	102	<1000	-	<100	-	<100	-	<100	-
OL-KR47_77_1			97.4	6.76	<1000	-	<100	-	<100	-	<100	-
OL-KR47_217_1	47 200	3470	26.2	0.409	<1000	-	<100	-	<100	-	<10	-
OL-KR40_786_1	92 200	7.07	19.4	2.12	<1000	-	<100	-	<100	-	<100	-

Sample	***mxaF***	**SD**	***narG1***	**SD**	***narG2***	**SD**	***nirK***	**SD**	***psvA***	**SD**	***ANME-1***	***ANME-2a***	***ANME-2c***
OL-KR45_295_1	1690	857	n.a.		n.a.		n.a.		n.a.		Neg.	Neg.	Neg.
OL-KR49_614_1	<100	-	4 610 000	2 340 000	<100	-	n.a.		160	70.1	Neg.	Neg.	Neg.
OL-KR47_77_1	<10	-	1 130 000	886 000	<100	-	<100	-	<100	-	Neg.	Neg.	Neg.
OL-KR47_217_1	<10	-	19 500	17 300	<100	-	1710	849	82 300	69 800	Neg.	Neg.	Pos.
OL-KR40_786_1	<10	-	444 000	55 600	<100	-	<100	-	2990	4070	Neg.	Neg.	Pos.

3.2 Drillhole OL-KR13_T360: effects of pumping on bacterial diversity and chemistry

It was hypothesized that drillhole pumping would influence the microbial population in the groundwater. The OL-KR13_T360 drillhole (length section 360.5–364.5 m, depth section 292.7–296.1 m) was therefore analysed over 48 h of pumping. Pumping started at 07:00 on 28 October 2008 at an average rate of 20 mL groundwater min^{-1}. Chemical (Table 3-3) and microbiological (Table 3-4) parameters were analysed daily as reported in the following sections. There was an increase in chloride and sulphate during pumping after the 10.4 L of water standing in the tubing was pumped out (Figure 3-13). This volume corresponds to the volume of the tube between the sampling section and the sampling point at the surface. Sulphate and chloride contents did not change before this volume was pumped out, reflecting the composition of the technical water used to fill the tube. Ferrous iron fluctuated, pH increased one unit from start of pumping to the last sampling, and sulphide appeared when the tube water (10.4 L) was pumped out.

3.2.1 Total number of cells

The average TNC from three previous analyses of OL-KR13_362 groundwater sampled using the PAVE system from open drillholes from 2004 to 2006 (n = 3) was 7.6 × 10^4 cells mL^{-1}, while the observed TNC from OL-KR13_T360 (using a multipacker system installed in the drillhole during the October 2008 pumping was approximately 1.0 × 10^5 cells mL^{-1}, close to previously observed numbers (Table 3-4). The TNC decreased significantly over the pumping period. The complete dataset including statistical details can be found in Table A-1.

Table 3-3. *The evolution of some chemical parameters in OL-KR13-T360 (–293 mbsl) when pumping water at a flow rate of 20 mL min^{-1}. The drillhole volume used to calculate turnover time, including the tube volume from the pumped sampling section to surface, was 28.53 L. Pumping started at 07:00, 28 October 2008 and ended at 08:10, 31 October 2008.*

Elapsed pumping time (min)	Pumped volume (L)	Drillhole volume turnover (times)	Chloride (mg L^{-1})	Ferrous iron (mg L^{-1})	pH	Sulphate (mg L^{-1})	Sulphide (mg L^{-1})
110	2.20	0.08	13.4	0.24	6.7	41.8	<0.01
290	5.80	0.20	13.5	0.10	6.8	41.9	<0.01
410	8.20	0.29	59.1	0.27	6.7	45.1	<0.01
530	10.60	0.37	18.5	0.20	6.9	44.2	<0.01
1440	28.80	1.01	531	0.43	7.7	88.2	0.03
1610	32.20	1.13	843	0.34	7.8	87.7	0.02
2870	57.40	2.01	1100	0.26	7.8	87.6	0.02
2950	59.00	2.07	1320	0.29	7.7	86.2	0.04

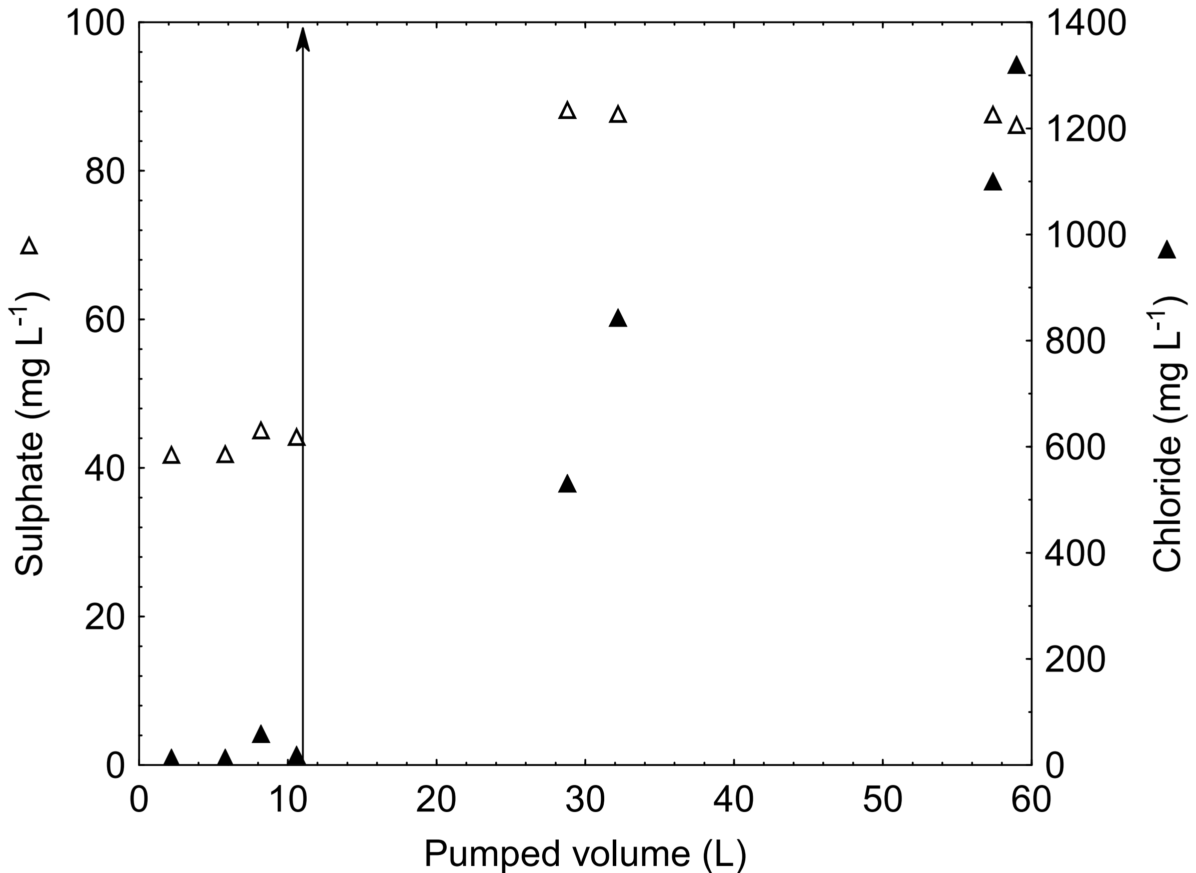

Figure 3-13. *The evolution of sulphate and chloride contents during the pumping of OL-KR13 T360, 28–31 October 2008. The arrow indicates the volume of the tube extending from the pumped sampling section to the ground surface where sampling was done.*

Table 3-4. *The evolution of microbiological parameters in OL-KR13_T360 (–293 mbsl) when pumping water at a flow rate of 20 mL groundwater min^{-1}. The drillhole volume includes the volume of the tube extending from the pumped sampling section to the surface and was 28.53 L. Pumping started at 07:00, 28 October 2008 and ended at 08:10, 31 October 2008. IRB = iron-reducing bacteria, SRB = sulphate-reducing bacteria, AM = autotrophic methanogens, apsA = adenosine-5′-phosphosulphate reductase alpha subunit.*

Sample number	Pumped volume (L)	Drillhole volume turnover (times)	(cells mL^{-1})				(standard units mL^{-1})			
			TNC	IRB	SRB	AM	16S *Bacteria*	16S *Archaea*	*apsA*-DNA	*apsa*-RNA
OL-KR13-1	3.60	0.13	28,000	13.0	130.0	<0.2	680,000	340	<1	200
OL-KR13-2	7.20	0.25	17,000	1.7	5.0	<0.2	800,000	200	11,000	<1
OL-KR13-3	9.60	0.34	19,000	2.3	130.0	<0.2	990,000	51	17,000	<1
OL-KR13-4	12.00	0.42	22,000				2,500,000	600	440	<1
OL-KR13-5	31.20	1.09	79,000	3.3	30	130	110,000	850	<1	<1
OL-KR13-6	33.60	1.18	15,000				120,000	1400	<1	<1
OL-KR13-7	58.80	2.06	62,000	1.3	8.0	3	150,000	1300	<1	<1

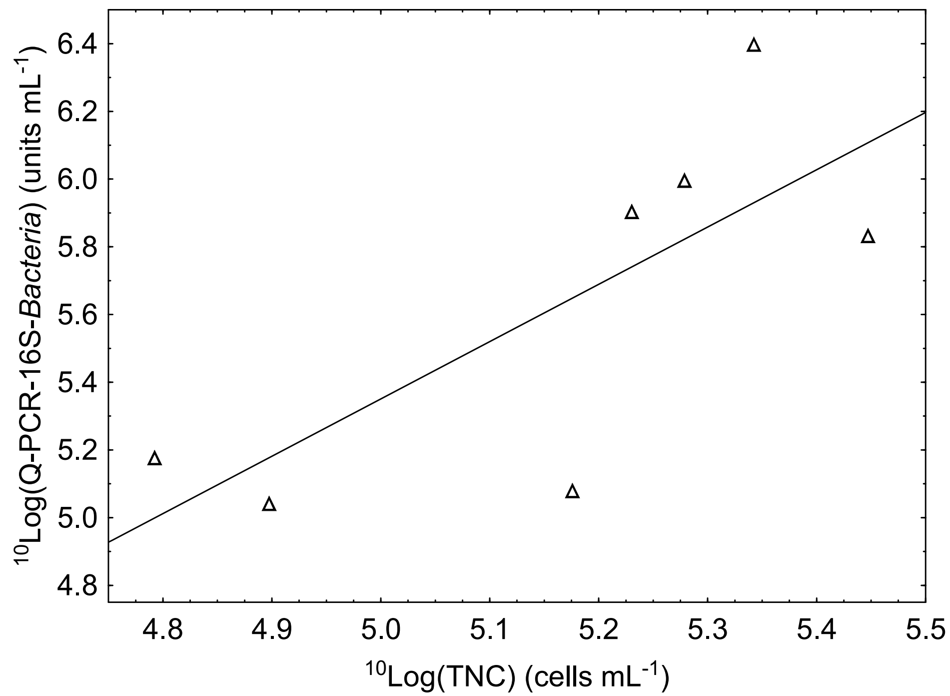

Figure 3-14. *The relationship between the total numbers of cells (TNC) counted using the microscope and the number of* Bacteria *cells analysed as 16S-RNA gene units using quantitative PCR (qPCR) during the pumping of OL-KR13_T360. Linear correlation line data are: Log(qPCR) = 1.69 × Log(TNC) – 3.11; r = 0.76, p = 0.049,* r^2 *= 0.5723.*

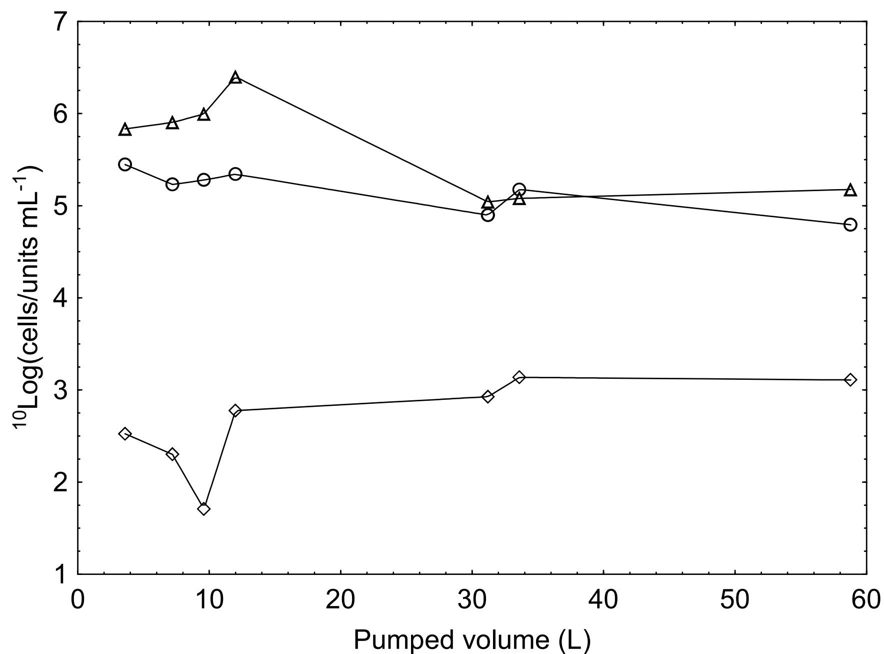

Figure 3-15. *The evolution of number of cells (TNC) (△) as evaluated using microscopy, and the number of standard cell units over the volume pumped from OL-KR13_T360 qPCR of the* Bacteria *16S-RNA gene (○) and qPCR of the* Archaea *16S-RNA gene (◇).*

3.2.2 Most probable number of cultivable anaerobic microorganisms

The MPN of IRB decreased over the pumping period, while the number of SRB varied without any trend related to pumping (Table 3-4). The largest number of SRB, 130 cells mL^{-1}, was found in sample OL-KR13-1. Significant numbers of AM appeared when groundwater from the pumped-out section reached the sampling point. Autotrophic methanogens were most abundant in OL-KR13-5, which contained 130 cells mL^{-1}

3.2.3 qPCR of biomass and SRB

The total biomass in OL-KR13 T360, as analysed using qPCR of the *Bacteria* 16S rRNA gene in the seven different pumped volumes, ranged from 1.1×10^5 to 2.5×10^6 cells mL^{-1}. These qPCR numbers correlated reasonably well with TNC numbers (Figure 3-14). As evaluated in detail in section 4.4, MPN and qPCR SRB results from OL-KR13:1-7 displayed trends similar to those from samples OL-KR13-1 to OL-KR13-7. The SRB were found to be most active in the first four samples (OL-KR13-1–4) using both MPN and the SRB qPCR analysis of *apsA*-DNA (Table 3-4). The qPCR on SRB *apsA*-DNA indicated the highest numbers of cells in the second and third samples, which then decreased to below the detection level in samples OL-KR13-5–7. The *Archaea* 16S rRNA gene unit values were lower than *Bacteria* 16S gene unit values in all samples. An increase in the *Archaea* unit numbers started in OL-KR13-4, when section water reached the sampling point, at 600 standard units mL^{-1}, increasing to 1400 standard units mL^{-1} in OL-KR13-6, which had the highest numbers of *Archaea* unit numbers. OL-KR13-7 also contained high *Archaea* biomass at 1300 standard units mL^{-1}. This indicated an increase in *Archaea* numbers as the pumping progressed, so these *Archaea* cells probably originated from the groundwater in the sampled drillhole aquifer and not from the tube-filling water or the water in the drillhole section. The OL-KR13-7 sample was positive in ANME-1, ANME-2a, and ANME-2c qPCR. Therefore, the groups ANME-1, -2a, and -2c seem to have been present in equal amounts in this sample, though a standard will be needed to calculate the exact numbers of ANME in standard units mL^{-1}. However, ANME has never been cultured, so pure cultures were unavailable as standards. At this stage we conclude that the OL-KR13-7 groundwater sample was positive for the three ANME groups; further studies are needed to be able to evaluate their numbers.

3.2.4 Diversity: cloning and sequencing of 16S rRNA genes in drillhole OL-KR13 T360

Using PCR, 16S rRNA genes from drillhole OL-KR13 T360 were amplified, after which they were cloned and sequenced (Table 2-3) for the identification of microorganisms present after various pumping durations. The analysis was performed using 16S rRNA gene primers for *Bacteria* and *Archaea*.

Sequences related to the domain *Bacteria*

Bacterial PCR, cloning, and sequencing were carried out on four samples (i.e., OL-KR13-1, -4, -6, and -7) to analyse microbial diversity as a function of pumped volume (Table 3-5). The depth of the drillhole section (–293 mbsl) coincides with a peak in the groundwater concentration of sulphide (see Figure 2d in Pedersen et al. 2008b). Sample OL-KR13-1 was dominated by bacteria related to the Beta-proteobacteria, Gamma-proteobacteria, and Flavobacteria domains, such as *Acidovorax defluvii*, *Pseudomonas*

fluorescens, *Flavobacterium granulii*, and *Rhodocyclaceae bacterium* FTL11 (Table 3-5). Sample OL-KR13-4 contained some bacteria found in OL-KR13-1 as well as clones unique to this sample. Four clones were observed in both OL-KR13-1 and OL-KR13-4. Sample OL-KR13-6 differed in sequence composition from OL-KR13-1 and OL-KR13-4. Sulphur-oxidizing-bacteria-related sequences such as *Thiobacillus* sp. D24TN were found and one clone belonged to the sulphate-reducing group *Dehalococcoides*. Most of the clones found were most similar to various uncultured bacteria, so detailed conclusions should not be drawn based on the results. However, there was a clear change in the sample diversity over the pumped volumes, with only a few overlapping observations of identical clones between samples. In OL-KR13-7, most of the analysed clones were related to SRB. Some displayed high identity to sulphate-reducing ANME partners, such as the uncultured bacterium clone BC_B2_3f (EU622295), the uncultured delta proteobacterium MERTZ_21CM_138 AF424243, and the uncultured bacterium CS_B042 AF419686. One OL-KR13-7 clone was related to the iron-oxidizing Gamma-proteobacteria *Siderooxidans lithoautotrophicus*. Others were related to uncultured and unknown bacteria.

Sequences related to the domain *Archaea*

Archaea PCR, cloning, and sequencing were carried out on four samples (OL-KR13-1, -4, -6, and -7) to analyse microbial diversity as a function of pumped volume. Some methanogens are assumed to be able to act as partners of ANME consortia and were expected to be present at this depth. A significant change in *Archaea* diversity over pumped volume was observed (Table 3-6). The samples OL-KR13-1 and OL-KR13-4 lacked detectable *Archaea*. Sample OL-KR13-6 contained two clone sequences belonging to different uncultured *Archaea* that were most similar to anaerobic methane oxidizers; these were found in two of eight clones. One clone from OL-KR13-7 was most similar to ANME *Archaea* (96 % id.) while another clone belonged to Euryarchaeota (Table 3-6).

Table 3-5. *Bacterial diversity as revealed by PCR, using* Bacteria *primers, cloning and sequencing of four groundwater samples from OL-KR13 T360 collected in series during pumping.*

Clone name	Phylogenetic group	Representative of closest match (accession number)
OL-KR13-15	Gammaproteobacteria	*Pseudomonas fluorescens* (AY947533)
OL-KR13-5	Betaproteobacteria	*Acidovorax defluvii* strain b268 (EU434475)
OL-KR13-7	Betaproteobacteria	*Acidovorax sp.* NA3 (EU910093)
OL-KR13-2	Betaproteobacteria	*Acidovorax defluvii* (Y18616)
OL-KR13-10	Flavobacteria	*Flavobacteria bacterium* KF030 (AB269814)
OL-KR13-11	Flavobacteria	*Flavobacterium granulii* (AB180738)
OL-KR13-14	Gammaproteobacteria	*Pseudomonas corrugata* (AF348508)
OL-KR13-21	Unclassified	Uncultured bacterium clone 2C228285 (EU800249)
OL-KR13-22	Unclassified	Uncultured bacterium clone 3C003586 (EU802167)
OL-KR13-41	Unclassified	Uncultured soil bacterium clone C011
OL-KR13-16	Betaproteobacteria	*Rhodocyclaceae bacterium* FTL11 (DQ451827)
OL-KR13-35	Bacterodetes	Uncultured *Cytophaga* sp. (AB189373)
OL-KR13-23	Betaproteobacteria	Uncultured bacterium clone BANW528 (DQ264506)
OL-KR13-37	Betaproteobacteria	Uncultured *Delftia* sp. clone GI5-007-G05 (FJ192817)
OL-KR13-1	Tenericutes	*Acholeplasma polakii* (AF031479)
OL-KR13-39	Deltaproteobacteria	Uncultured *Desulfobacteraceae* (AJ582688)
OL-KR13-25	Synergistetes	Uncultured bacterium clone CSB87 (DQ677365)
OL-KR13-43	Betaproteobacteria	*Verminephrobacter eiseniae* EF01-2 (CP000542)
OL-KR13-13	Actinobacteria	*Propionibacterium acnes* isolate WD1 (AY642054)
OL-KR13-3	Betaproteobacteria	*Acidovorax defluvii* (Y18616)
OL-KR13-4	Betaproteobacteria	*Acidovorax defluvii* (Y18616)
OL-KR13-12	Betaproteobacteria	*Polaromonas naphthalenivorans* CJ2 (CP000529)
OL-KR13-18	Betaproteobacteria	*Thiobacillus* sp. D24TN (EU685841)
OL-KR13-36	Deltaproteobacteria	Uncultured *Dehalococcoides* sp. clone 32IIISN
OL-KR13-26	Gammaproteobacteria	Uncultured bacterium clone EV818SWSAP14 (DQ773063)
OL-KR13-31	Gammaproteobacteria	Uncultured bacterium clone Won86 (0714) (DQ839504)
OL-KR13-20	Unclassified	Uncultured bacterium 051011_S1_W_T_SDP_134 (FJ524892)
OL-KR13-28	Unclassified	Uncultured bacterium clone Hados.Sed.Eubac.7. (AB355076)
OL-KR13-34	Unclassified	Uncultured bacterium, clone M6A-713 (AM991274)
OL-KR13-40	Firmicutes	Uncultured organism clone MAT-CR-M8-G11 (EU245968)
OL-KR13-8	Alphaproteobacteria	*Brevundimonas mediterranea* (AJ244706)
OL-KR13-9	Deltaproteobacteria	*Desulfoarculus baarsii* (AF418174)
OL-KR13-24	Deltaproteobacteria, ANME	Uncultured bacterium clone BC_B2_3f (EU622295)
OL-KR13-38	Deltaproteobacteria, ANME	Uncultured deltaproteobacterium (AF424243)
OL-KR13-17	Gammaproteobacteria	*Siderooxidans lithoautotrophicus strain* ES-1 DQ386264
OL-KR13-33	Potential ANME partner	Uncultured bacterium CS_B042 (AF419686)
OL-KR13-32	Spirochaetes	Uncultured bacterium clone ZZ12C6 (AY214182)
OL-KR13-19	Unclassified	Uncultured bacteria clone X35 (DQ083107)
OL-KR13-27	Unclassified	Uncultured bacterium clone FCPN695 (EF516216)
OL-KR13-30	Unclassified	Uncultured bacterium clone MD2898-B4 (EU386048)
OL-KR13-29	Unclassified, SRB?	Uncultured bacterium clone L2B_031) (EF551853)
OL-KR13-42	Verrucomicrobia	Uncultured *Verrucomicrobia* bacterium MVP-92 (DQ676386)
Sum		

***Table 3-5.** continued Bacterial diversity as revealed by PCR, using* Bacteria *primers, cloning and sequencing of four groundwater samples from OL-KR13 T360 collected in series during pumping. The numbers of identical clones and of observations are shown for each drillhole.*

Identity (%)	Accession number	OL-KR13-1	OL-KR13-4	OL-KR13-6	OL-KR13-7	Sum of observations
99	FJ851563, FJ851550	1	1			2
99	FJ851561, FJ851551	1	3			4
99	FJ851555, FJ851559	1	4			5
92	FJ851549	1				1
99	FJ851554	1				1
98	FJ851552	1				1
99	FJ851557	1				1
100	FJ851606	1				1
91	FJ851605	1				1
94	FJ851558	1				1
98	FJ851553, FJ851556	4	2			6
99	FJ851565		1			1
99	FJ851564		1			1
94	FJ851560		1			1
96	FJ851566		1			1
98	FJ851610		2			2
100	FJ851608, FJ851597		3	1		4
82	FJ851609		6			6
100	FJ851598			1		1
99	FJ851568			1		1
93	FJ851576			1		1
72	FJ851600			1		1
95	FJ851562			1		1
95	FJ851574			1		1
92	FJ851570, FJ851586			1	1	2
97	FJ851577			1		1
99	FJ851569			1		1
90	FJ851573			1		1
93	FJ851571			1		1
92	FJ851567			3		3
99	FJ851581				1	1
92	FJ851594				1	1
97	FJ851579				2	2
95	FJ851582				1	1
96	FJ851585				1	1
95	FJ851583				1	1
99	FJ851578				1	1
87	FJ851584				1	1
95	FJ851595				1	1
94	FJ851587				1	1
91	FJ851580				2	2
95	FJ851593				1	1
		14	**25**	**15**	**15**	**69**

Table 3-6. *Archaea diversity as revealed by PCR, using Archaea primers, cloning and sequencing of four groundwater samples from OL-KR13 T360 collected in series during pumping. The numbers and distribution of unique clones over the samples are shown.*

Drill-hole	Phylo-genetic group	Clone name	Representative of closest match in GenBank (accession number)	Iden-tity (%)	Accession number of clones	OL KR 13-1	OL KR 13-4	OL KR 13-6	OL KR 13-7	Sum
OL-KR13-6	Un-classified	OL-KR13-44	Uncultured candidate division OD1 bacterium clone SRC61 (AY193293)	*90*	FJ851596			1		1
OL-KR13-6	*Archaea* (potential ANME)	OL-KR13-45	Uncultured *Archaea* clone MOB4-5 (DQ841225)	*92*	FJ851599			1		1
OL-KR13-6	*Archaea* (potential ANME)	OL-KR13-46	Uncultured *Archaea* clone MH1492_B10B (EU155957)	*97*	FJ851601			1		1
OL-KR13-7	Un-classified	OL-KR13-47	Uncultured bacterium clone EV818SWSAP14 (DQ773063)	*92*	FJ851592				6	6
OL-KR13-7	ANME	OL-KR13-48	Uncultured *Archaea* clone 5H3_A21 (DQ301872)	*96*	FJ851589				1	1
OL-KR13-7	Eury-archaeota	OL-KR13-49	Uncultured euryarchaeote SPG12_153_163_A7 (FJ487471)	*89*	FJ851591				1	1
Sum								**3**	**8**	**11**

ANME cultures from OL-KR13-7

Most probable number cultures with methane for ANME were set up for the OL-KR13-7 samples. Two MPN dilutions (1×10^0 and 3×10^0) from the ANME MPN were turbid, which is indicative of culture growth, and were selected for further study. Table 3-7 shows the results of sequencing the obtained clones. ANME methanogens and SRB were found. They seemed relatively well enriched, especially in 2008-10-30-OL-KR13-7:1 (dilution 3×10^0), since we found both ANME and sulphate reducers in this culture. Other microorganisms found in the two MPN cultures from OL-KR13-7 were various sulphate-reducers known to form clusters with ANME *Archaea* (Lösekann et al. 2007; Pedersen et al. 2008a). These observations suggest that methanogenic *Archaea* and SRB involved in anaerobic methane oxidation were present in the MPN cultures from the OL-KR13_7 samples.

Table 3-7. *The diversity as revealed by PCR, using ANME* Archaea *primers, cloning and sequencing for MPN cultures collected from OL-KR13-7 samples.*

Cultivated sample	No. of clones	Microbial group	Closest relative in the database	Identity, %	Length, bp
2008-10-30-OL-KR13:7:1-1×10^0-1	4	ANME	Uncultured *Archaea* gene for 16S rRNA, partial sequence, clone: YWA05 AB294253 (*Methanolobus taylorii* U20154, 97% id.)	100	950
2008-10-30-OL-KR13:7:1-1×10^0-12	2	ANME	Uncultured *Archaea* clone PL-7A3 AY570661 (*Methanolobus taylorii* 96%)	99	901
2008-10-30-OL-KR13:7:1-3×10^0-1	6	ANME	Uncultured *Archaea* clone PL-7A3 AY570661 (*Methanolobus taylorii* 95% U20154)	99	801
2008-10-30-OL-KR13:7-3×10^0-1	8	Clostridia; *Desulfosporos inus*	Uncultured bacterium clone C3 EU234214	99	886
2008-10-30-OL-KR13:7-3×10^0-3	1	Bacteroidia	*Paludibacter propionicigenes* AB078842	96	959

3.3 ONKALO groundwater: drillholes ONK-KR1-3 and ONK-PVA1-2

The complete dataset including statistical details can be found in Table A-1 to Table A-3.

3.3.1 Total number of cells

The total number of cells in groundwater from the ONKALO drillholes was within the range of 6.2×10^3 to 4.9×10^5 cells mL^{-1} (Table 3-8). The highest TNC was found in drillhole ONK-KR3 and this observation correlated with the qPCR results for the 16S rRNA *Bacteria* gene.

3.3.2 Biomass and activity based on 16S rRNA and *apsa* RNA qPCR

Biomass was analysed in terms of total number of cells, as described above (3.3.1). These enumerations agreed with the qPCR results for the 16S rRNA gene for most but not all drillholes. Biomass based on qPCR results for groundwater from the ONKALO drillholes was within the range of 3.55×10^4 to 1.63×10^6 cells mL^{-1}; the highest qPCR 16 rRNA value was found in ONK-KR3, which also had the highest TNC value (Table 3-8). SRB activity as analysed using qPCR *apsa* RNA was highest in ONK-PVA1 (1.10×10^5 cells mL^{-1}) and also high in ONK-KR1 (2.20×10^4 cells mL^{-1}) and ONK-KR2 (2.90×10^4 cells mL^{-1}). SRB activity was at least two orders of magnitude lower in ONK-KR3 and almost absent from ONK-PVA2 compared with the other four analysed ONKALO drillholes (Figure 3-16). These numbers agreed with the trends observed earlier during studies of ONKALO groundwater using cloning and sequencing (Pedersen et al. 2008a).

__Table 3-8.__ Results for samples collected in ONKALO, 2008-09-09-2008-09-11.

Drillhole	Sampling date	(cells mL^{-1})			(standard units mL^{-1})		
		Total number of cells	MPN SRB	MPN AM	qPCR 16S rRNA gene *Bacteria*	qPCR 16S rRNA gene *Archaea*	qPCR *apsa* RNA
ONK-KR1	2008-09-09	44,000	-	-	36,000	-	22,000
ONK-KR2	2008-09-09	6200	-	-	63,000	-	29,000
ONK-KR3	2008-09-09	490,000	-	-	1,600,000	-	1000
ONK-PVA1	2008-09-09	51,000	-	-	48,000	-	110,000
ONK-PVA2	2008-09-09	79,000	-	-	130,000	-	35
ONK-PVA3 :1 L	2008-09-09	87,000	-	-	41,000	-	15
ONK-PVA3:2 L	2008-09-09	35,000	-	-	42,000	-	6200
ONK-PVA3: 14 L	2008-09-09	n.d.	-	-	330,000	-	10
ONK-PVA3:175 L	2008-09-09	12,000	-	-	26,000	-	710
ONK-PP128-1	2008-09-10	n.d.	0.7	0.2	610,000	59	2600
ONK-PP128-2	2008-09-10	26,000	<0.2	<0.2	85,000	61	410
ONK-PP128-3	2008-09-11	48,000	0.4	<0.2	130,000	47	7600

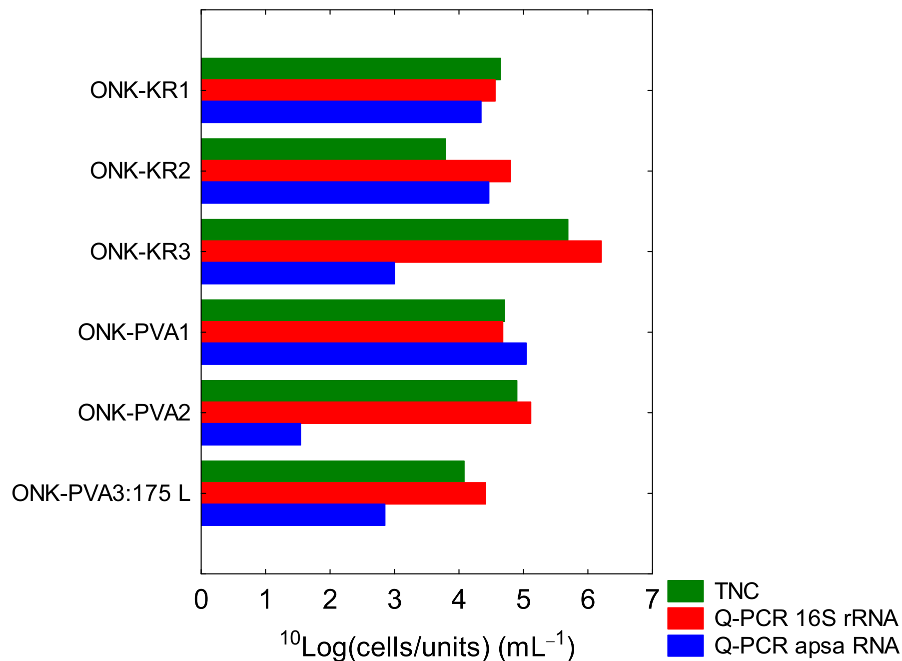

__Figure 3-16.__ The total number of cells and qPCR 16S rRNA gene determination of total number and SRB activity in groundwater analysed as apsa *RNA from six drillholes in ONKALO.*

3.4 ONKALO groundwater: drillhole ONK-PVA3 draining

A draining experiment was performed to investigate whether draining had any effect on numbers of cells and biomass in ONKALO drillholes. Samples were therefore collected in series during a draining event.

3.4.1 Total number of cells

The total number of cells (Figure 3-17) in groundwater from ONKALO drillhole ONK-PVA3 was within the range of 1.2×10^3 to 8.7×10^4 cells mL^{-1}. The highest number was found in the first sample collected after 1 L of draining, and the total numbers of cells then declined with drained volume. This was also observed using qPCR on the 16S rRNA gene (Figure 3-17). However, there was a small increase in SRB activity as indicated by the *apsa* value after 14 L of draining.

3.4.2 Biomass and activity based on 16S rRNA *Bacteria* gene and *apsa* mRNA qPCR

Biomass generally decreased over time when draining ONK-PVA3. The biomass analysed as 16S rRNA gene was highest after 14 L of draining and lowest after 175 L of draining, ranging from 2.56×10^4 to 3.30×10^5 standard units mL^{-1} (Figure 3-17). The number of active SRB as analysed in terms of *apsa* RNA was highest after 2 L of draining, when it reached 6×10^3 standard units mL^{-1}, and low at the other three drained volumes (Figure 3-17).

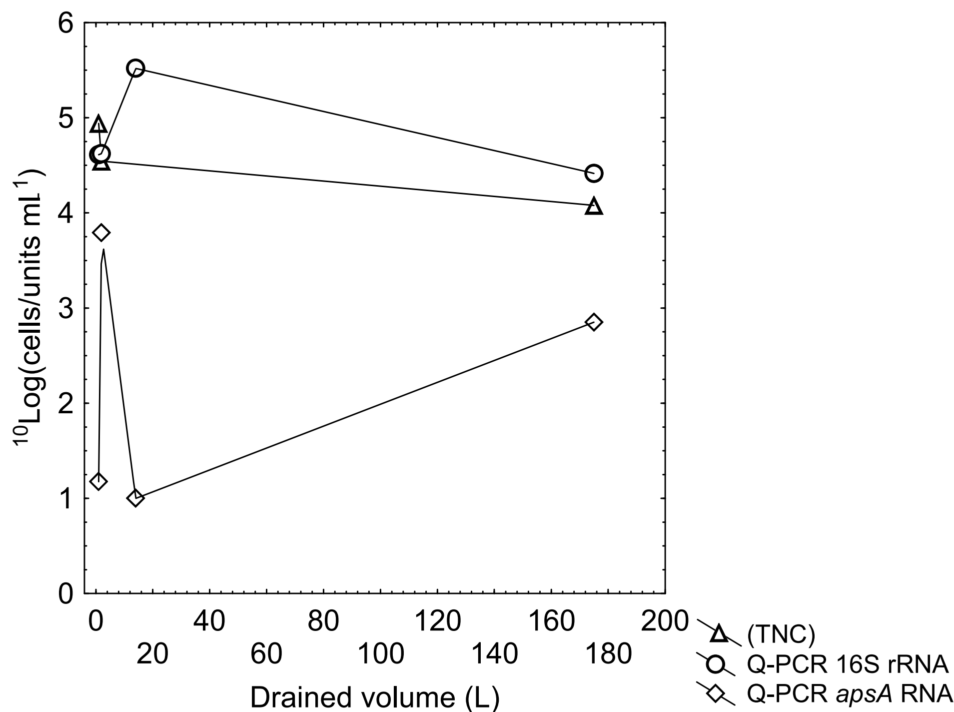

Figure 3-17. *The effect of draining ONK-PVA3 on the TNC, the amount of 16S rRNA Bacteria gene, and the* apsa *RNA activity.*

3.5 Draining of drillhole ONK-PP128

The complete dataset including statistical details can be found in Table A-1 to Table A-3.

3.5.1 Total number of cells

The total number of cells in groundwater drained from ONK-PP128 ranged from 2.6×10^4 to 4.8×10^4 cells mL^{-1} (Table 3-8). The first sample was very contaminated with concrete from adjacent injection activity, so the TNC could not be counted due to interference from the concrete in the fluorescence microscope.

3.5.2 Most probable number of SRB, AM, and ANME

The MPN of SRB and methanogens indicated low numbers of SRB in the first and third samples, with 0.7 and 0.4 cells mL^{-1}, respectively (Table 3-8). Autotrophic methanogens (AM) were found in the first sample with 0.2 cells mL^{-1}. The three ANME MPN were below detection limit (<0.2 cells mL^{-1}).

3.5.3 Biomass and activity based on 16S rRNA *Bacteria* gene, 16S rRNA *Archaea* gene, and *apsa* mRNA qPCR

Total biomass measured using 16S rRNA gene qPCR for domain *Bacteria* ranged from 8.5×10^4 standard units mL^{-1} in ONK-PP128-2 to 6.1×10^5 standard units mL^{-1} in ONK-PP128-1. ONK-PP128-3 had a somewhat lower number than ONK-PP128-1, with 1.3×10^5 standard units mL^{-1} (Table 3-8).

ONK-PP128 biomass was also analysed in terms of the number of copies of the 16S rRNA gene universally found in domain *Archaea* (Table 3-8). The number of *Archaea* cells was near the detection level. Activity of SRB was analysed using qPCR for the *apsa* RNA. The highest number of active SRB was found in sample ONK-PP128-3, in which 7600 standard units mL^{-1} were detected. Sample ONK-PP128-1 had the second highest number, 2600 standard units mL^{-1}, while ONK-PP128-2 contained 410 standard units mL^{-1}.

Diversity: cloning and sequencing of 16S rRNA genes

Cloning was carried out to analyse for the existence of DNA sequences similar to ANME belonging to domain *Archaea* and to analyse for their sulphate-reducing partners. ONK-PP128 at the 297-m level was the first ONKALO drillhole studied for this purpose. However, ANME signatures have been observed earlier in Olkiluoto in other drillholes (Pedersen et al. 2008a). One clone found in ONK-PP128-3 water was similar to the uncultured *Archaea* clone SBAK-mid-46 (accession number DQ640143), 1010 bp being affiliated with an 83 % identity to this sequence found at GenBank (Table 3-9). This identity was very low, but no sequence in GenBank was more similar to this one. The SRB *Chromobacterium violaceum* was also found and it could be a potential ANME partner. The cloning efficiency for PP128 groundwater was low, probably due to disturbance of the molecular protocols by the considerable concrete contamination of the sampled groundwater. The last sample, number 3, was fairly free of concrete and was the only sample that returned some relevant, although limited, clone data.

Table 3-9. *Sequences found by cloning the groundwater collected from the ONK-PP128 drillhole at a dept of 297 m on three occasions after the start of draining (samples ONK-PP128-1–3).*

Sample	No. of id. clones	Phylum	Closest relative	Identity, %	Length, bp
2008-09-11-ONK-PP-128-3 65:1-2	1	ANME	Uncultured *Archaea* clone SBAK-mid-46 DQ640143	83	1010
2008-09-11-ONK-PP-128-3 65:1-6	1	SRB	*Chromobacterium violaceum* AE016825	76	

3.5.4 GenBank accession numbers

The sequences from each drillhole were submitted to GenBank. OL-KR13 data were submitted to the GenBank database and given the accession numbers FJ851549-FJ851587 for the bacterial sequences and FJ851588-FJ851610 for the *Archaea* sequences. ONK-PP128 sequences were very few and were therefore not submitted to GenBank.

3.5.5 ONKALO 2009

ONK-PVA1, ONK-PVA2, ONK-PVA4, and ONK-PVA5 groundwater

The results of qPCR analysis of groundwater from ONKALO drillholes are shown in Table 3-10. The analysis detected *Bacteria*, *Archaea*, some aerobic MOB, and NRB. The results correlated reasonably well between drillholes, but the *nirK* analysis seems to overestimate the numbers of NRB, and the 16S rRNA gene analysis returned unusually low numbers. Possible explanations for these results are evaluated in the discussion.

ONK-PVA6 and ONK-PVA7 groundwater and fracture surface material biofilms

The results of qPCR for fracture surface materials from the drilling of ONK-PVA6 and ONK-PVA7 were low or below detection. The results above detection coincided with drilling water results, suggesting possible drill water contamination. Possible explanations for the results are evaluated in the Discussion section.

Table 3-10. *qPCR results for various genes in ONKALO groundwater samples. The table shows the numbers of standard units mL^{-1} for each of the primers and standard cells used as described in Table 2-4 .*

Drillhole	***Bacteria***	**SD**	***Archaea***	**SD**	***Eukarya***	**SD**	***apsA***	**SD**	***fthfs***	**SD**	***pmoA***	**SD**
ONK-PVA1	215	3	169	2.1	n.a.		<0.05	-	<0.05	-	144	46.7
ONK-PVA2	1 940	316	64	12.6	n.a.		<0.05	-	<0.05	-	<0.05	-
ONK-PVA4	175	10	0.0926	0.012	n.a.		0.0602	0.06	<0.05	-	<0.05	-
ONK-PVA5	24	1			n.a.		<0.05	-	<0.05	-	<0.05	-
ONK-PVA6	9	1	0.0173	0.0006	n.a.		<0.05	-	<0.05	-	<0.05	-

Sample	***mxaF***	**SD**	***narG1***	**SD**	***narG2***	**SD**	***nirK***	**SD**	***psvA***	**SD**	***ANME-1***	***ANME-2a***	***ANME-2c***
ONK-PVA1	4.57	1.06	n.a.		n.a.		200	59.5	<0.05	-	n.a	n.a	n.a
ONK-PVA2	1.74	0.779	n.a.		n.a.		67 800	21 600	770	156	n.a	n.a	n.a
ONK-PVA4	0.363	0.049	n.a.		n.a.		5 870	6 510	105	22.4	n.a	n.a	n.a
ONK-PVA5	0.084	0.029	n.a.		n.a.		<0.05	-	<0.05	-	n.a	n.a	n.a
ONK-PVA6	<0.05	-	n.a.		n.a.		<0.05	-	<0.05	-	Neg.	Neg.	Neg.

Table 3-11. *qPCR results for various genes of fracture surface material from two ONKALO drill cores and drill water samples. The table shows numbers of standard units mL^{-1} for each of the primers and standard cells used as described in Table 2-4.*

Sample		16S Bacteria		16S Archaea		apsA		fthfs		pmoA		mxaF		ANME-1		ANME-2a		ANME-2c	
		Relative amount	Standard deviation	Relative amount	Standard deviation (n)	Relative amount	Standard deviation	Relative amount	Standard deviation (n)	Relative amount	Standard deviation	Relative amount	Standard deviation (n)	Amplification	(n)	Amplification	(n)	Amplification	(n)
ONK-PVA6 fracture material	units cm^{-2}	<100	-	807	355 (14)	<100	-	<100	-	<100	-	341	303	Neg.	-	Neg.	-	Pos.	(7)
ONK-PVA7 fracture material	units cm^{-2}	<100	-	567	328 (7)	<100	-	568	290 (3)	<100	-	397	291	Neg.	-	Neg.	-	Pos.	(5)
Drill water	units mL^{-1}	<100	-	12	5.5 (3)	<100	-	<100	-	<100	-	3.8	5.2	Neg.	-	Neg.	-	Neg.	-

3.6 Dissolved gas in groundwater

The total gas volume increased with depth, as was found in previous years. There was great variability in total gas volume with depth, down to approximately 300 m (Figure 3-18), also consistent with the results from 2005–2007. This variability was present in the nitrogen content as well (Figure 3-19). The problem with leakage between the PAVE pressure compartment and the sample compartment discussed previously (Pedersen et al. 2008a) can be assumed to have caused some of the variability in both total gas and nitrogen amounts. As a rule of thumb, nitrogen may occasionally have been overestimated because of contamination of the samples from the pressure compartment. Due to this potential problem, the nitrogen gas in the pressure compartment was replaced with neon gas beginning in 2009. The sample results were thereafter corrected for any volume of neon leaking from the pressure compartment. This new approach has as yet been used for too few samples to be able to evaluate its effectiveness. The results of the analysis of gas in deep groundwater in 2008 and 2009 have been merged with the results from 2005–2007 in all figures to compare reproducibility and to check for changes in composition and amounts. In addition, a first set of gas data from ONKALO that was obtained in November 2009 has been included.

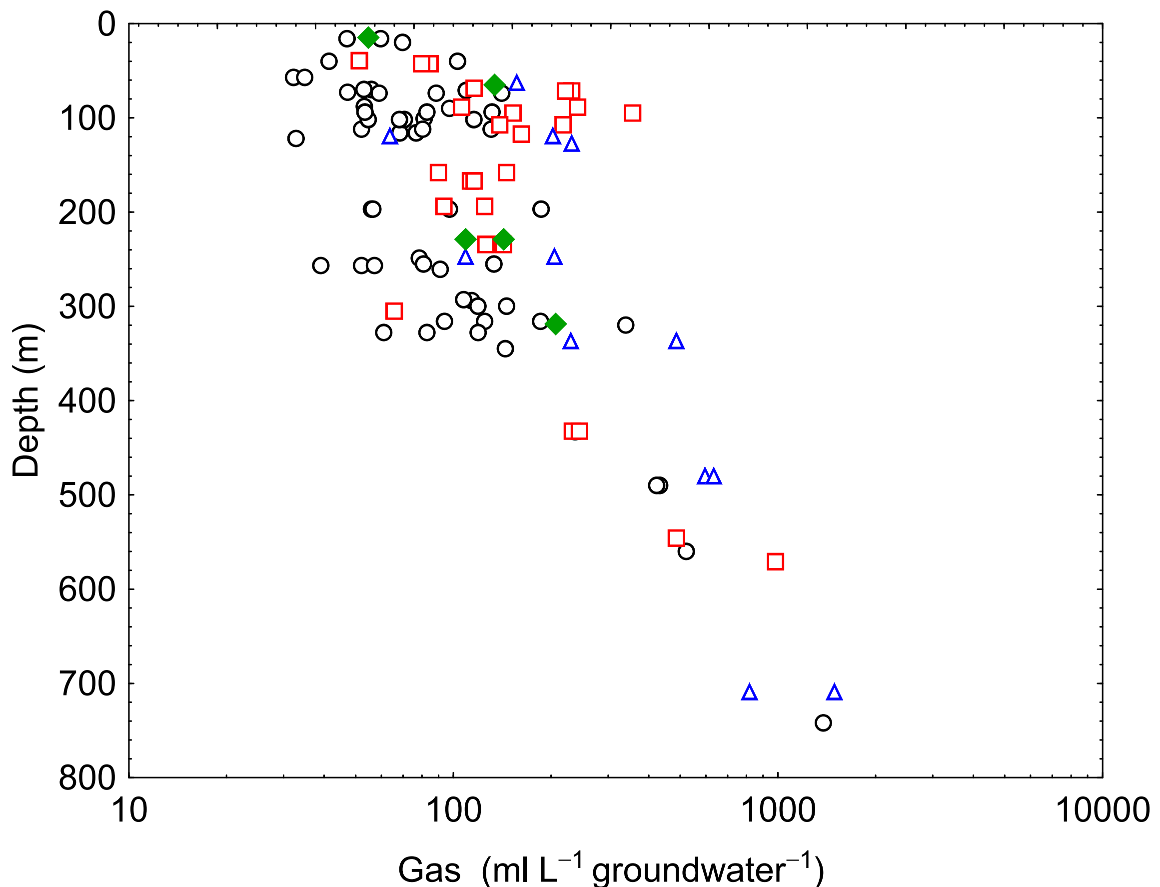

Figure 3-18. *The total amount of extractable dissolved gas in Olkiluoto and ONKALO groundwater sampled from 2005 to 2009. Olkiluoto data: Black circles indicate 2005–2007 results; red squares, 2008; blue triangles, 2009. Green diamonds indicate groundwater data from ONKALO, sampled in 2009.*

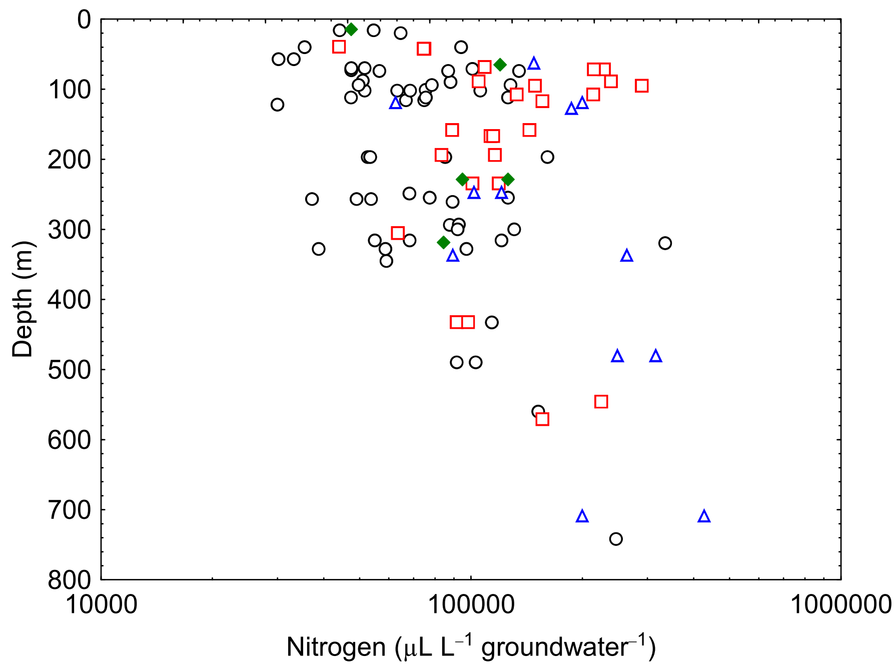

Figure 3-19. *The total amount of extractable nitrogen gas in Olkiluoto and ONKALO groundwater sampled from 2005 to 2009. Olkiluoto data: Black circles indicate 2005–2007 results; red squares, 2008; blue triangles, 2009. Green diamonds indicate groundwater data from ONKALO, sampled in 2009.*

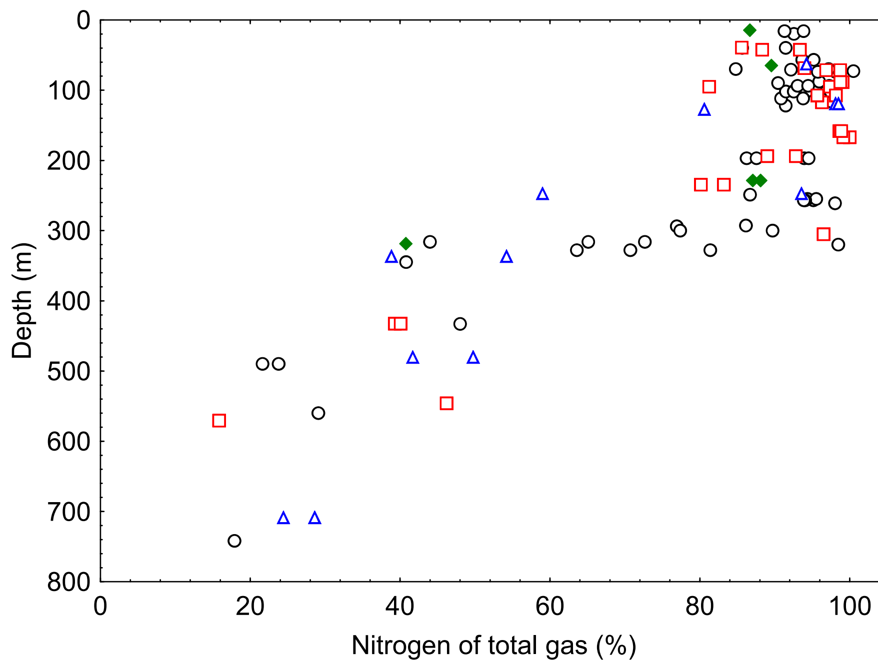

Figure 3-20. *Nitrogen as a percentage of the total amount of gas in Olkiluoto and ONKALO groundwater sampled from 2005 to 2009. Olkiluoto data: Black circles indicate 2005–2007 results; red squares, 2008; blue triangles, 2009. Green diamonds indicate groundwater data from ONKALO, sampled in 2009.*

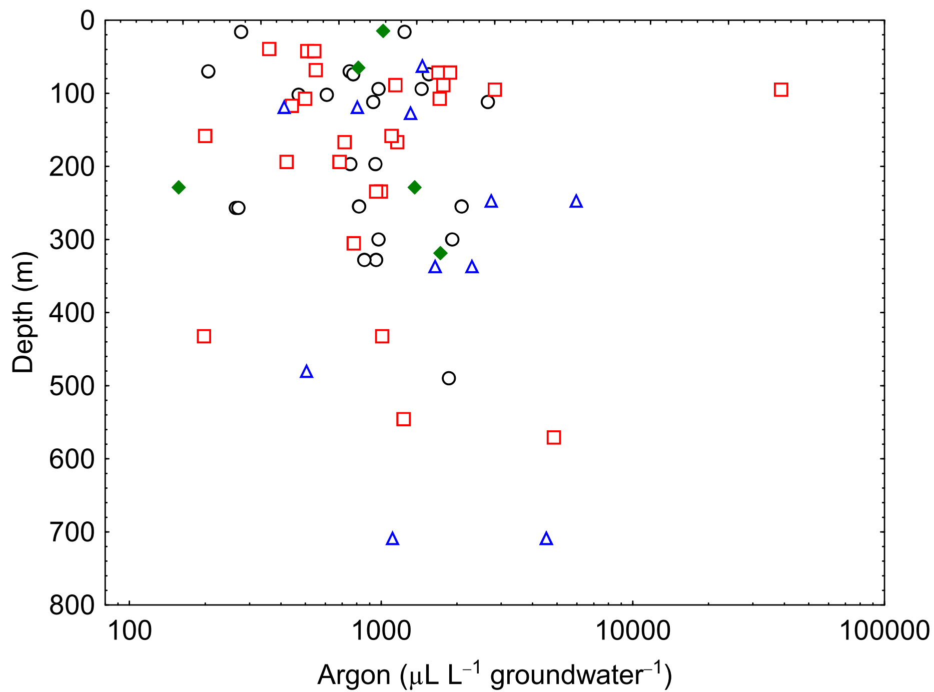

Figure 3-21. *The total amount of extractable argon gas in Olkiluoto and ONKALO groundwater sampled from 2005 to 2009. Olkiluoto data: Black circles indicate 2005–2007 results; red squares, 2008; blue triangles, 2009. Green diamonds indicate groundwater data from ONKALO, sampled in 2009.*

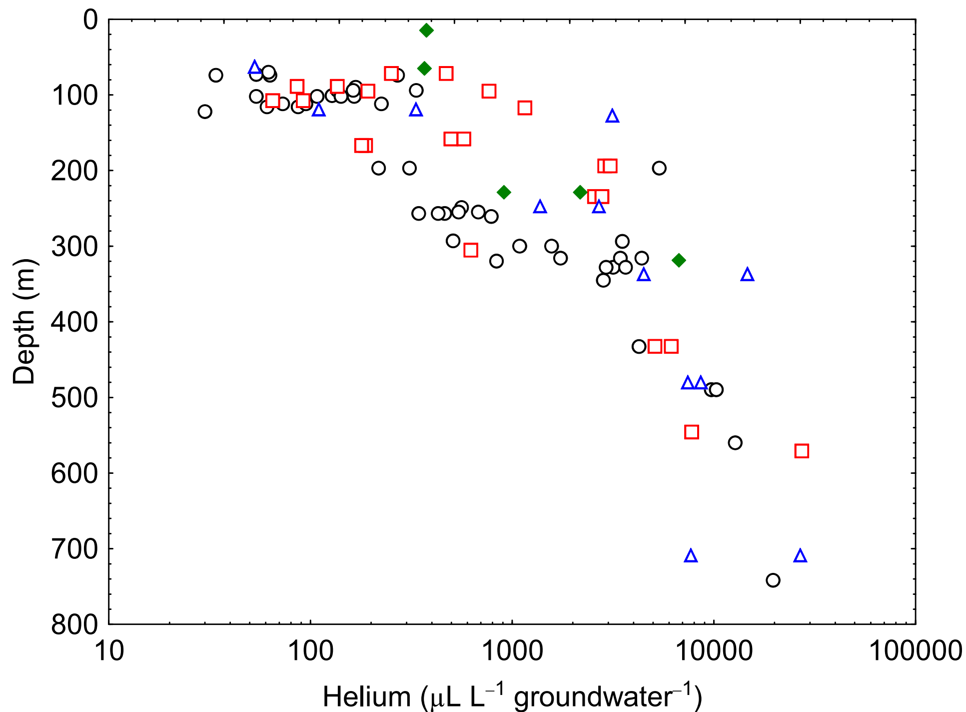

Figure 3-22*. The total amount of extractable helium gas in Olkiluoto and ONKALO groundwater sampled from 2005 to 2009. Olkiluoto data: Black circles indicate 2005–2007 results; red squares, 2008; blue triangles, 2009. Green diamonds indicate groundwater data from ONKALO, sampled in 2009.*

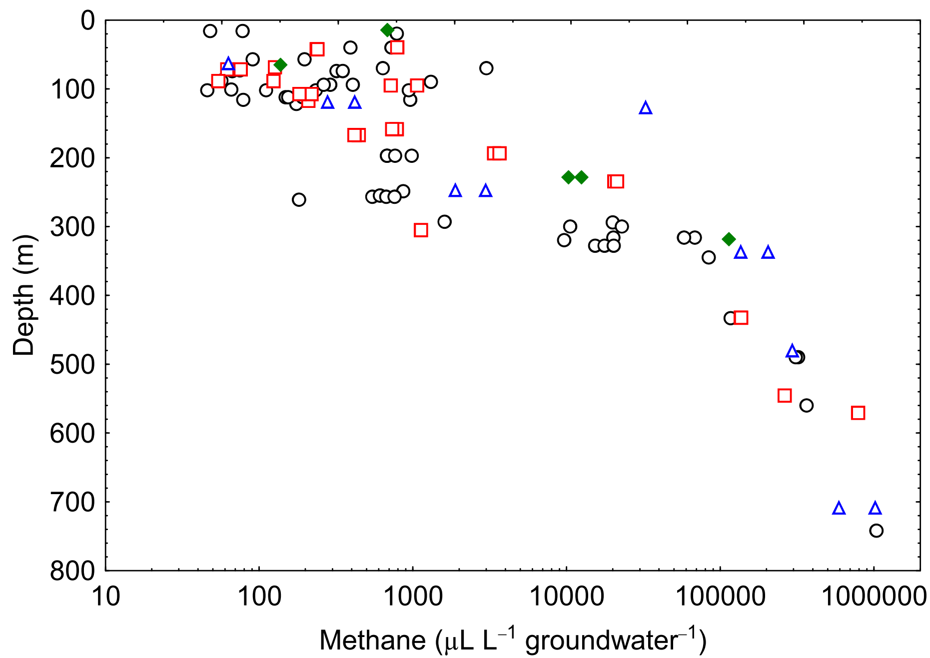

Figure 3-23. *The total amount of extractable methane gas in Olkiluoto and ONKALO groundwater sampled from 2005 to 2009. Olkiluoto data: Black circles indicate 2005–2007 results; red squares, 2008; blue triangles, 2009. Green diamonds indicate groundwater data from ONKALO, sampled in 2009.*

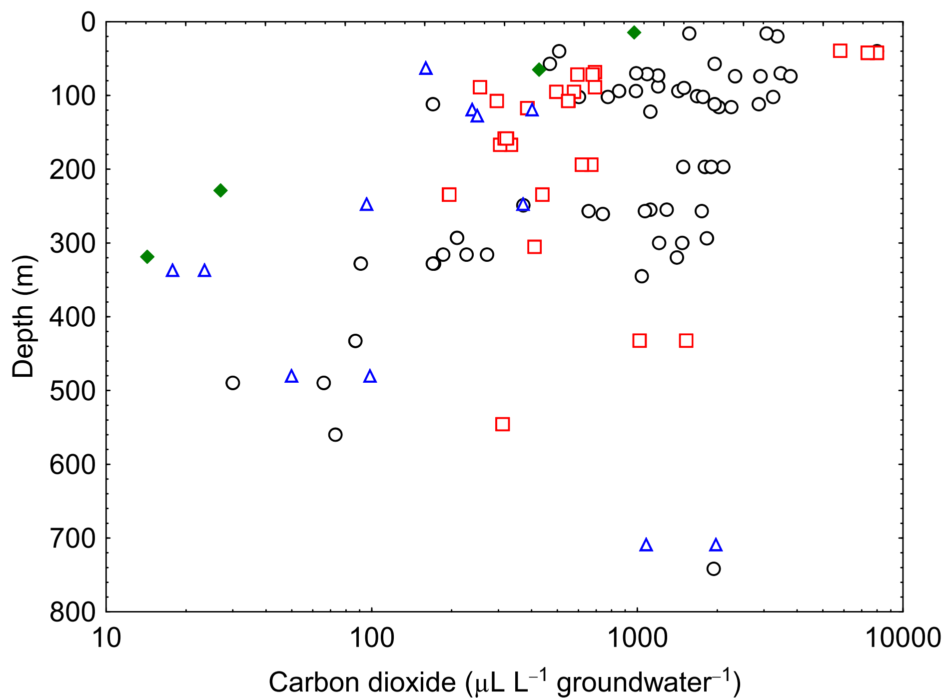

Figure 3-24. *The total amount of extractable carbon dioxide gas in Olkiluoto and ONKALO groundwater sampled from 2005 to 2009. Olkiluoto data: Black circles show 2005–2007 results; red squares, 2008; blue triangles, 2009. Green diamonds indicate groundwater data from ONKALO, sampled in 2009.*

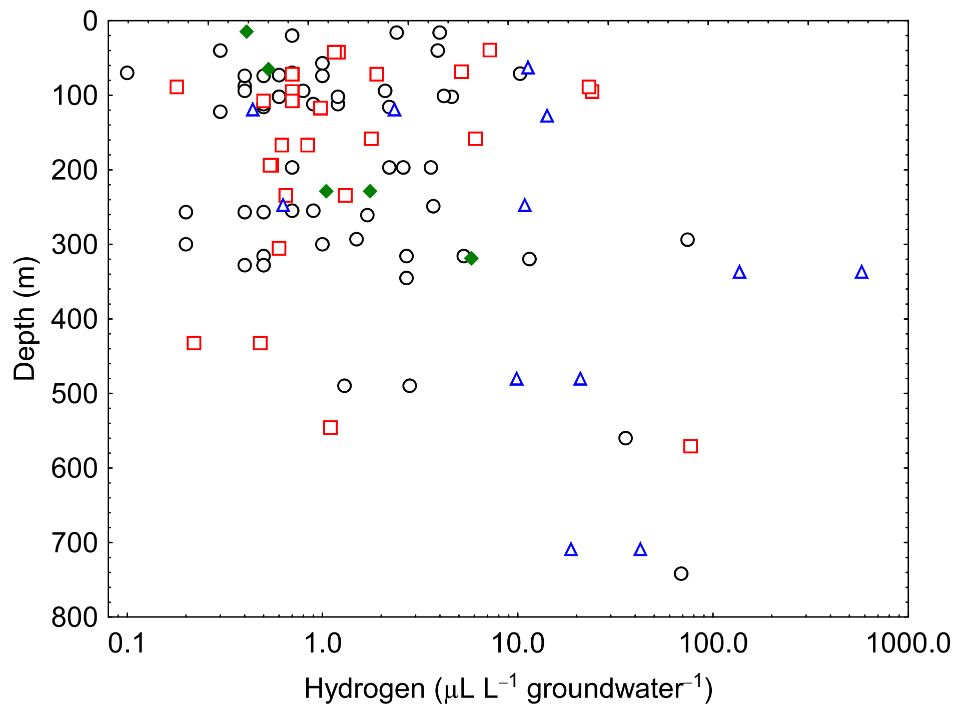

Figure 3-25. *The total amount of extractable hydrogen gas in Olkiluoto and ONKALO groundwater sampled from 2005 to 2009. Olkiluoto data: Black circles indicate 2005–2007 results; red squares, 2008; blue triangles, 2009. Green diamonds indicate groundwater data from ONKALO, sampled in 2009.*

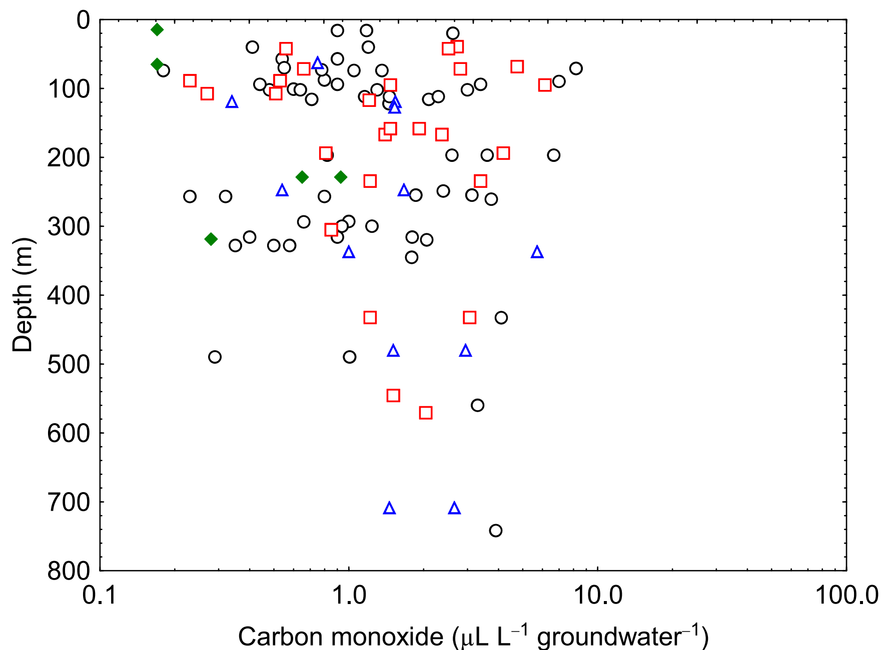

Figure 3-26. *The total amount of extractable carbon monoxide gas in Olkiluoto and ONKALO groundwater sampled from 2005 to 2009. Olkiluoto data: Black circles indicate 2005–2007 results; red squares, 2008; blue triangles, 2009. Green diamonds indicate groundwater data from ONKALO, sampled in 2009.*

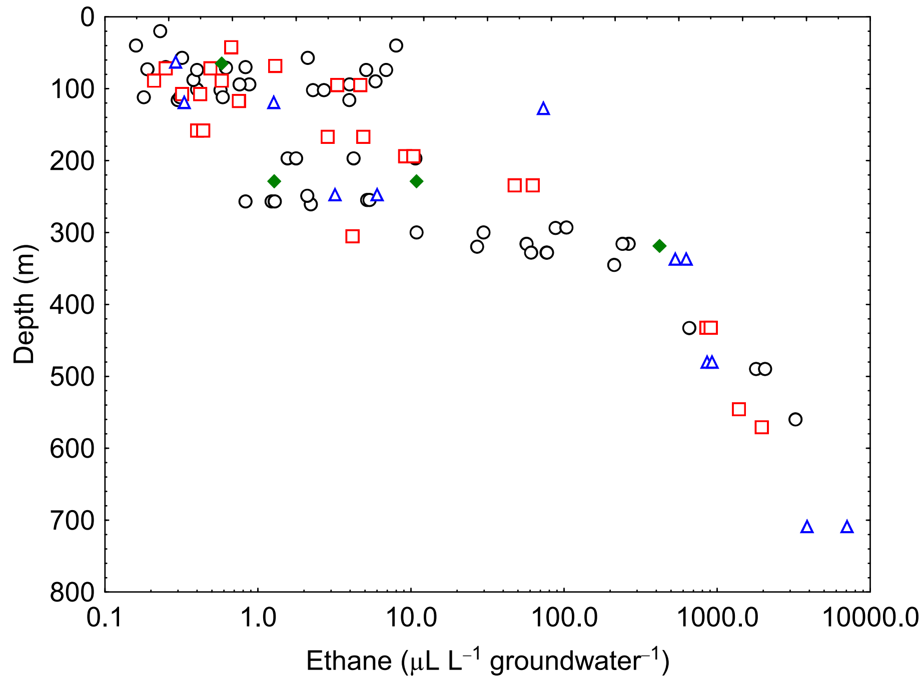

Figure 3-27. *The total amount of extractable ethane gas in Olkiluoto and ONKALO groundwater sampled from 2005 to 2009. Olkiluoto data: Black circles indicate 2005–2007 results; red squares, 2008; blue triangles, 2009. Green diamonds indicate groundwater data from ONKALO, sampled in 2009.*

4 DISCUSSION

4.1 Evaluation of applied analysis methods

4.1.1 Comparison of biomass-related quantitative analytical methods

Four biomass-related methods were used to analyse groundwater samples: TNC counts cell numbers, ATP is a measure of biomass, cultivation is a measure of microbe diversity and numbers, and qPCR counts the numbers of primer-specific types of microorganisms. It has previously been demonstrated that TNC and ATP values correlate in Fennoscandian Shield groundwater. Eydal and Pedersen (2007) demonstrated that the amount of ATP in groundwater samples reflects the number of cells as well as their biovolume and activity. This relationship was again found when the 18 deep groundwater analyses of 2008–2009 were added to 25 previously obtained values (Figure 4-1). There was previously a tendency towards increasing variability in the ATP values with increasing TNC numbers, which probably reflects different growth stages. Growing microorganisms contain more ATP per cell than do microorganisms in starvation stages. The risk of starvation increases when increasing numbers of microbes must share a fixed amount of energy (Kjelleberg et al. 1987). Therefore, the possibility of variation between the stages of starvation and growth will increase with increasing cell numbers. However, this effect is mitigated by the activity of bacteria-attacking viruses, so-called phages. Large numbers of phages have been found in deep groundwater (Kyle et al. 2008) and they are known to control population density (Eydal et al. 2009).

In 2007, qPCR using primers specific to various groups of microorganisms was added to some of the groundwater investigations (Pedersen et al. 2008a). The numbers of analyses using different primers is as yet too limited for correlation analysis, except for qPCR using *Bacteria*-specific 16S rRNA gene primers. The results from pumping out OL-KR13 (Table 3-4), the deep drillhole data from 2009 (Table 3-2), and the results from draining ONKALO drillholes (Table 3-8) correlated significantly with TNC data (Figure 4-2).

Taken altogether, the four methods for biomass-related analysis have been successfully developed. The outputs from the different analyses have been demonstrated to correlate well with each other (Figure 4-1 to Figure 4-2). As well, the methods focus on different aspects of microbial cells: TNC analyses whole cells using a microscope, ATP analyses a cell component using a biochemical method, MPN is based on cultivation, and qPCR analyses DNA (genes) and RNA (gene expression). The range of analytical focus encompassed by these methods ensures that the biomass-related information in this and previous reports from Olkiluoto and ONKALO is reliable and reflects a diverse range of the biomass-related characteristics of the analysed microorganisms.

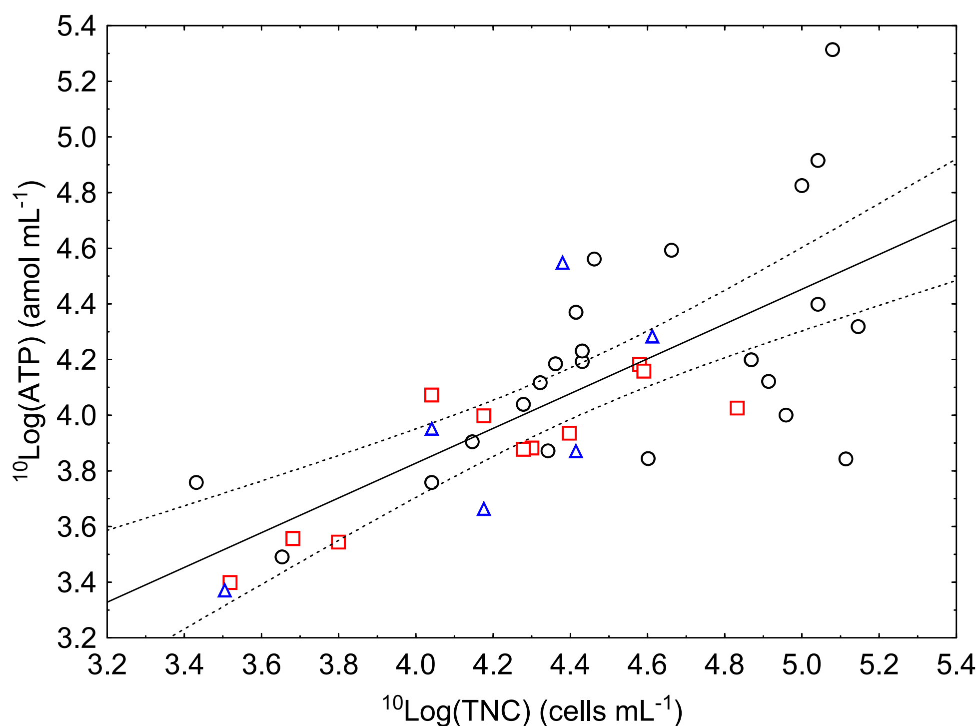

Figure 4-1. *The relationship between total numbers of cells (TNC) in deep Olkiluoto groundwater (Figure 3-1) and ATP concentrations (Figure 3-2). The least square regression line for TNC versus ATP is shown ($^{10}Log(ATP) = 0.6 \times {}^{10}Log(TNC) + 1.41$; $r = 0.69$, $p = 0.00001$, $n = 39$). Dashed lines indicate the 95 % confidence interval. Data: 2005–2007, black circles; 2008, red squares; 2009, blue triangles.*

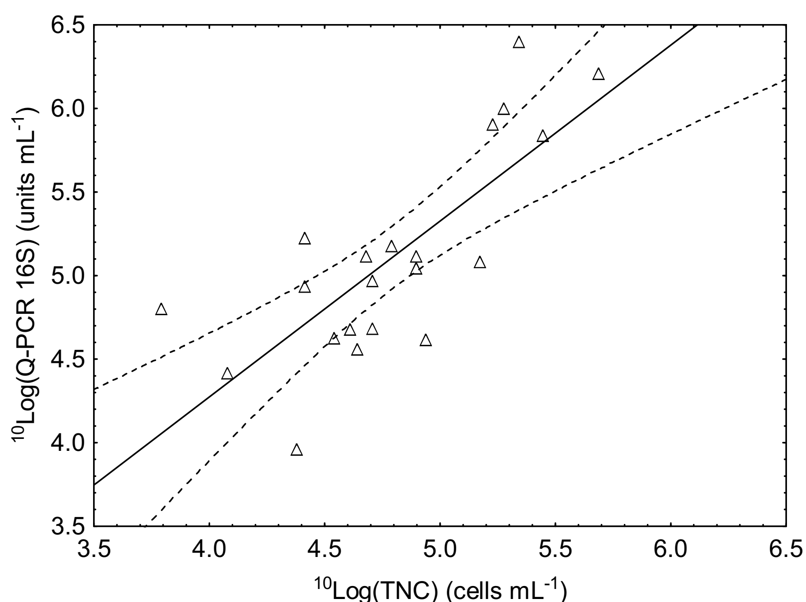

Figure 4-2. *The relationship between total number of cells (TNC) and qPCR data for samples from Olkiluoto and ONKALO. The least square regression line for TNC versus qPCR is shown ($^{10}Log(qPCR) = 1.05 \times {}^{10}Log(MPN) + 0.06$; $r = 0.77$, $p = 0.0001$, $n = 21$). Dashed lines indicate the 95 % confidence interval.*

4.1.2 qPCR of phylogenetic groups

Calibrating the 16S rRNA gene qPCR results with the TNC results indicated good agreement with a high correlation coefficient and low *p*-value (Figure 4-2). As judged from the small intercept value at TNC = 0, qPCR estimated the cell numbers with good precision. The numerical outcome of qPCR analyses relates to the type of standard DNA that is amplified concomitant with the samples. Each qPCR analysis is performed separately using different standard species (Table 2-4). Here, the sulphate-reducing bacterium *D. aespoeensis* was used as the standard for the 16S rRNA and *apsA* genes. This microorganism has one gene each of 16S rRNA and *apsA*, as judged from its annotated whole genome sequence (GenBank accession number ADDI00000000). When applied to natural samples of unknown population diversity, there may be some discrepancy between the obtained qPCR values for 16S rRNA and the TNC. This would mainly be due to possibly higher numbers of 16S rRNA gene copies in various species and strains in the analyzed populations relative to the standard used for qPCR (Lee et al. 2009). However, the correlation between TNC and qPCR 16S rRNA gene analysis (Figure 4-2) suggests that the qPCR of the 16S rRNA gene reflected the TNC reasonably well, within the obtained standard deviations.

In some cases, like that of the qPCR of NRB (Table 3-2), qPCR estimated much higher numbers than were found using the 16S rRNA gene qPCR. There are several possible explanations for these results. The qPCR of NRB was done using *Pseudomonas fluorescens* as the standard. This bacterium has five copies of the 16S rRNA gene, meaning that the qPCR value presented as the "standard unit" represents five genes per cell. If the bacteria in the sample have fewer copies per cell than do the standard species, the value of standard units will underestimate the actual cell number, and vice versa. Some bacteria may have up to 15 16S rRNA gene copies (Lee et al. 2009) per cell. The application of qPCR analyses is still under development, so the results obtained thus far should be interpreted as relative rather than absolute.

Inspecting the data for MPN of NRB in samples analysed using qPCR in sample OL-KR49_614_1 indicates the importance of evaluating all performed biomass determination results together in a relative way. The MPN of NRB was 50 000 cells mL^{-1} (Table A-2), the highest MPN of NRB ever found in groundwater samples from deep drillholes (Figure 3-5). The ATP and CHAB results were the highest values obtained in the 2008–2009 period (Table 3-1), while TNC indicated a low number. It appears as though the TNC determination underestimated the biomass. The reason for this is unknown, but the most plausible explanation is technical error during analysis. The qPCR results for this sample indicated a low 16S rRNA gene value, though the qPCR results for NRB (*narG1*) indicated very large numbers. Although it appears as though the qPCR analysis overestimated the NRB number in sample OL-KR49_614_1, the results did correlate in relative terms with the MPN results, which also indicated a very high NRB number. This case thus exemplifies the importance of applying more than just one biomass analysis to a single sample, and making conclusions based on the integrated interpretation of all data. Interpreting this sample is complicated, because the applied analyses point in different directions. TNC and qPCR 16S rRNA results were low, but the ATP, CHAB, NRB MPN, and qPCR *narG1* results were unusually high, MPN NRB being at least 10 times greater than commonly found in deep groundwater (Figure 3-5). Only two other deep samples have displayed such high numbers, i.e., OL-

KR6_422 (30 000 cells mL^{-1}) and OL-KR40_282 (24 000 cells mL^{-1}) (Figure 3-3). However, unlike these two samples, OL-KR49_614_1 was almost devoid of other microorganisms cultivable with MPN. It appears that NRB are the most common physiological group in Olkiluoto deep groundwater. This does not necessarily mean that nitrate reduction is ongoing; many bacteria are facultative nitrate reducers and can shift between using oxygen, nitrate, iron(III), and manganese(IV) as their terminal electron acceptor. More detailed investigation and understanding of possible metabolic traits associated with the detected microorganisms calls for analysis of microorganism diversity using cloning and sequencing methods, as discussed next.

4.1.3 Cloning and sequencing

The existence of large international databases of DNA sequence data makes it possible to sample and analyse the diversity of microbial populations in any environment. The most commonly used sequence is the ribosomal 16S rRNA gene. This gene is very conservative and present in all *Bacteria* and *Archaea* microorganisms. The corresponding ribosomal gene in *Eukarya* is named the 18S rRNA. The use of these ribosomal genes in analysing diversity is well established, and is extensive in research and for diagnostic purposes. In many cases in which environmental DNA is extracted and sequenced, the obtained sequences differ somewhat from sequences in the database. Therefore, the identity is usually reported in terms of the closest relative in the database. It is, in fact, rather unusual to find 100 % identity, as the genetic variability and hence diversity of the microbial world is almost infinite. Still, identities of 97.5 % and above are usually good enough to draw some general conclusions about the phylogenetic position of an organism in the tree of life. For example, SRB constitute a coherent group of microorganisms with respect to their 16S rRNA sequences, so they are easy to detect using 16S analysis. A drawback of cloning and sequencing is that the method tends to be biased towards dominant sequences. Hybridizing target sequences to a universal 16S rRNA gene with microarrays has been demonstrated to provide a rapid and comprehensive view of prokaryotic community composition that detects both dominant sequences and sequences not detected by cloning and sequencing (DeSantis et al. 2007). Microarrays are still relatively new in microbial ecology, but their use is rapidly increasing.

4.2 Deep Olkiluoto groundwater microbiology

After the pre-investigation phase, the deep groundwater of Olkiluoto has been investigated for microbiology since 2005, generating a set of data comprising physiological groups of microorganisms, together with their numbers and biomass. In 2008–2009, 18 complete datasets were added to the database that now contains 43 sets of microbiology data. The microbiology data have been divided into three groups in the Results section with respect to sampling dates, i.e., 2005–2007, 2008, and 2009. Many variance-creating factors, such as time, depth, groundwater type, drillhole age, flow conditions, and sampling methodology may contribute to the variability of the microbiological dataset. Detailed analysis over time and between sampling positions is complicated and thus outside the scope of this report. However, we can make a preliminary estimate of the range of the new datasets from 2008 and 2009. The drillholes sampled and analysed in 2008 and 2009 are all more peripherally situated

than those sampled in 2005–2007, all but OL-KR43 being found east-north-east of the previously sampled drillholes (Figure 2-1).

The TNC and ATP biomass data correlated as was previously the case (Figure 4-1). Unlike in the previous sampling period (2005–2007), the peak in values at approximately 300 m was not found. Many of the 43 sample sets were distributed between different drillholes (cf. Figure 3-3), so it is probably irrelevant to make detailed interpretations about any possible relationship between obtained microbiology values and depth. However, drillhole OL-KR47 was sampled at seven depths, ranging from 62.5 m to 708 m, for the first time permitting the evaluation of a depth relationship in a single drillhole. There were very low values for all analysed physiological groups and for biomass at a depth of 336 m (Figure 4-3), though samples above and below this depth displayed higher values. Clearly, a depth–microorganism relationship was absent from this drillhole; instead, each sampled level contained different numbers of microorganisms. Fully understanding the microbial diversity and distribution in deep Olkiluoto groundwater requires the evaluation of geochemistry, gases, and hydrology, emphasizing the availability to sources of energy for microbial activity over time. The data in Figure 4-3 indicate nicely how well the various analysis results correlate, as most of the curves display similar trends with depth. TNC, ATP, CHAB, and MPN analyses all use very different laboratory methods and principles. The correlation between their results is a good indication that the information from each of the analyses indeed reflects true groundwater conditions, as discussed in an introductory way in section 4.1.1.

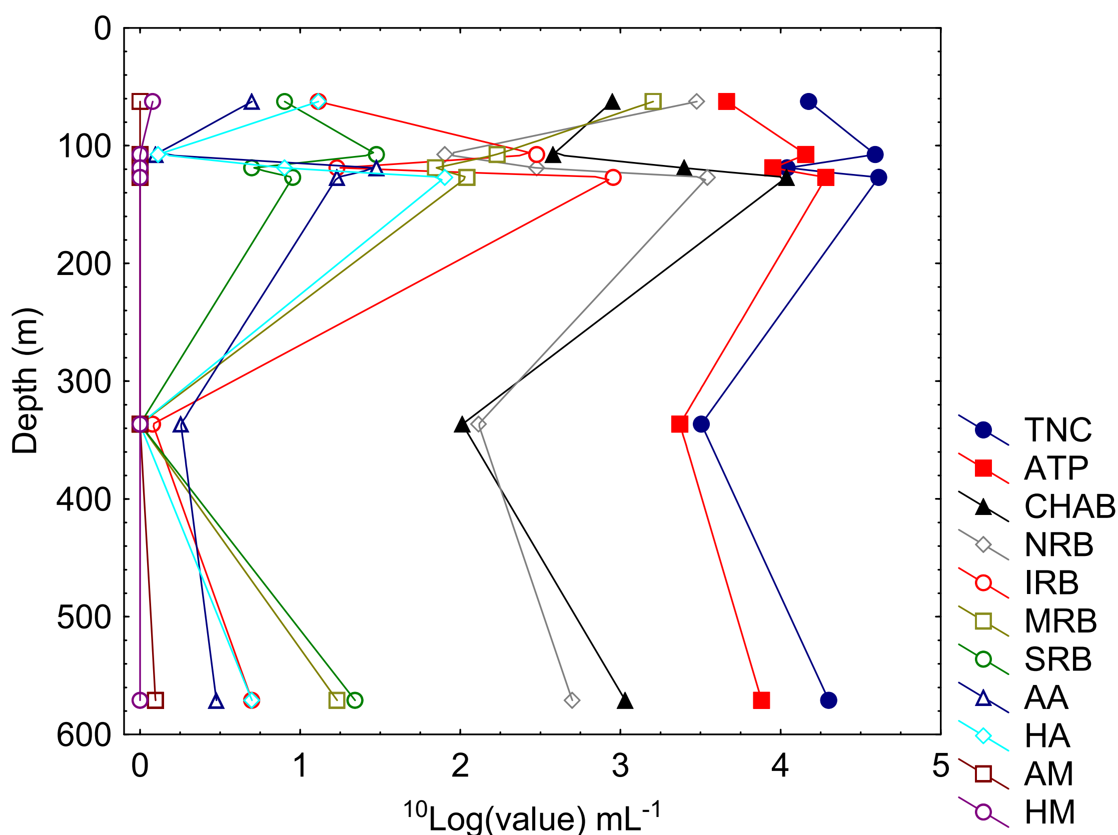

Figure 4-3. *The depth distributions of seven complete biomass and cultivation datasets obtained from OL-KR47 samples taken over approximately one year.*

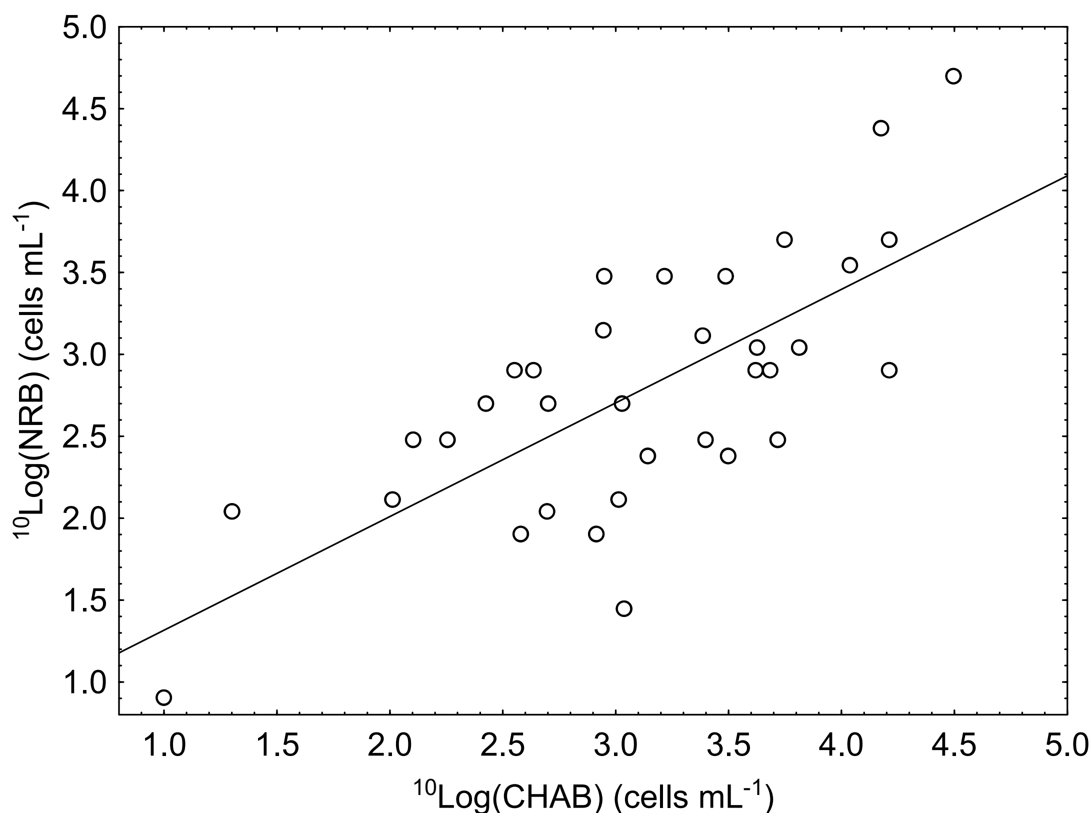

Figure 4-4. *The relationship between NRB and CHAB data from deep groundwater samples from Olkiluoto. NRB = 0.6935 × CHAB + 0.62; r = 0.636, p = 0.0001. The data point from the deepest drillhole, OL-KR40_786_1, was excluded from the correlation; see text for explanation.*

Correlation analysis of the microbiology dataset revealed the correlation between TNC and ATP (Figure 4-1) and between TNC and qPCR (Figure 4-2), as discussed above. The NRB and CHAB data also displayed good correlation (Figure 4-4), though otherwise correlations were absent. Most NRB are facultative anaerobes: they will respire using oxygen, if present; otherwise, they can switch to nitrogen in nitrate as the electron acceptor in their respiration. The MPN of NRB is determined in an oxygen-free environment in anaerobic tubes, while CHAB cultivation is performed in air with oxygen. The obtained correlation indicates that, if oxygen is intruding at depth in Olkiluoto groundwater, microorganisms able to respire and thereby remove oxygen are present at all depths in Olkiluoto. One exception may be the deepest drillhole, OL-KR40_786_1, which contained 1300 NRB mL^{-1} but only 3 CHAB mL^{-1}. These NRB may therefore be obligate anaerobes, but one observation is not enough to draw a proper, general conclusion. In other words, the bacteria cultivable using the MPN method for NRB can also be cultivated using the CHAB method, which means that the same microorganisms are possibly being counted twice. As expected, the results of the remaining MPN analyses did not correlate. The analysed bacteria all use different electron acceptors and are adapted to different growth conditions. For example, IRB and SRB generally cannot switch from ferric iron to sulphate, or vice versa, so IRB and SRB are only detected and counted once in their respective media.

The MPN results for IRB and MRB obtained in 2008 and 2009 did overlap with previous data (Figure 3-6 and Figure 3-7). Three of the new samples were obtained from depths below 300 m and their values were all low. There are still only five observations of IRB and MRB from below a depth of 300 m, none containing more than approximately 10 cells mL^{-1}, while the range of observations from above 300 m in depth is from 0.2 to more than 1000 cells mL^{-1}. Three of the 2008–2009 SRB data points from a depth of approximately 300 m were the highest values so far obtained for SRB from below the shallow groundwater zone (0–20 m). These data coincide with previous high SRB values at this depth (Figure 3-8) and with peak ATP values (Figure 3-2). New SRB data from below a depth of 300 m were low relative to data from shallower depths. The AA values were generally lower than those observed before 2008 (Figure 3-9). The reasons for this shift remain to be established, though it may reflect a response to a similar decrease in detected hydrogen concentrations, which were generally approximately 10 times lower in 2008–2009 than in 2005–2007 (Figure 3-25). Autotrophic acetogens rely on hydrogen as their main source of energy. A similar situation may apply for the AM, which are also dependent on hydrogen as an energy source. The 2008–2009 values for AM were all, except one, below detection (0.2 cells mL^{-1}). Data for HA from 2008–2009 overlapped previous data, while the HM were generally below detection. Detailed analysis of the relationships between the microbiological dataset and all other available information about groundwater in Olkiluoto may reveal important dependencies that will be helpful in the modelling of microbial processes in deep Olkiluoto groundwater.

4.3 ONKALO groundwater microbiology

Numbers of microorganisms in ONKALO drillholes were analysed as done previously with TNC and for the first time with qPCR. The TNC and qPCR results correlated and were in the range of numbers previously found in ONK-KR1-3 and ONK-PVA1-3 (Pedersen et al. 2008a). When analysed for MPN in 2007, SRB were found in ONK-PVA1-3, while qPCR of *apsa* RNA in 2008 detected SRB in drillholes ONK-KR1-3 and ONK-PVA1-3 (Figure 3-16). The qPCR analyses from 2009 were not very successful: many targets were below detection limits, and those that were detectable were found in much smaller amounts than found previously. It is at present not clear why this should be the case. Substances in the groundwater may have exerted an inhibition effect due to the increase in filtered volume from 6 mL to 1000–2500 mL. This increase was introduced to increase the sensitivity of the analyses. Another possibility is that the filtering technique was inappropriate. Precipitates may have made it difficult to remove cells from the filters to the extraction liquids. In a similar study, but using a different primer for SRB (dissimilatory sulphate reductase gene, *dsrAB*), the authors analysed ONK-PVA3 water and found 3.8×10^3 gene copies mL^{-1} (Itävaara et al. 2008). They did not detect any inhibiting effect of the groundwater. This number corresponds well with that reported here for the ONK-PVA3 sample from 2008 (Table 3-8). The qPCR methodology will be developed to include internal standards that will reveal any inhibiting effect. Until then, the 2009 qPCR values from ONKALO should be treated as relative rather than absolute.

However, the results from 2009 can still be interpreted qualitatively. In addition to previous results, the 2009 investigation detected *Archaea* in all analysed samples, which

suggests that methanogens were present in the groundwater. *Archaea* genes were found in ONK-PP128 as well (Table 3-8). Methane-oxidizing bacteria were found in ONK-PVA1 and methylotrophs were found in all samples but ONK-PVA6 (Table 3-10), in line with previous findings in shallow groundwater (Pedersen 2008). These groups of microorganisms oxidize methane with oxygen and thereby constitute an important component of the processes that reduce oxygen in surface water and rainwater that infiltrate the groundwater. The *nirK* gene was found in drillholes ONK-PVA1, -2, and -4, suggesting the presence of NRB able to reduce nitrite. The presence of SRB was indicated in ONK-PVA4. NRB were also detected in ONK-PVA2 and 4 using the *psvA* gene primer pair. NRB are clearly present in ONKALO groundwater, as demonstrated previously (Pedersen et al. 2008a). Taken together, the qPCR analysis performed in 2009 yielded important qualitative results concerning the microbial diversity in ONKALO groundwater, in agreement with previously obtained results.

4.4 The effect of pumping and draining drillholes

The standard procedure for sampling drillholes usually includes pumping until chemical parameters such as salinity and pH have stabilized. Previously, the long-term pumping of a shallow borehole (PVP4A) had displayed little influence on the microbiology results (Pedersen 2008). Pumping of OL-KR6_422 in 2007, however, indicated that pumping time significantly affected the SRB population (cf. Figure 3-12 in Pedersen et al. 2008a). In addition, data from pumping and draining a series of drillholes in Laxemar (Bergelin et al. 2010) and the Äspö Hard Rock Laboratory (Pedersen 2005) indicated that different pumped/drained volumes correlated with considerable variability in microbiology results, including SRB numbers and activity. It has become clear that microbiological datasets may vary depending on time of pumping or draining. Therefore, several sampling activities were performed in time series in 2008 to further evaluate the relationships between pumping/draining times and microbiology results.

4.4.1 Drillhole OL-KR13 T360

The effect of pumping a drillhole was studied by analysing chemistry and microbial diversity. The drillhole section OL-KR13_T360 was equipped with new tubing and a clean pump. The pumping system was filled with surface technical water. By analysing the change in chemistry and microbial diversity, it became possible to compare the outcome of the microbial diversity protocols with the results of the chemistry analysis. It was hypothesized that there should be a significant shift in diversity as the surface water in the tubing was replaced with groundwater from the pumped-out depth of 293 m, which proved to be the case. The chemistry results indicated that the sulphate concentration stabilized after approximately 30 L were pumped out while salinity levelled out after 55 L. Samples OL-KR13-1 to -4 thus comprised tubing water mixed with groundwater, while samples OL-KR13-6 and -7 appeared to consist mostly of groundwater.

A detailed analysis of the diversity results over time indicated that, while the drillhole was being drained, the diversity changed from sequences mostly belonging to the beta-proteobacteria, gamma-proteobacteria, and flavobacteria (e.g., *Acidovorax defluvii*, *Pseudomonas fluorescens*, and *Flavobacterium granulii*) in samples OL-KR13-1 to -4,

to less of these groups and more sulphur-oxidizing and sulphate-reducers in sample OL-KR13-6 (Table 3-5). In sample OL-KR13-7, most of the genes were affiliated with SRB, some of which were most closely related to sulphate-reducing ANME partners such as the uncultured bacterium clone BC_B2_3f (EU622295), the uncultured delta-proteobacterium MERTZ_21CM_138 AF424243, and the uncultured bacterium CS_B042 AF419686. One clone belonged to the iron-oxidizing gammaproteobacteria *Siderooxidans lithoautotrophicus*. Sequences affiliating with *Archaea* appeared after prolonged pumping in the OL-KR13-6 and -7 samples. This is in line with the qPCR results, which displayed the largest *Archaea* values in these two samples (Table 3-4). Taken altogether, the diversity results for the pumping of drillhole KR13 indicated that there was a significant shift in the microorganisms populating different water types. The surface water used to fill the pumping tube differed from the types of water found in the drillhole groundwater. This is good news, because the results indicate that microorganisms not relevant to the groundwater sample (in the tube surface water) were washed out during pumping and replaced with indigenous microorganisms from the sampled groundwater system. The issue of the possible contamination of groundwater samples with irrelevant microorganisms from tubes and pumps can therefore be regarded as strongly mitigated by a period of pumping, until the sample contains only groundwater from the sampled fracture system. However, we have to keep in mind that in this case the tubes were recently installed and probably clean; using tubes that have been in use for a long time may influence the results differently.

4.4.2 Drillholes ONK-PVA-3 and ONK-PP128

The two drillholes chosen for studies of the effect of draining on a tunnel drillhole displayed similar trends. The ONK-PVA-3 drillhole located in ONKALO first displayed an increase in total biomass that decreased after 14 L of groundwater were drained (Figure 3-17). The 16S rRNA gene qPCR analysis indicated that total biomass first increased somewhat during draining and then decreased (Table 3-8). The qPCR analysis of *apsA* rRNA indicated variable numbers of active SRB during the drainage. These results indicate that the duration of drillhole drainage affects the diversity and biomass of microorganisms detected in the groundwater system. The results of draining PP128 are difficult to evaluate, due to the very heavy cement contamination of the first and to some extent the second sample. There was no obvious trend relative to draining volume (Table 3-8).

The pumping tests conducted in drillholes OL-KR6_422, OL-KR13 T360, and ONK-PVA3 conclusively demonstrated the importance of understanding the mechanisms that control microbial diversity, activity, and numbers as a function of sampling methodology, including pumping and draining. Flow rates during sampling, volumes let out before the sample is taken, and configuration of drillhole equipment are factors that must be further investigated. The microbiology analyses deal with life processes that may rapidly change as a function of sampling disturbance. Sampling of life material is a much more delicate matter to design, carry out, and interpret than is chemistry or geology sampling.

4.5 Biofilms on fracture surfaces in groundwater conducting aquifers

An interface is the boundary between two phases in a heterogeneous system. Solid–liquid interfaces are very important in microbial ecology as they influence microbial life in various ways (Marshall 1976). Microorganisms attach within minutes to most inanimate solid surfaces immersed in natural water (Bitton and Marshall 1980), where they may grow to form biofilms (Characklis and Marshall 1990). Microorganisms have been found in most subsurface environments where life is possible in terms of temperature and water availability (Fredrickson and Fletcher 2001). Like most other investigated underground environments, groundwater from deep aquifers in Olkiluoto is populated by significant numbers of microorganisms (Pedersen et al. 2008b). The solid-to-liquid ratio of subsurface environments is strongly displaced to the solid side, with very large surface areas relative to the available volumes of water. The existence of subsurface aquifers populated by attached microorganisms is very likely. A recent investigation of drillcores retrieved in the Äspö Hard Rock laboratory confirmed that biofilms grow on water-conducting fracture surfaces (Jägevall et al. 2010).

Drilling and retrieving cores that intersect natural aquifers would make it possible to investigate fracture surfaces for natural biofilms. Evidence of fossilized biofilms was obtained from such cores using transmission electron microscopy (Pedersen et al. 1997). Due to the strong autofluorescence of most minerals, detecting living biofilm microorganisms using fluorescence methods is impossible or severely disturbed. However, nucleic acid analysis should reveal the presence and diversity of fracture surface biofilms if nucleic acids are sampled, extracted, and quantified using qPCR. Cloning and sequencing can reveal diversity, as they have previously done for biofilm microorganisms. In ONKALO, two drillhole cores were inspected for the possible presence of biofilms. It was assumed that the sampled fracture surface material (FSM) came from fractures that intersected water-conducting fractures. The FSM was sampled for nucleic acids by means of DNA extraction.

The FSM samples were collected from a heterogeneous fracture system with varied aperture size. As biofilms tend to develop where there is water flow, positions exposed to little or no groundwater flow could be expected to have less biomass than positions with flowing groundwater. The flow situation before drilling and sampling the intersected aquifers, however, was impossible to analyze. Any increase in fracture water flow after core retrieval can be used to infer that water-conducting fractures have been intersected, but the groundwater flow from drillhole ONK-PVA6 was tiny while ONK-PVA-7 was dry. In addition, fracture surfaces in hard rock may be partly closed and groundwater flow therefore channelled (i.e., rock makes contact at some positions and lets water through at others), so it was very difficult to determine where groundwater was running and where fracture surfaces were in contact after drill core fractures were opened post-drilling. Therefore, it is plausible that all the FSM samples may have been collected from fracture positions with low or no water flow and very little or no biomass. This was of course certainly true for the ONK-PVA7 sampling site, which was dry, but as the flow was very small in ONK-PVA6, the chance that the FSM samples came from a water-conducting part is small. Flow logging was not possible before sampling, so the obtained results indicate that the FSM was contaminated with drilling water and there was no clear evidence that biofilms were sampled (Table 3-11).

4.6 DNA signatures of ANME in Olkiluoto and ONKALO groundwater

Scientists have long observed methane, sulphate, sulphide, and carbon dioxide profiles in anaerobic aquatic sediments that strongly suggested the presence of active ANME (Zehnder and Brock 1980; Thomsen et al. 2001). It was not until recently, however, that the ANME microorganisms underlying this process were identified (Boetius et al. 2000). It was obvious that strong methane and sulphate gradients meet at several locations at a depth of 300 m in Olkiluoto (Pedersen 2008). Furthermore, it was obvious from ATP determinations and from the MPNs of various physiological groups of bacteria that microbial abundance and activity both peak at these sample locations. Finally, sulphide concentrations were also very high at the same locations. Of the sites evaluated and discussed earlier (see Pedersen 2008), OL-KR6_422 (328-m depth), OL-KR10_326 (316-m depth), and OL-KR13_360 (292-m depth) had the greatest potential for pronounced anaerobic methane oxidation; these three sampling locations had high concentrations of ATP and DOC and high MPNs of NRB, SRB, AA, and HA relative to those at other deep groundwater sampling locations. The drillhole OL-KR40_282 (255 m), sampled and analysed in 2007, also belongs to this group because of its similar high results for TNC, ATP, and CHAB analyses (Table 3-1) and similar high MPNs (Figure 3-3).

Anaerobic methane oxidation is a process that must be studied when designing a nuclear waste repository, because one product of the oxidation is sulphide, which is corrosive to the copper waste-storage canisters. The two major groups of microorganisms involved in this process are anaerobic methanotrophs and SRB belonging to specific phylogenetic groups. Previous microbiological studies have suggested that anaerobic methane oxidation is ongoing in Olkiluoto (Pedersen et al. 2008a). In September 2008, samples were collected from the 300-m level in ONKALO and the first ANME signatures were found in ONK-PP128 by means of cloning and sequencing (Table 3-9). A combined draining and ANME signature study was later carried out at OL-KR13, in which further DNA sequencing and qPCR provided more evidence of the presence of anaerobic methane oxidation processes in deep Olkiluoto groundwater. An example of an ANME organism was the uncultured *Archaea* clone 5H3_A21 (DQ301872), which displayed 96% identity to our sequence; a detected sulphate reducer that could be a partner to this ANME was *Desulfoarculus baarsii* (AF418174). These were both found in sample OL-KR13 T360-7. *Archaea* were also found using *Archaea* 16S rRNA gene qPCR in samples OL-KR13 T360-6 and -7. These results are simply suggestive in nature, and do not confirm that the process of anaerobic methane oxidation is actually ongoing. *In situ* experiments are planned at a depth of approximately 300 m in ONKALO, in which the process rate of anaerobic methane oxidation will be addressed in a sulphate-reduction experiment (SURE) project (Aalto et al. 2009).

4.7 Dissolved gas

The amount of dissolved gases analysed in 2008–2009 generally overlapped previous gas analysis results (Figure 3-18 to Figure 3-27). A detailed analysis of gas distribution and concentration over time and depth is outside the scope of this microbiology report. However, some gases, such as hydrogen, methane, and carbon dioxide, are related to microbiological processes. As noted previously, hydrogen concentration appears to have

decreased in 2008–2009 versus previous recorded levels (4.2). Methane levels, on the other hand, overlapped previously detected levels. Carbon dioxide levels were generally lower than found previously. These differences may be because the 2008–2009 samples were collected from new drillholes located farther from the ONKALO area than were the drillholes analysed previously. The gas samples from ONKALO are too few to permit any conclusions about differences from or similarities to data from surface drillholes. A preliminary observation is that dissolved gases in the ONKALO groundwater do not differ significantly in composition or amount from what has been found in Olkiluoto drillholes.

REFERENCES

Aalto, P., Aaltonen, I., Ahokas, H., Andersson, J., Hakala, M., Hellä, P., Hudson, J., Johansson, E., Kemppainen, K., Koskinen, L., Laaksoharju, M., Lahti, M., Lindgren, S., Mustonen, M., Pedersen, K., Pitkänen, P., Poteri, A., Snellman, M., and Ylä-Mella, M. 2009, Sulphate reduction under deep bedrock conditions, *Programme for Repository Host Rock Characterisation in the ONKALO (ReRoC)*, Posiva Working Report no. 21-27. Posiva Oy, Eurajoki, Finland.

Andersson, J., Ahokas, H., Hudson, J.A., Koskinen, L., Luukkonen, A., Löfman, J., Keto, V., Pitkänen, P., Mattila, J., Ikonen, A.T.K., and Ylä-Mella, M. 2007, 7. Hydrogeochemistry, *Olkiluoto Site Description 2006*, 225–290. Posiva Report 2007-3.

Ben-Dov, E., Brenner, A., and Kushmaro, A. 2007, Quantification of sulfate-reducing bacteria in industrial wastewater, by real-time polymerase chain reaction (PCR) using dsrA and apsA genes, Microbial Ecology, 54, no. 3, 439–451.

Bergelin, A., Pedersen, K., and Wallin, B. 2010, Investigation of sulphide in core drilled boreholes KLX06, KAS03 and KAS09 at Laxemar and Äspö. Chemical-, microbiological- and dissolved gas data from groundwater in four borehole sections, 1–80. P-rapport P-10-18, Svensk Kärnbränslehantering AB, Stockholm.

Bitton, G. and Marshall, K.C. 1980, *Adsorption of Microorganisms to Surfaces*. John Wiley & Sons, New York, 439 pp.

Boetius, A., Ravenschlaug, K., Schubert, C.J., RIckert, D., Widdel, F., Gleseke, A., Amann, R., Jörgensen, B.B., Witte, U., and Pfannkuche, O. 2000, A marine microbial consortium apparently mediating anaerobic oxidation of methane, Nature, 407, 623–626.

Characklis, W.G. and Marshall, K.C. 1990, *Biofilms*. Wiley, New York, 796 pp.

DeSantis, T.Z., Brodie, E.L., Moberg, J.P., Zubieta, I.X., Piceno, Y.M., and Andersen, G.L. 2007, High-density universal 16S rRNA microarray analysis reveals broader diversity than typical clone library when sampling the environment, Microbial Ecology, 53, 371–383.

Eydal, H.S.C., Jägevall, S., Hermansson, M., and Pedersen, K. 2009, Bacteriophage lytic to *Desulfovibrio aespoeensis* isolated from deep groundwater, The ISME Journal, 3, 1139–1147.

Eydal, H.S.C. and Pedersen, K. 2007, Use of an ATP assay to determine viable microbial biomass in Fennoscandian Shield groundwater from depths of 3–1000 m, Journal of Microbiological Methods, 70, 363–373.

Fredrickson, J.K. and Fletcher, M. 2001, *Subsurface Microbiology and Biogeochemistry*. Wiley-Liss, New York, 341 pp.

Ginzinger, D.G. 2002, Gene quantification using real-time quantitative PCR: An emerging technology hits the mainstream, Experimental Hematology, 30, 503–512.

Girguis, P.R., Cozen, A.E., and De Long, E.F. 2005, Growth and population dynamics of anaerobic methane-oxidizing archaea and sulfate-reducing bacteria in a continuous-flow bioreactor, Applied and Environmental Microbiology, 71, no. 7, 3725–3733.

Greenberg, A.E., Clesceri, L.S., and Eaton, A.D. 1992, Estimation of bacterial density, in: *Standard methods for the examination of water and wastewater 18th ed.* American Public Health Association, Washington, DC, pp. 9–49.

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

Haveman, S.A. and Pedersen, K. 2002, Distribution of culturable anaerobic microorganisms in Fennoscandian Shield groundwater, FEMS Microbiology Ecology, 39, 129–137.

Henry, S., Baudoin, E., López-Gutiérrez, J.C., Martin-Laurent, F., Brauman, A., and Philippot, L. 2004, Quantification of denitrifying bacteria in soils by nirK gene targeted real-time PCR, Journal of Microbiological Methods, 59, 327– 335.

Hobbie, J.E., Daley, R.J. and Jasper, S. 1977, Use of nucleopore filters for counting bacteria by fluorescence microscopy, Applied and Environmental Microbiology, 33, 1225–1228.

Horz, H.P., Yimga, M.T., and Liesack, W. 2001, Detection of methanotroph diversity on roots of submerged Rice plants by molecular retrieval of pmoA, mmoX, mxaF, and 16S rRNA and ribosomal DNA, including pmoA-based terminal restriction fragment length polymorphism profiling, Applied and Environmental Microbiology, 67, 4177–4185.

Itävaara, M., Vehkomäki, M.L., and Nousiainen, A. 2008, Sulphate-reducing bacteria in groundwater samples from Olkiluoto – Analyzed by Quantitative PCR, Posiva working report 2008-82, 45 pp. Posiva oy, Eurajoki, Finland.

Jägevall, S., Rabe, L., and Pedersen, K. 2010, Deep granitic rock aquifers of the Äspö Hard Rock Laboratory Sweden harbor indigenous biofilms. Microbial Ecology (in press).

Kjelleberg, S., Hermansson, M., Mården, P., and Jones, G.W. 1987, The transient phase between growth and nongrowth of heterotrophic bacteria, with emphasis on the marine environment, Annual Review of Microbiology, 41, 25–49.

Kyle, J.E., Eydal, H.S.C., Ferris, F.G., and Pedersen, K. 2008, Viruses in granitic groundwater from 69 to 450 m depth of the Äspö hard rock laboratory, Sweden, The ISME Journal, 2, 571–574.

Lane, D.J. 1991, 16S/23S rDNA sequencing, in: Stackebrandt, E. and Goodfellow, M. (eds.) *Nucleic Acid Techniques in Bacterial Systematics*, pp. 115–175. John Wiley & Sons, West Sussex, UK.

Lane, D.J., Pace, B., Olsen, G.J., Stahl, D.A., Sogin, M.L., and Pace, N.R. 1985, Rapid determination of 16S ribosomal RNA sequences for phylogenetic analyses, Proceedings of the National Academy of Sciences of the United States of America, 82, 6955–6959.

Lee, Z.M.-P., Bussema, C., and Schmidt, T.M. 2009, rrnDB: documenting the number of rRNA and tRNA genes in bacteria and archaea, Nucleic Acids Research, 37, D489–D493

Lösekann, T., Knittel, K., Nadalig, T., Fuchs, B., Niemann, H., Boetius, A., and Amann, R. 2007, Diversity and abundance of aerobic and anaerobic methane oxidizers at the Haakon Mosby Mud Volcano, Barents Sea, Applied and Environmental Microbiology, 73, 3348–3362.

Lundin, A. 2000, Use of firefly luciferase in ATP-related assays of biomass, enzymes, and metabolites, Methods in Enzymology, 305, 346–370.

Lundin, A., Hasenson, M., Persson, J., and Pousette, Å. 1986, Estimation of biomass in growing cell lines by adenosine triphosphate assay, Methods in Enzymology, 133, 27–42.

Marshall, K.C. 1976, in: *Interfaces in Microbial Ecology*, pp. 1–156. Harvard University Press, Cambridge, MA.

Mossialos, D., Ochsner, U., Baysse, C., Chablain, P., Pirnay, J.P., Koedam, N., Budzikiewicz, H., Fernández, D.U., Schäfer, M., Ravel, J., and Cornelis, P. 2002, Identification of new, conserved, non-ribosomal peptide synthetases from fluorescent pseudomonads involved in the biosynthesis of the siderophore pyoverdine, Molecular Microbiology, 45, 1673–1685.

Nazarenko, I., Lowe, B., Darfler, M., Ikonomi, P., Schuster, D., and Rashtchian, A. 2002, Multiplex quantitative PCR using self-quenched primers labeled with a single fluorophore, Nucleic Acids Research, 30, e37

Orphan, V.J., House, C.H., Hinrichs, K.U., McKeegan, K.D., and DeLong, E.F. 2002, Multiple archaeal groups mediate methane oxidation in anoxic cold seep sediments, Proceedings of the National Academy of Sciences of the United States of America, 99, 7663–7668.

Pedersen, K. 1997, Microbial life in granitic rock, FEMS Microbiology Reviews, 20, nos. 3–4, 399–414.

Pedersen, K. 2005, MICROBE Analysis of microorganisms and gases in MICROBE groundwater over time during MINICAN drainage of the MICROBE water conducting zone, *Äspö Hard Rock Laboratory: International Progress Report IPR-05-29*, 26 pp. Swedish Nuclear Fuel and Waste Management Co., Stockholm.

Pedersen, K. 2006, Microbiology of transitional groundwater of the porous overburden and underlying shallow fractured bedrock aquifers in Olkiluoto 2004, Finland, POSIVA Working Report 2006-09, 40 pp. Posiva Oy, Eurajoki, Finland.

Pedersen, K. 2007, Microbiology of transitional groundwater of the porous overburden and underlying shallow fractured bedrock aquifers in Olkiluoto 2005, Finland. October 2005–January 2006, POSIVA Working Report 2007-20, 46 pp. Posiva Oy, Eurajoki, Finland.

Pedersen, K. 2008, Microbiology of Olkiluoto groundwater 2004–2006, POSIVA Report 2008-2, Posiva Oy, Eurajoki, Finland.

Pedersen, K., Arlinger, J., Eriksson, S., Hallbeck, M., Johansson, J., Jägevall, S., and Karlsson, L. 2008a, Microbiology of Olkiluoto groundwater. Results and interpretations 2007, POSIVA Working Report 2008-34, Posiva Oy, Eurajoki, Finland

Pedersen, K., Arlinger, J., Hallbeck, A., Hallbeck, L., Eriksson, S., and Johansson, J. 2008b, Numbers, biomass and cultivable diversity of microbial populations relate to depth and borehole-specific conditions in groundwater from depths of 4 to 450 m in Olkiluoto, Finland, The ISME Journal, 2, 760–775.

Pedersen, K. and Ekendahl, S. 1990, Distribution and activity of bacteria in deep granitic groundwaters of southeastern Sweden, Microbial Ecology, 20, 37–52.

Pedersen, K., Ekendahl, S., Tullborg, E.-L., Furnes, H., Thorseth, I.-G., and Tumyr, O. 1997, Evidence of ancient life at 207 m depth in a granitic aquifer, Geology, 25, 827–830.

Pedersen, K., Nilsson, E., Arlinger, J., Hallbeck, L., and O'Neill, A. 2004, Distribution, diversity and activity of microorganisms in the hyper-alkaline spring waters of Maqarin in Jordan, Extremophiles, 8, 151–164.

Smith, C.J., Nedwell, D.B., Dong, L.F., and Osbor, A.M. 2007, Diversity and abundance of nitrate reductase genes (narG and napA), nitrite reductase genes (nirS and nrfA), and their transcripts in estuarine sediments, Applied and Environmental Microbiology, 73, no. 11, 3612–3622.

Thomsen, T.R., Finster, K. and Ramsing, N.B. 2001, Biogeochemical and molecular signatures of anaerobic methane oxidation in a marine sediment, Applied and Environmental Microbiology, 67, no. 4, 1646–1656.

Widdel, F. and Bak, F. 1992, Gram-negative, mesophilic sulphate-reducing bacteria, in: Balows, A., Truper, H.G., Dworkin, M., Harder, W. and Schleifer, K.-Z. (eds.), *The Prokaryotes*, vol. 4, pp. 3352–3378. Springer-Verlag, New York

Zehnder, A. and Brock, T. 1980, Anaerobic methane oxidation: Occurrence and ecology, Applied and Environmental Microbiology, 39, no. 1, 194–204.

Zhang, T. and Fang, H.H.P. 2006, Applications of real-time polymerase chain reaction for quantification of microorganisms in environmental samples, Applied Microbiology and Biotechnology, 70, 281–289.

A. APPENDIX

Table A-1. *Biomass determinations for groundwater in Olkiluoto and ONKALO, sampled in 2008 and 2009. TNC = total number of cells, SD = standard deviation, n = number of observations, CHAB = cultivable heterotrophic aerobic bacteria, and ΣMPN = sum of all most probable number of cells values (see Tables A-2 and A-3).*

Drillhole	Sampled (Y-M-D)	Depth (m)	TNC (cells mL^{-1})	SD	n	ATP (amol mL^{-1})	SD	n	CHAB (cells mL^{-1})	SD	n	CHAB/ TNC (%)	ATP/ TNC	ΣMPN/ TNC (%)
KR41_213_1	2008-02-12	193.9	68,000	6500	3	10,600	1070	9	1650	172	3	2.43	0.156	5.30
KR41_257_2	2008-03-03	234.6	38,000	14,000	3	15,200	1460	9	5230	757	3	13.76	0.400	6.56
KR43_96_1	2008-03-05	68.6	25,000	12,000	2	8600	1100	9	1030	152	3	4.12	0.344	0.55
KR43_214_1	2008-05-06	167.1	11,000	5900	3	11,800	1620	9	4830	2040	3	43.91	1.073	7.46
KR6_393_1	2008-05-07	305.2	15,000	4000	3	9930	708	9	6500	1000	3	43.33	0.662	13.89
KR46_175_1	2008-05-26	158.3	6300	1600	3	3500	920	9	20	10	3	0.32	0.556	1.91
KR46_131_1	2008-07-01	117.3	4800	3100	3	3600	880	9	357	177	3	7.44	0.75	17.52
KR46_82_1	2008-09-23	71.6	3300	740	3	2500	1000	9	267	35	3	8.09	0.76	17.92
KR47_131_1	2008-09-30	105.0	39,000	7300	3	14,400	1040	9	380	72	3	0.97	0.37	1.49
KR47_708_1	2008-11-17	571.0	20,000	240	3	7610	1580	9	1070	186	3	5.35	0.38	2.76
KR45_117_1	2008-12-01	95.2	19,000	4400	3	7540	1880	9	4170	321	3	21.95	0.40	4.28
KR47_413_1	2009-01-07	336.5	3200	2400	3	2350	667	9	103	25	3	3.22	0.73	4.09
KR47_145_1	2009-02-17	118.90	11,000	2300	3	8960	2630	9	2500	721	3	22.73	0.81	3.91
KR45_295_1	2009-04-14	247.00	26,000	4300	3	7440	1510	9	5600	800	3	21.54	0.29	21.90
KR49_614_1	2009-06-29	480.00	24,000	3000	3	35,300	4880	9	31,400	1010	3	130.83	1.47	208.34
KR47_77_1	2009-09-29	62.50	15,000	1900	3	4610	227	9	890	46	3	5.93	0.31	30.93

Drillhole	Sampled (Y-M-D)	Depth (m)	TNC (cells mL^{-1})	SD	n	ATP (amol mL^{-1})	SD	n	CHAB (cells mL^{-1})	SD	n	CHAB/ TNC (%)	ATP/ TNC	ΣMPN/ TNC (%)
KR47_217_1	2009-11-17	126.90	41,000	8700	3	19,200	4690	9	10,900	1970	3	26.59	0.47	11.26
KR40_786_1	2009-12-08	708.70	51,000	7600	3	-	-	-	3.33	5.77	3	0.01	0.00	2.55
OL-KR13 T360_1	2008-10-28	294.0	280,000	19,000	3	-	-	-	-	-	-	-	-	-
OL-KR13 T360_2	2008-10-28	294.0	170,000	9100	3	-	-	-	-	-	-	-	-	-
OL-KR13 T360_3	2008-10-28	294.0	190,000	14,000	3	-	-	-	-	-	-	-	-	-
OL-KR13 T360_4	2008-10-28	294.0	220,000	18,000	3	-	-	-	-	-	-	-	-	-
OL-KR13 T360_5	2008-10-29	294.0	79,000	16,000	3	-	-	-	-	-	-	-	-	-
OL-KR13 T360_6	2008-10-29	294.0	150,000	8600	3	-	-	-	-	-	-	-	-	-
OL-KR13 T360_7	2008-10-30	294.0	62,000	9600	3	-	-	-	-	-	-	-	-	-
ONK-KR1	2008-09-09	7.1	44,000	11,000	3	-	-	-	-	-	-	-	-	-
ONK-KR2	2008-09-09	30.5	6200	740	3	-	-	-	-	-	-	-	-	-
ONK-KR3	2008-09-09	44.8	490,000	240,000	3	-	-	-	-	-	-	-	-	-
ONK-PVA1	2008-09-09	14.6	51,000	14,000	3	-	-	-	-	-	-	-	-	-
ONK-PVA2	2008-09-09	65.2	79,000	6300	3	-	-	-	-	-	-	-	-	-
ONK-PVA3 :1L	2008-09-09	78.5	87,000	23,000	3	-	-	-	-	-	-	-	-	-
ONK-PVA3:2L	2008-09-09	78.5	35,000	8800	3	-	-	-	-	-	-	-	-	-
ONK-PVA3: 14L-	2008-09-09	78.5	-	-	-	-	-	-	-	-	-	-	-	-
ONK-PVA3:175 L	2008-09-10	78.5	12,000	3200	3	-	-	-	-	-	-	-	-	-
ONK-PP128-1	2008-09-10	297	-	-	-	-	-	-	-	-	-	-	-	-
ONK-PP128-2	2008-09-10	297	26,000	3200	3	-	-	-	-	-	-	-	-	-
ONK-PP128-3	2008-09-10	297	48,000	5900	3	-	-	-	-	-	-	-	-	-

Table A-2. *The most probable numbers of nitrate-, iron-, manganese-, and sulphate-reducing bacteria (NRB, IRB, MRB, and SRB, respectively) in groundwater from Olkiluoto and ONKALO sampled in 2008 and 2009. L and U limits are the 95% confidence values.*

Drillhole	Sampled (Y-M-D)	Depth (m)	NRB (cells mL^{-1})	L limit	U limit	IRB (cells mL^{-1})	L limit	U limit	MRB (cells mL^{-1})	L limit	U limit	SRB (cells mL^{-1})	L limit	U limit
KR41_213_1	2008-02-12	193.9	3000.0	1000.0	12,000.0	70.0	30.0	210.0	500.0	200.0	2000.0	13.0	5.0	39.0
KR41_257_2	2008-03-03	234.6	300.0	100.0	1200.0	1100.0	400.0	3000.0	50.0	20.0	170.0	240.0	100.0	940.0
KR43_96_1	2008-03-05	68.6	130.0	50.0	390.0	5.0	2.0	17.0	2.3	0.9	8.6	0.0		
KR43_214_1	2008-05-06	167.1	800.0	300.0	2500.0	17.0	7.0	48.0	2.2	0.9	5.6	0.8	0.3	2.4
KR6_393_1	2008-05-07	305.2	1100.0	400.0	3000.0	80.0	30.0	250.0	220.0	100.0	580.0	500.0	200.0	1700.0
KR46_175_1	2008-05-26	158.3	110.0	40.0	300.0	0.0			1.3	0.5	3.8	0.0		
KR46_131_1	2008-07-01	117.3	800.0	300.0	2500.0	7.0	3.0	21.0	17.0	7.0	48.0	0.2	0.1	1.1
KR46_82_1	2008-09-23	71.6	500.0	200.0	1700.0	2.3	0.9	8.6	50.0	20.0	170.0	0.2	0.1	1.1
KR47_131_1	2008-09-30	105.0	80.0	30.0	250.0	300.0	100.0	1300.0	170.0	70.0	480.0	30.0	10.0	120.0
KR47_708_1	2008-11-17	571.0	500.0	200.0	1700.0	5.0	2.0	17.0	17.0	7.0	48.0	22.0	10.0	58.0
KR45_117_1	2008-12-01	95.2	800.0	300.0	2500.0	7.0	3.0	21.0	1.4	0.6	3.5	1.7	0.7	4.6
KR47_413_1	2009-01-07	336.5	300	100	1200	17.0	7.0	48.0	70.0	30.0	210.0	5.0	2.0	15.0
KR47_145_1	2009-02-17	118.5	300	100	1200	17.0	7.0	48.0	70.0	30.0	210.0	5.0	2.0	15.0
KR45_295_1	2009-04-14	247.0	5000.0	2000.0	17,000.0	0.4	0.1	1.7	0.7	0.2	2.1	600.0	300.0	1800.0
KR49_614_1	2009-06-29	480.0	50,000.0	20,000.0	200,000.0	0.4	0.1	1.5	1.4	0.6	3.5	0.0		
KR47_77_1	2009-09-29	62.5	3000.0	1000.0	13,000.0	13.0	3.0	39.0	1600.0	600.0	5300.0	8.0	3.0	25.0
KR47_217_1	2009-11-17	126.9	3500.0	1600.0	8200.0	900.0	300.0	2900.0	110.0	40.0	300.0	9.0	4.0	25.0
KR40_786_1	2009-12-08	708.7	1300.0	500.0	3900.0	0.2	0.1	1.1	0.0			1.4	0.6	3.5

Drillhole	Sampled (Y-M-D)	Depth (m)	NRB (cells mL^{-1})	L limit	U limit	IRB (cells mL^{-1})	L limit	U limit	MRB (cells mL^{-1})	L limit	U limit	SRB (cells mL^{-1})	L limit	U limit
OL-KR13 T360_1	2008-10-28	294.0	-	-	-	13.0	5.0	39.0	-	-	-	130.0	50.0	390.0
OL-KR13 T360_2	2008-10-28	294.0	-	-	-	1.7	0.7	4.6	-	-	-	5.0	2.0	17.0
OL-KR13 T360_3	2008-10-28	294.0	-	-	-	2.3	0.9	8.6	-	-	-	5.0	2.0	15.0
OL-KR13 T360_4	2008-10-28	294.0	-	-	-				-	-	-			
OL-KR13 T360_5	2008-10-29	294.0	-	-	-	3.3	1.5	7.7	-	-	-	30.0	10.0	120.0
OL-KR13 T360_6	2008-10-29	294.0	-	-	-				-	-	-			
OL-KR13 T360_7	2008-10-30	294.0	-	-	-	1.3	0.5	3.8	-	-	-	8.0	3.0	25.0
ONK-KR1	2008-09-09	7.1	-	-	-	-	-	-	-	-	-	-	-	-
ONK-KR2	2008-09-09	30.5	-	-	-	-	-	-	-	-	-	-	-	-
ONK-KR3	2008-09-09	44.8	-	-	-	-	-	-	-	-	-	-	-	-
ONK-PVA1	2008-09-09	14.6	-	-	-	-	-	-	-	-	-	-	-	-
ONK-PVA2	2008-09-09	65.2	-	-	-	-	-	-	-	-	-	-	-	-
ONK-PVA3 :1L	2008-09-09	78.5	-	-	-	-	-	-	-	-	-	-	-	-
ONK-PVA3:2L	2008-09-09	78.5	-	-	-	-	-	-	-	-	-	-	-	-
ONK-PVA3: 14L-	2008-09-09	78.5	-	-	-	-	-	-	-	-	-	-	-	-
ONK-PVA3:175 L	2008-09-10	78.5	-	-	-	-	-	-	-	-	-	-	-	-
ONK-PP128-1	2008-09-10	297	-	-	-	-	-	-	-	-	-	0.7	0.2	2.1
ONK-PP128-2	2008-09-10	297	-	-	-	-	-	-	-	-	-	<0.2	-	-
ONK-PP128-3	2008-09-10	297	-	-	-	-	-	-	-	-	-	0.4	0.1	1.5

Table A-3. *The most probable numbers of autotrophic acetogens (AA) and methanogens (AM), heterotrophic acetogens (HA), heterotrophic methanogens (HM), and methane-oxidizing bacteria (MOB) in groundwater from Olkiluoto and ONKALO. L and U limits are the 95 % confidence values.*

Drillhole	**Sampled (Y-M-D)**	**Depth (m)**	**AA (cells mL^{-1})**	**L limit**	**U limit**	**HA (cells mL^{-1})**	**L limit**	**U limit**	**AM (cells mL^{-1})**	**L limit**	**U limit**	**HM (cells mL^{-1})**	**L limit**	**U limit**
KR41_213_1	2008-02-12	193.9	8.0	3.0	25.0	11.0	4.0	30.0	<0.2	-	-	2.3	0.9	8.6
KR41_257_2	2008-03-03	234.6	2.6	1.2	6.5	800.0	300.0	2500.0	<0.2	-	-	<0.2	-	-
KR43_96_1	2008-03-05	68.6	0.0			0.0			<0.2	-	-	<0.2	-	-
KR43_214_1	2008-05-06	167.1	0.0			0.2	0.1	1.1	<0.2	-	-	<0.2	-	-
KR6_393_1	2008-05-07	305.2	13.0	5.0	39.0	170.0	70.0	480.0	<0.2	-	-	<0.2	-	-
KR46_175_1	2008-05-26	158.3	1.1	0.4	2.9	8.0	3.0	25.0	<0.2	-	-	<0.2	-	-
KR46_131_1	2008-07-01	117.3	2.6	1.2	6.5	14.0	6.0	36.0	<0.2	-	-	<0.2	-	-
KR46_82_1	2008-09-23	71.6	9.0	4.0	25.0	30.0	10.0	130.0	<0.2	-	-	<0.2	-	-
KR47_131_1	2008-09-30	105.0	0.8	0.3	2.4	1.3	0.5	3.8	<0.2	-	-	<0.2	-	-
KR47_708_1	2008-11-17	571.0	3.0	1.0	11.0	5.0	2.0	17.0	0.2	0.1	1.0	<0.2	-	-
KR45_117_1	2008-12-01	95.2	0.2	0.1	1.1	2.3	0.9	8.6	<0.2	-	-	<0.2	-	-
KR47_413_1	2009-01-07	336.5	30.0	10.0	130.0	8.0	3.0	25.0	0.0			0.0		
KR47_145_1	2009-02-17	118.5	30.0	10.0	130.0	8.0	3.0	25.0	0.0			0.0		
KR45_295_1	2009-04-14	247.0	2.7	1.2	6.7	90.0	30.0	290.0	0.0			0.0		
KR49_614_1	2009-06-29	480.0	0.0			0.0			0.0			0.0		
KR47_77_1	2009-09-29	62.5	5.0	2.0	17.0	13.0	5.0	39.0	0.0			0.2	0.1	1.1
KR47_217_1	2009-11-17	126.9	17.0	8.0	41.0	80.0	30.0	250.0	0.0			0.0		
KR40_786_1	2009-12-08	708.7	0.0			0.0			0.2	0.1	1.0	0.0		

Drillhole	Sampled (Y-M-D)	Depth (m)	AA (cells mL^{-1})	L limit	U limit	HA (cells mL^{-1})	L limit	U limit	AM (cells mL^{-1})	L limit	U limit	HM (cells mL^{-1})	L limit	U limit
OL-KR13 T360_1	2008-10-28	294.0	-	-	-	-	-	-	<0.2	-	-	-	-	-
OL-KR13 T360_2	2008-10-28	294.0	-	-	-	-	-	-	<0.2	-	-	-	-	-
OL-KR13 T360_3	2008-10-28	294.0	-	-	-	-	-	-	<0.2	-	-	-	-	-
OL-KR13 T360_4	2008-10-28	294.0	-	-	-	-	-	-				-	-	-
OL-KR13 T360_5	2008-10-29	294.0	-	-	-	-	-	-	130.0	50.0	390.0	-	-	-
OL-KR13 T360_6	2008-10-29	294.0	-	-	-	-	-	-				-	-	-
OL-KR13 T360_7	2008-10-30	294.0	-	-	-	-	-	-	3.0	1.0	12.0	-	-	-
ONK-KR1	2008-09-09	7.1	-	-	-	-	-	-	-	-	-	-	-	-
ONK-KR2	2008-09-09	30.5	-	-	-	-	-	-	-	-	-	-	-	-
ONK-KR3	2008-09-09	44.8	-	-	-	-	-	-	-	-	-	-	-	-
ONK-PVA1	2008-09-09	14.6	-	-	-	-	-	-	-	-	-	-	-	-
ONK-PVA2	2008-09-09	65.2	-	-	-	-	-	-	-	-	-	-	-	-
ONK-PVA3 :1L	2008-09-09	78.5	-	-	-	-	-	-	-	-	-	-	-	-
ONK-PVA3:2L	2008-09-09	78.5	-	-	-	-	-	-	-	-	-	-	-	-
ONK-PVA3: 14L-	2008-09-09	78.5	-	-	-	-	-	-	-	-	-	-	-	-
ONK-PVA3:175 L	2008-09-10	78.5	-	-	-	-	-	-	-	-	-	-	-	-
ONK-PP128-1	2008-09-10	297	-	-	-	-	-	-	0.2	0.1	1.0	-	-	-
ONK-PP128-2	2008-09-10	297	-	-	-	-	-	-	<0.2	-	-	-	-	-
ONK-PP128-3	2008-09-10	297	-	-	-	-	-	-	<0.2	-	-	-	-	-