Working Report 2008-82

Sulphate-Reducing Bacteria in Ground Water Samples from Olkiluoto – Analyzed by Quantitative PCR

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December 2008

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ABSTRACT

The GEOFUNC Project is connected to safety and risk assessment research on the final disposal site of nuclear waste disposal.

Sulphate-reducing micro-organisms, which are the major group of micro-organisms of concern, are able to cause copper corrosion by converting sulphate to sulphide, which is a very corrosive agent. The quantitative assessment of sulphate reducers by the qPCR method, which is based on sulphite reductase gene quantification, is related to the transformation of sulphate to sulphide. In this project we developed external and internal standard methods for our qPCR method in order to evaluate the quantitative amount and efficiency of DNA extractions and PCR inhibition possibly caused by high salt or iron concentrations. These standards are needed in quantifying and estimating the real quantity of sulphate reducers on the basis of their functional genes.

Sulphate reducers were detected in all the samples studied. Based on this small dataset, no clear relationships were found between the geochemistry and the numbers of sulphate reducers, and the phenomena should be studied in more detail with more variable drillhole waters in the future

Keywords: Sulphate-reducing bacteria, quantitative PCR, OLKILUOTO, ONKALO

Sulfaatinpelkistäjät Olkiluodon pohjavesinäytteissä - analysoitu kvantitatiivisella PCR-tekniikalla

TIIVISTELMÄ

Tutkimushankkeessa GEOFUNC on tavoitteena molekyylibiologisten monitorointi-menetelmien soveltaminen ja kehittäminen korkea-aktiivisen jätteen loppusijoituksen turvallisuusanalyysien tueksi. Tässä projektissa tutkittiin vesinäytteitä ONKALOSTA, Olkiluodon tutkimustunnelista ja loppusijoitusalueen kairarei'istä. Vertailuna tutkittiin myös Palmottun, luonnollisen uraanialueen kairareikää 387.

Pääasiallinen mikrobiryhmä, joka on noussut huolenaiheeksi on sulfaatinpelkistäjien esiintyminen ja aktiivisuus, koska ne ovat merkittävin mikrobiryhmä, jotka voivat välillisesti aiheuttaa kuparin korroosiota. Sulfaatinpelkistäjien osoittaminen qPCR menetelmällä perustuen sulfiittireduktaasigeenin kvantitointiin liittyy suoraan sulfaatin pelkistymiseen sulfidiksi.

Tässä projektissa verrattiin menetelmiä, jotka tunnistavat sulfiittireduktaasi geenialueen molemmista päistä, jotta voitiin valita menetelmä jatkotutkimuksiin. Lisäksi projektissa kehitettiin sekä ulkoinen että sisäinen standardi tuntemattomien näytteiden sulfaatin-pelkistäjien kvatitatiivisuuden osoittamiseksi ja DNA eristysmenetelmän tehokkuuden ja PCR inhibition selvittämiseksi. Standardit tarvitaan, jotta voidaan arvioida sulfaatin-pelkistäjien todellisia pitoisuuksia.

Sulfaatinpelkistäjiä tunnistettiin kaikissa tutkituissa näytteissä qPCR menetelmällä. Olkiluodon pohjavesinäytteissä oli huomattavasti vähemmän sulfaatinpelkistäjiä kuin Palmottu vesinäytteissä. Olkiluodon pohjavesien geokemiallisissa analyyseissä ei todetu selkeää yhteyttä sulfaatinpelkistäjien esiintymiseen. Tämä tutkimus kaipaa lisä-selvityksiä suuremmalla näytemäärällä sekä olosuhteiltaan erilaisten kairareikien tutkimuksella.

Palmottun suuret sulfaatinpelkistäjämäärät ovat todennäköisesti yhteydessä pintavesien kulkeutumiseen kairareikään, jonka geokemialliset olosuhteetkin olivat muuttuneet useiden suureiden osalta. Huolimatta siitä, että näytteissä oli happea, voitiin niissä todeta suuria sulfaatinpelkistäjä määriä. Lukuisissa tutkimuksissa onkin todettu, että vaikka sulfaatinpelkistäjiä on pidetty erittäin anaerobisina mikrobiryhminä voivat ne toimia myös vyöhykkeissä joissa happea on läsnä.

Avainsanat: sulfaatin pelkistäjät, mikrobit, kvantitatiivinen PCR, OLKILUOTO, ONKALO

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PREFACE

This work was carried out at VTT Biotechnology during 1.8.2007-30.8.2008. The contact person at Posiva Oy was Anne Lehtinen, and at VTT Merja Itävaara.

The scientists who have contributed to this project are Maija-Liisa Vehkomäki and Aura Nousiainen.

We thank Petteri Pitkänen from VTT for valuable discussions, as well as the personnel of Posiva.

nomenclature ABBREVIATIONS

amplicon= amplified gene fragment

DAPI= 4',6-diamidino-2-phenylindole is a fluorescent stain that binds strongly to DNA

dsDNA= double stranded DNA

DNA sequence= the order of nucleotide molecules in DNA

DNA polymerases= enzymes required for DNA replication

External standard = a standard dilution series of a known gene to quantify the gene copy number of the unknown sample

hybridization= reaction in which two single stranded DNA fragments form a double stranded DNA molecule (dsDNA)

Internal standard = a method to assess the loss of DNA in the extraction procedures or PCR inhibition

melting temperature= temperature at which the double stranded DNA opens and the two single-stranded DNA fragments separate

nucleotides= nucleotides are the structural units of RNA and DNA

PCR polymerase chain reaction = amplification method for fragments of DNA

primers= oligonucleotides used to target the amplicon of interest, specific detection of gene fragments

probe= a fragment of DNA or RNA of variable length (usually 100-1000 bases long), which is used to detect, in DNA or RNA samples, the presence of nucleotide sequences (the DNA target) that are complementary to the sequence in the probe

synthetic oligonucleotides=short, single stranded pieces of DNA

qPCR quantitative polymerase chain reaction= quantitative method for PCR

1 INTRODUCTION

1.1 The deep subsurface microbial world and repository safety

Geobiochemical processes in deep subsurface bedrock and groundwater have been extensively studied during recent years (Hallbeck and Pedersen, 2008; Kotelnikova and Domingo, 1998; Stroes-Gascoyne and West, 1997; Pedersen, 1997, 1998; Motamedi et al., 1996; Pedersen, 2000, Pedersen et al., 2000; Nazina et al., 2004). The microbiological risks associated with the long-term safety of nuclear waste disposal have also been taken seriously in the Finnish disposal programme. The major microbiological risks have been estimated to be due to the corrosion of construction materials, and the mobilization of radionuclides or changes in their behaviour and migration in the final disposal site (Pedersen, 2008). Corrosion may be caused by micro-organisms that use the metals as electron acceptors, or due to indirect changes in local conditions that allow chemical corrosion to take place. Nutrients, water and energy sources will affect the rate of biocorrosion (West et al., 2002).

The major questions raised concerning the microbial safety of final disposal site are: How deep can micro-organisms survive in the bedrock and in groundwater. How do the geomicrobial processes affect geochemistry, and can they cause copper corrosion? What are the long-term effects on geochemistry? It is well known that the geological environments can change due to microbial activity. Can bentonite clay compaction be penetrated by micro-organisms? Are there biodegradable materials present in the tunnel structures? If so, how do these organic materials degrade, and how does the biodegradation process affect microbial processes and biocorrosion?

The questions still to be resolved also include the energy metabolism of deep subsurface microbial communities and autotrophic chemolithototrophic processes.

1.2 Survival of micro-organisms in extreme environments

Micro-organisms have been found in extreme environments where other organisms are not able to survive. Living microbes exist almost everywhere where there is water and a suitable temperature to support life. The temperature limits for micro-organisms are wide and hyperthermophilic micro-organisms have been cultured from temperatures as high as 113^{o}C (Stetter, 1996). On the other hand, living micro-organisms have also been detected in permafrost at extremely low temperatures. Intra terrestrial life and cultivable micro-organisms have been successfully isolated from a depth of 5278 m in the bedrock of Gravenberg in Sweden, where the temperature was 65-75^{o}C (Gold, 1992). It has been estimated that bacterial ecosystems, independent of the sun-driven surface ecosystems, exist both in granitic and basaltic rock at depths as deep as 3.5 kilometers (Pedersen, 1993; Stevens et al., 1995).

Why are certain species of micro-organisms present at such sites? This is not a coincidence. The environmental conditions, such as redox conditions, nutrients, electron acceptors and gases, provide suitable conditions for micro-organisms. Micro-organisms

also change their environment and produce metabolites that make the conditions more suitable for themselves and also for other micro-organisms. Micro-organisms in deep subsurface environments have learnt to live together and to benefit from each other's metabolic products in order to survive. This kind of succession is occurring continuously in the environment. Micro-organisms are known to be able to leach minerals, as well as to precipitate them depending on the environmental conditions. The major requirements for micro-organisms are the presence of electron acceptors for energy-driven cellular processes and water.

The ability of micro-organisms to tolerate high radiation doses and high temperatures has been studied in research programmes carried out in Canada on the long-term safety of radioactive wastes (Stroes-Gascoyne and West, 1997). Radiation and desiccation studies with micro-organisms have resulted in the finding that micro-organisms can tolerate high gamma radiation up to 10kGy - 15 kGY (Pitonzo et al., 1999a and1999b). Other radiation work with sulphate-reducing bacteria was performed by West, (1995) who found a tolerance of up to 10k Gy for over 40 hours.

Moisture is important for microbial activity, and the effects of moisture content on the presence of microbes in bentonite clay have been investigated in an in situ experiment in which nuclear wastes were surrounded by a compacted buffer consisting of 50% bentonite and 50% of sand (Stroes-Gascoyne et al., 1997). At the end of the experiment, microbes could only be cultured from the buffer material in which the water activity (aw) was greater than 0.96.

1.3 Microbial processes at different depths in deep subsurface conditions

Torsvik et al. (1990, 1998, 2002) have estimated that 10.000 bacterial species may be present in one gram of surface soil. However, Gans et al. (2005) recently estimated that the number of different species in soil might be even over one million. The number and diversity of species are highly dependant on the amount and type of organic compounds and electron acceptors present in the environment. In principle, the geochemical zones and processes are much more complex than is generally described.

The surface soil layers contain large amounts of organic matter, and aerobic biodegradation processes are prevailing. Aerobic metabolism involves enzymes that selectively degrade individual classes of compound. Aerobic respiration is the most energy efficient mechanism for the degradation of organic matter and therefore represents the first biogeochemical zone. Micro-organisms that use oxygen as their terminal electron acceptor completely oxidize a wide variety of natural and synthetic organic compounds to carbon dioxide water and biomass

In the absence of oxygen, organic matter is decomposed by anaerobic degradation processes in which several microbial species degrade the organic matter to acetates and other volatile fatty acids. Further degradation of these compounds results in the

formation of methane, together with intermediate products such as hydrogen and carbon dioxide (Kristensen et al., 1995).

When going deeper into the soil and towards the bedrock, microbes gradually consume all the available oxygen and, in the absence of oxygen, other redox processes occur. In deep geological environments the total amount of organic matter is low.

After the depletion of oxygen, nitrate is used as a terminal electron acceptor if available. Dissimilatory nitrate reduction occurs at the depth were oxygen is used up. Nitrate may also be reduced to organic nitrogen by assimilatory nitrate reduction (Tiedje et al., 1982; Konhauser et al., 2002).

After the depletion of nitrate, the reduction of managanese oxides becomes the most efficient bacterial repository process (Santschi et al., 1990). Manganese reduction results in the dissolution of manganese oxides to form soluble Mn^{2+}, which may diffuse upwards and reprecipitate as fresh Mn oxides at the sediment surface (Burdige, 1993). Downward diffusion of Mn^{2+} may result in the formation of rhodocrosite ($MnC0_3$) or other manganese carbonates such as kutnathorite and manganoan calcite (Aller and Rude, 1988).

The major source of energy below the zones where nitrate and managanese reduction occurs, and where these compounds have been reduced, is iron reduction. Several species of microorganism, such as *Geobacter metallireducens* (Lovley and Phillips, 1988), *Shewanella putrefaciens*, are responsible for iron reduction. In addition, several species are also known to be involved in sulphate reduction, such as *Desulfuromonas sp.* and *Desulfuvibrio sp*. Iron reduction is considered to have the most significant effect on sediment mineralogy. Amorphous to poorly crystalline iron hydroxides, such as ferrihydrite and lepidocrocite, are considered to be the most important source of Fe (III) for iron reduction. More crystalline Fe (III) oxides (hematite and goethite) are also reduced by micro-organisms and act as energy sources for micro-organisms (Roden and Zachara, 1996).

The reduction of ferric iron minerals results in increased amounts of dissolved ferrous iron in pore water. If the ferrous iron moves upwards it will be reoxidized, but most of it will be precipitated as iron sulphides when moving downwards into deeper environments. Sulfate-reducing bacteria (SRB) are responsible for this reaction. The metastable iron monosulphides such as greigite and mackinawite are precursors of pyrite (FeS_2).

Sulphate reduction occurs only after the total depletion of all the other terminal electron acceptors. It has been estimated that sulphate reduction is responsible for organic matter degradation in anoxic conditions, mainly due to the availability of sulphate and of sulphate-reducing bacteria (SRBs). After the sulphide concentrations increase and become very toxic to SRBs, the detoxification of sulphide may occur by iron through the formation of insoluble iron monosulphides (FeS) and eventually prite (Konhauser et al., 2002). Sulphate reduction and sulphidation occur below the nitrate-reducing, managanese-reducing and iron-reducing zones (Konhauser et al., 2002).

Methanogenesis is due to several species of bacteria which use CO_2 and H_2 as terminal electron acceptors.

The major redox processes and electron acceptors are illustrated in Figure 1.

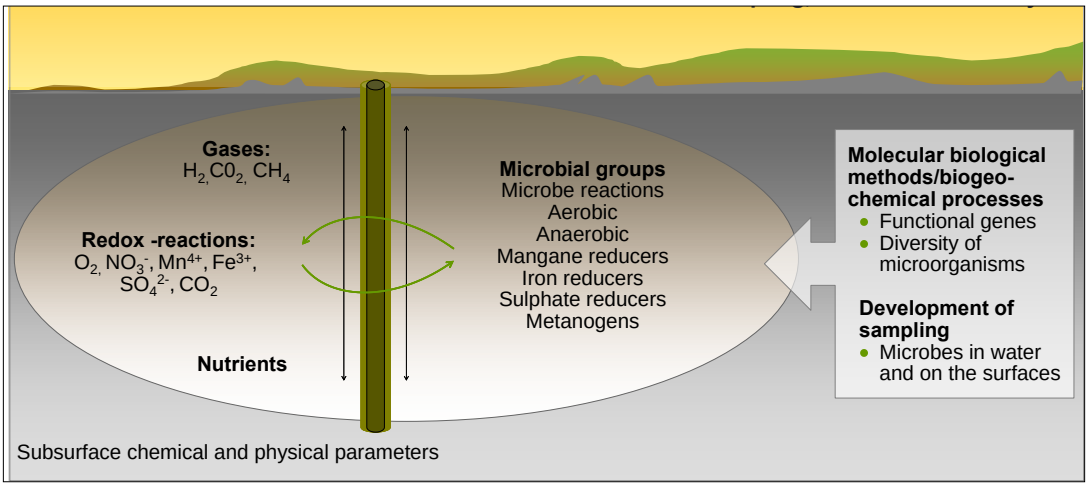


Figure 1. *Electron acceptors and gases formed in deep subsurface aquifers.*

1.4 The sulphur cycle in ecosystems

The bulk of the sulphur present in the earth's crust is in the form of sulphate (gypsym, $CaSO_4$) and sulphide minerals (pyrite, FeS_2) that constitute rocks.

In nature three sulphur oxidation states form the major cycle: +6 sulphate SO_4 which is the most abundant form of sulphur and energetically stable, elemental sulphur (S^0), -2: sulphhydryl (R-SH) and sulphide (S^{2-}). Sulphide mainly occurs as a result of microbial action, but it is also formed during volcanic activity.

Sulphur is an essential element for all living organisms because it is a constituent of many amino acids and enzymes. The assimilated sulphur is released gradually during the decomposition of living biota and, under anaerobic conditions, sulphide is formed during desulphurylation. However, sulphide is not only formed in the decomposition of organic molecules because dissimilatory sulphate reducing micro-organisms reduce SO_4 to sulphide. Sulphide can also be easily oxidized to sulphate in the presence of oxygen. Intermediate oxidation states may be formed, such as thiosulphate (S_2O_3) or elemental sulphur S^0, which also might serve as electron acceptors for microrganisms that are unable to reduce sulphate to sulphide (Sorokin, 1972).

1.5 Sulphate-reducing bacteria

More than 150 species of sulphate-reducing bacteria have been detected, and they are divided into 40 genera (Garrity et al., 2004).

Sulphate-reducing bacteria (SRB) play a significant role in the mineralization of organic matter in anaerobic environments and in the biogeochemical cycling of sulphur (Dar et al., 2000). Under anaerobic conditions, SRB use sulphate as a terminal electron acceptor in the degradation of organic matter, resulting in the production of sulphide which is a highly reactive, corrosive, and very toxic compound. Sulphate-reducing bacteria are mainly found in anoxic environments, but they can also be found in the interfaces of oxic/anoxic environments (Loy, 2003). Even if most of the SRBs are degraders of organic matter, there are also autotrophic species which are able to fix CO_2 and use hydrogen as an energy source in their metabolic processes. Some SRB species may be able to fix nitrogen and reduce phosphite to phosphate (Schink and Friedrich, 2000). In addition to the ability of SRBs to reduce NO_3 to NO_2, oxygen has also been shown to be a potential electron acceptor at low concentrations (Rabus et al., 2006) despite the fact that SRBs have been considered as strictly anaerobic species (Lovley and Phillips, 1994).

Sulphate-reducing bacteria utilize a variety of inorganic sulphur compounds as electron acceptors such as elemental sulphur (S^0), sulphite (SO_3), thiosulphate ($S_2O_3^{2-}$). In the absence of sulphate or other inorganic electron acceptors, many SRBs can change their metabolism and degrade organic matter. In addition, syntrophic associations with H_2 consuming micro-organisms have also been found (Rabus et al., 2006).

Sulphate-reducing bacteria (SRB) are a diverse group of anaerobic bacteria that can use sulphate as a terminal electron acceptor (Wagner et al., 1998; Daly et al., 2000). SRBs have been divided into six different taxonomic groups based on their phylogenetic diversity (Figure 2).

1. *Desulfotomaculatum*
2. *Desulfobulbus*
3. *Desulfobacterium*
4. *Desulfobacter*
5. *Desulfococcus – Desulfonema – Desulfosarcina*
6. *Desulfovibrio – Desulfomicrobium*

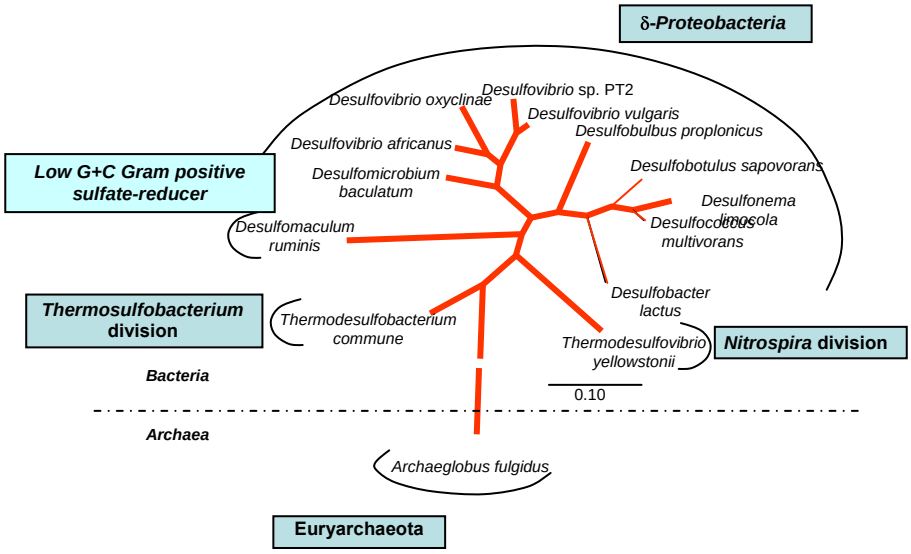


Figure 2. *Evolution tree of sulphate-reducing bacteria based on 16S rRNA diversity (Wagner et al. 1998).*

Most of the SRB isolates within the deltaproteobacteria belong to the orders of *Desulfobacterales*, *Syntrophotobacterales* and *Desulfovibrionales*. The low G+C gram-positive SRB genera are *Desulfotomaculatum* and *Desulfosporosinus*, and they belong to the *Firmicutes phylum*. In the class *Nitrospira* only two of the species in the genus *Thermosulfovibrio* can use sulphate as a terminal electron acceptor. *Thermodesulfobacteriaceae*, which earlier contained only one sulphate reducing genus *Thermodesulfobacterium*, has gained a new member from *Geothermobacterium ferrireducens*. Some sulphate reducers are also found in the domain of *Archaea* (Loy, 2003).

Sulphate reduction is considered as one of the main threats to long term nuclear waste disposal. Therefore special emphasis has been given to molecular methods for the detection of these processes.

1.6 Functional genes in sulphate reduction

Functional genes code for proteins catalyzing various biogeochemical processes such as the carbon, nitrogen and sulphur cycles. Sulphate reduction is catalyzed by the dissimilatory sulphate reductase gene (*dsrAB*), which is present in micro-organisms able to convert sulphate to sulphide (Karkhoff-Schweizer et al., 1995, Karr et al., 2005). The *dsrAB* gene sequence is highly conserved across the sulphate-reducing bacteria and Archea (Figure 3). As an enzyme coding gene, *dsrAB* is also a good target for the identification and enumeration of populations with a specific metabolic potential in a wide range of environments in which the traditional culture-dependent methods cannot be used.

The size of the *dsrAB* gene is 1.9 kb, and it consists of α and β subunits (Wagner et al., 1998).

The activity, diversity and number of sulphate-reducing micoorganisms can be studied by analyzing the *dsrAB* gene with different molecular methods. In the present project we have applied quantitative PCR based on the functional gene *dsrAB*.

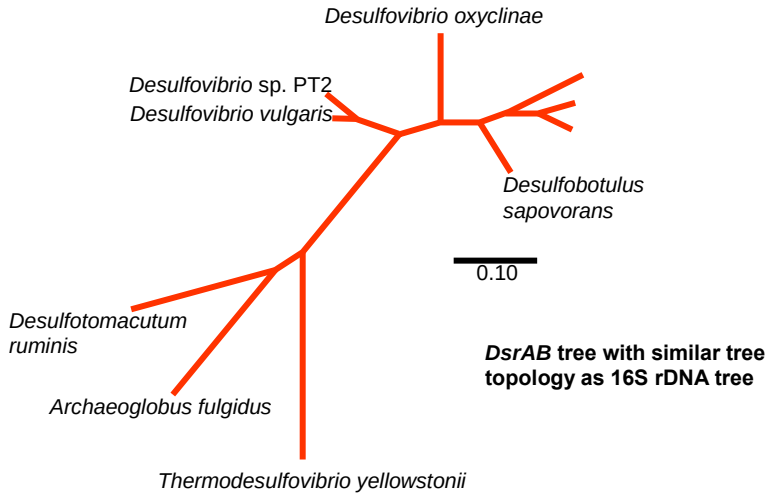


Figure 3. *DsrAB gene and diversity (Wagner et al., 1998).*

1.7 Short introduction to the real-time qPCR method used in this work

Generally the amount of DNA is too small to be studied without first amplification of the target. PCR the polymerase chain reaction is a method for amplifying small amounts of DNA using a pair of synthetic oligonucleotides which target a specific gene of interest (Shipley, 2006).

During the polymerase chain reaction (PCR), the target DNA sequence is amplified over a number of denaturation-annealing-extension cycles. In conventional PCR, only the final concentration of the amplified DNA region is measured. In contrast, in real–time qPCR technology the amplification of a specific DNA sequence takes place in the presence of a DNA-binding fluorescent dye, and the amplification product is detected concurrently as it is formed via the generation of an associated fluorescent signal. The fluorescence intensity emitted during this process reflects the target concentration in real time. Specialized qPCR instruments detect changes in target-associated fluorescence and plot them as a function of the PCR cycle number. The cycle at which the fluorescence signal is most closely associated with the initial copy number of the target is reported as the crossing point, C_p (synonymous to the threshold cycle, Ct). Under optimal conditions the fluorescent signal changes are directly proportional to the initial target input. Absolute quantification is achieved by plotting samples of unknown

concentration against a standard curve generated from a dilution series of template DNA of known concentration Mackay (2007).

1.8 External and internal standards in evaluation of the quantity of sulphate reducing bacteria

In quantitative studies all the steps must be thoroughly standardized in order to achieve reliable, comparable results. DNA samples taken at different sites, depths and even on different dates can be completely different because the DNA extraction efficiency depends on many factors. Adsorption of DNA on soil particles is the most widely acknowledged problem (Ikeda et al., 2008). The need to estimate the DNA extraction efficiency and to evaluate PCR inhibition in the samples are important because they may cause errors in data quantification. Inhibition in PCR can also be due to the presence of salts, iron, humic acids etc. (England et al., 2005). Owing to the difference in DNA extraction efficiency between the samples, as well as to potential PCR inhibition, it was necessary to develop a method for evaluating these differences.

2 TASK DEFINITION

Because microbial related sulphate reduction is considered as one of the risks for the long term safety of nuclear waste disposal in the bedrock, methods are needed to study the presence and function of deep groundwater sulphate-reducing microbial communities. The aim of this project was to establish a quantitative PCR method based on the functional gene involved in sulphate reduction, and to quantitate sulphate-reducing micro-organisms in the groundwater samples from boreholes on Olkiluoto Island.

3 MATERIALS AND METHODS

3.1 Samples

3.1.1 SRB pure cultures

Cultures of four species of sulphate–reducing bacteria representing different phylogenetic groups of SRB *Desulfobacterium autotrophicum, Desulfobulbus propionicus, Desulfosarcina variabilis* and *Desulfovibrio vulgaris* were ordered from DSMZ (German Collection of Micro-organisms and Cell Cultures), an international culture collection.

Total DNA was isolated from the cells and was used as positive controls. *Desulfobacterium autotrophicum* was used to prepare an external standard for qPCR, which was then used for quantification of the sulphate reducers in the environmental samples.

3.1.2 Groundwater samples from Palmottu Nummi-Pusula

Groundwater samples from Palmottu, which is a natural uranium site located at Nummi-Pusula (Blomqvist et al., 1995; 2000, Pedersen and Haveman, 1999), were studied for comparison with the Olkiluoto samples.

The sampling campaign was carried out in the GEOMOL Project (KYT research program) during summer 2006. The sampling was performed with a pressurized tube system in collaboration with the Geological Survey of Finland. The sampling procedure is described in detail in the research report Itävaara et al., 2006. VTT-R-02178-07.

Several samples were taken during groundwater pumping in order to clean the drillhole (samples 832-835, Table 1). Sample 844 is ‘a packer sample’ and was sampled at the depth of 170 m. The sample was taken after two packers had been installed to close the drillhole at both ends and the space between the packers had been pumped empty and then allowed to fill up with the groundwater originating from the bedrock fractures. Sample 842 was taken at the depth of 170 m before the packer was installed.

In order to study the source of possible microbial contamination, several swab samples were taken by pulling a sterile cotton swab along the surface of the sampling tube and the drillhole opening. The DNA was then extracted from the cotton swab and the studies performed in the same way as for the water samples. The samples were numbered 1, 2, 4 and 7. More detailed information about the sampling and other analyses can be found in the research report Itävaara et al. 2008 (VTT-R-01952-08).

Table 1. *Samples from Palmottu, Nummi-Pusula (drillhole 387).*

Sample Nro	Description Depth of the samples
832	20 m-26 m (pumping water)
833	80-109 m packer sample
834	pumping water
835	pumping water
842	170 m pumping water
843	110 m pumping water sample
844	170 m packer sample
1	swab sample, surface of the drillhole opening
2	river water, sampled close to the drillhole
4	swab sample, surface of the pumping tube
7	swab tube

3.1.3 Groundwater samples from Olkiluoto

Four samples from the Olkiluoto final disposal site were studied. Sample ONK-PVA3/19.6.07 was taken from the groundwater observation station, which is located on a drainage 964, at 83 m depth. The sample was taken directly from the packered section by connecting it via an injection needle to a head space flask closed with a butyl rubber cap and aluminium band which was made anaerobic by nitrogen gasification.

The other samples were taken by the PAVE pressurized sampling device from drillholes OL-KR8/556.1-561 m (2.10.07), OL-KR42/175-179 m (28.11.2007), OL-KR43/96-102 m (25.2.2008) at the end of 2007 and during spring 2008. The PAVE sampler was not cleaned with Freebact solution before sampling in OL-KR42/175-179 m.

The samples were transported refrigerated at +5ºC to VTT for the microbial and molecular biological analysis. At VTT the water samples were filtered through 0.22 µm sterile filters and stored frozen at -80^{o}C until analyzed.

3.2 Physical and chemical analysis of groundwater samples

3.2.1 Olkiluoto samples

The data concerning geophysical and geochemical measurements were analyzed by Posiva Oy's personnel and by an external laboratory contracted by Posiva.

3.3 Microbiological analysis

3.3.1 Number of micro-organisms in the samples

The total number of micro-organisms in the groundwater samples was determined by staining and microscoping the cells. When staining cells for calculating cell numbers, it is always important to disperse the cells so that they do not clump together but instead are spread evenly over the filter where they can be easily counted. For DAPI staining 0.1 M *tetranatrium pyrophosphate* (Riedel-Haën) was added to two parallel groundwater samples as a dispergent. After the dispersal step, DAPI staining liquid (Sigma) was added to the two samples. The water samples were mixed carefully and left to react for 20 minutes in the dark. After this the samples were filtrated through a 0.2 µm polycarbonate filter to catch the cells. Filtered cells were then inspected by epifluorescence microcope (Olympus BX 60, Olympus Corporation) under fluorescent light using 100 x magnification. The cells were counted by taking images of the microscopic views with a camera connected to the microsope-computer. 20 different randomly chosen microscope viewscreen images were recorded, and the cells were counted on each viewscreen (Figure 4). The number of cells in the sample was then calculated on the basis of the magnification factor, filtered volume and the surface area of the filter used.

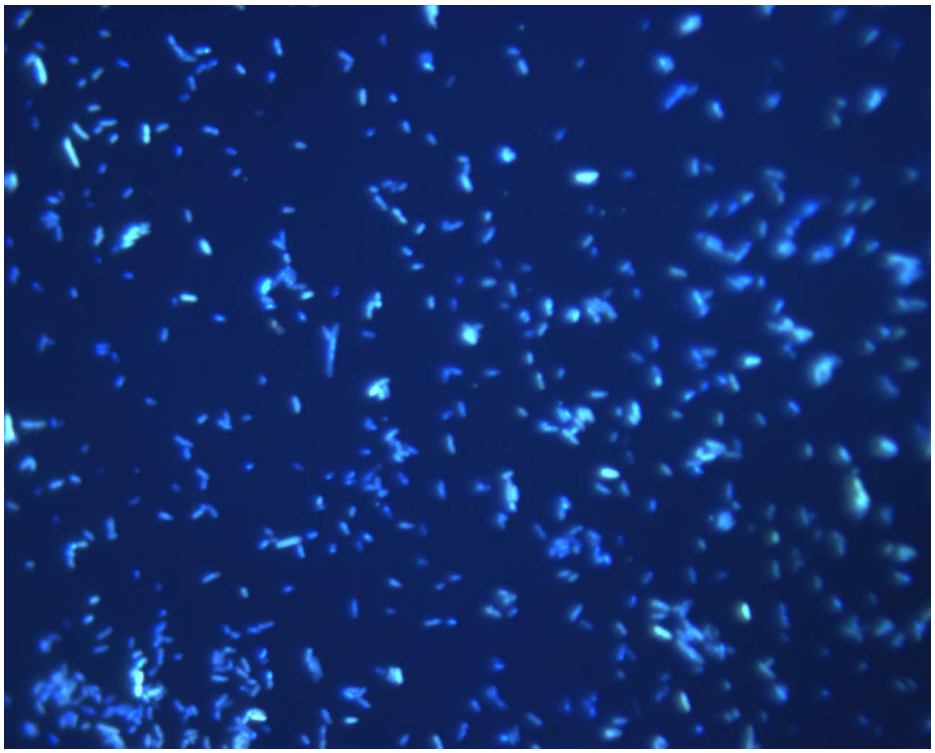


Figure 4. *DAPI stained cells. The cells are visualized as blue fluorescent dots. The cells in the picture are Eschericia coli cells used to prepare the internal standard for qPCR.*

The results were calculated according to the formula

B = (N*Af)/(d* Af *G*Ag)

Where
B = number of cells/ml
N = total number of cells counted
Af = functional area of the filter (283.53 mm^2)
D = dilution factor
Af = volume of the filtered sample
G = number of viewscreens counted
Ag = area of the viewscreen (0.0201 mm^2 at 100 x magnification)

Another staining method was used to visualize the proportions of living and dead cells in the groundwater. The commercial Live/Dead staining kit (BacLight Bacterial Viability Kit, Molecular Probes) includes two different fluorescent stains. The SYTO®9 green-fluorescent stain stains both living and dead bacteria. The red-fluorescent propidium iodide dye enters only cells with a ruptured cell wall, and reduces the SYTO®9 fluorescence when both dyes are present. Thus, a combination of these two dyes makes it possible to distinguish between living cells with intact cell walls and dead cells (Figure 5).

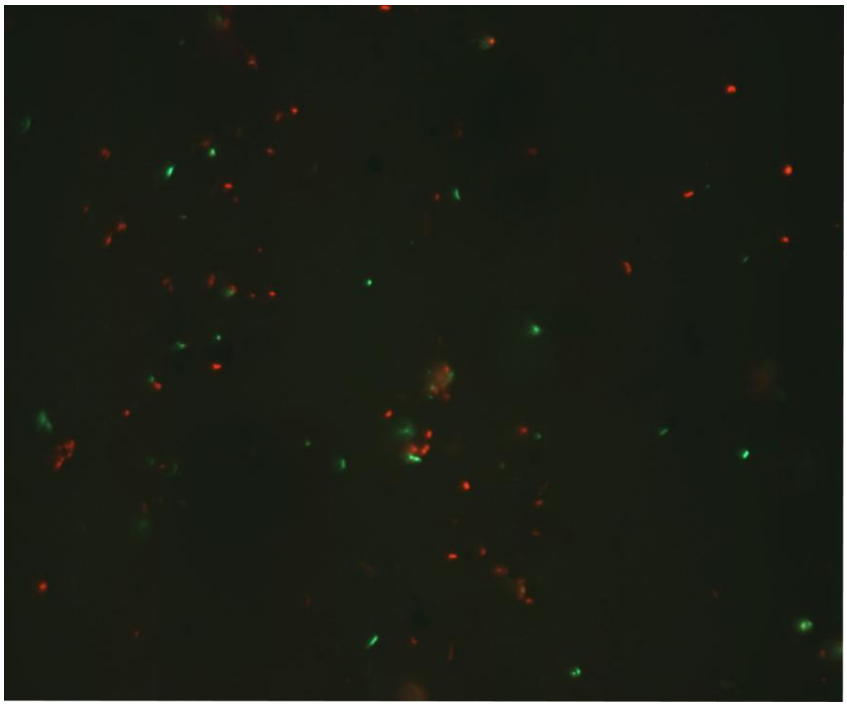


Figure 5. *Live/dead staining of the microbial cells. The red dots are dead micro-organisms and the green coloured ones are alive. The sample is from a pumping water sample (832) taken at Palmottu, Nummi-Pusula.*

3.3.2 Cultivation

E. coli cells cloned with IgMorph gene for preparation of the internal standard were cultivated on LB broth with *ampicillin* for six hours at 37^{o}C. Bacterial growth was subsequently stopped by cooling the nutrient broth on ice, and dividing the broth into smaller units.

3.4 Molecular methods for detecting micro-organisms

3.4.1 DNA extraction and isolation

Prior to nucleic acid extraction the microbial biomass in the water samples was concentrated by filtering or centrifugation and stored at -80^{o}C until DNA extraction. Total DNA was extracted by using the PowerSoil® DNA Isolation Kit (Mo Bio Laboratories, Inc.), in which cell lysis occurs mechanically and chemically, and the released genomic DNA is captured and washed on a silica membrane. The total DNA eluted from the membrane was used in the qPCR analysis.

3.4.2 External standard curve for quantification of sulphate reducers

A 1.9 kb fragment of the *dsrAB* gene from a sulphate-reducing bacterium, *Desulfobacterium autotrophicum*, was used to prepare a plasmid dilution series and a standard curve for estimating the quantity of sulphate reducers in the sample. The *dsrAB* gene fragment was PCR amplified from the genomic DNA of *Desulfobacterium autotrophicum* with forward primer DSR1F (5'-acscactggaagcacg-3') and reverse primer DSR4R (5'-gtgtagcagttaccgca-3').

The gene was cloned into a plasmid vector pCR® 2.1-TOPO® (Invitrogen) and the plasmid obtained was named pK5DSRAB (Figure 6). The success of transferring the gene to the plasmid was verified by sequencing.

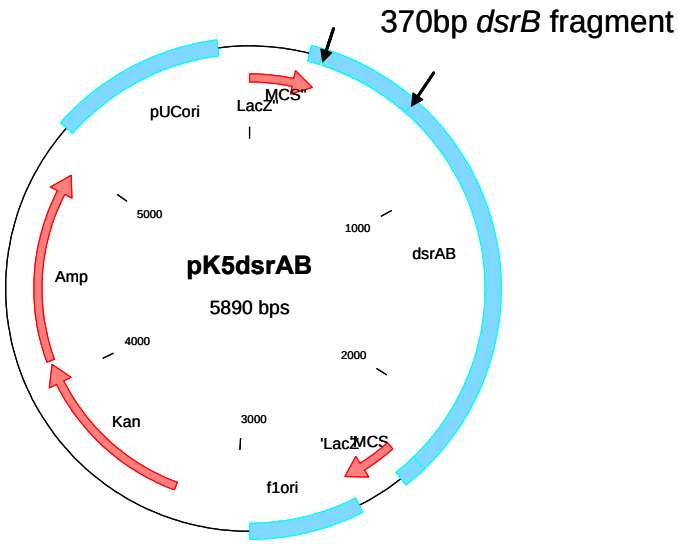


Figure 6. *Plasmid pK5DSRab. The 370bp fragment of the dsrB gene is shown between the arrows.*

3.4.3 qPCR method development

The qPCR method is based on amplification of a 370 bp fragment of the *dsrB* gene in the presence of a fluorescent dye that binds to double-stranded DNA. The *dsrB* gene fragment encodes the β subunit of the dissimilatory sulphite reductase enzyme.

The *dsrB* specific primers were forward primer DSRp2060F (5'-caacatcgtycayacccaggg-3'), and reverse primer DRS4R (5'-gtgtagcagttaccgca-3') (Foti et al., 2007). These primers amplify about 370bp fragment portion of the *dsrB* gene.

Quantification of the *dsrB* copy number in the extracted DNA was performed in a LightCycler™ (Roche) capillary-based instrument. The amplification was done with DyNAmo™ Capillary SYBR® Green qPCR Kit (Finnzymes). SYBR Green I is a fluorophore dye that binds specifically to double-stranded DNA and shows enhanced fluorescence when bound. The amplification was performed over 37 to 45 cycles, with one cycle consisting of denaturation (10s at 95°C), annealing (20s at 55°C) and

extension (20s at 72^{o}C). The amplification reaction (20 µl) contained 1X SYBR® Green master mix, *dsrB* specific primers at a final concentration of 0.5 µM each, 2µl of template DNA and ddH_2O. The no template control (ntc) reaction contained all the reagents for amplification but no template DNA.

3.4.4 Melting curve analysis of the qPCR end products

A melting curve analysis was performed after each qPCR run in order to check the specificity of the amplified product.

When the temperature is gradually increased, there is a sharp decrease in SYBR Green I fluorescence as the PCR product undergoes denaturation at a characteristic melting temperature (Tm). The melting point of the product depends mainly on the base composition and length of the DNA, and therefore specific products can be distinguished from the non-specific products by the difference in their melting temperatures.

3.4.5 Agarose gel electrophoresis of the qPCR end products

The size of the qPCR end products were examined electrophoretically on 12 % agarose gels (SeaKem® LE Agarose, Lonza) in Tris-acetae buffer.

3.4.6 Internal standard- DNA extraction efficiency and PCR inhibition

The internal standard was developed for estimating the DNA extraction efficiency and PCR inhibition in the samples. The standard gene can be any gene that is not present in micro-organisms because standardization is based on quantifying the standard gene separately. The internal standard is added as a whole cell preparation to the sample prior to DNA extraction. After the DNA extraction, it is possible to estimate the loss of DNA in the extraction procedures by quantifying the standard gene in the samples by quantitative PCR.

The internal standard was prepared by cloning the IgMorph gene into DH5α *E. coli* cells. The clone was then cultivated in a liquid broth until the desired cell number had been reached. The cells were harvested in the exponential growth phase. The medium was then divided into 30 µl volumes in 200 tubes.

The cell number in the internal standard was approximated by enumerating the cells by means of DAPI staining. DAPI staining is described in Chapter 3.3.1. DNA from the internal standard was extracted with the same method as will be used to extract DNA from environmental samples, and the IgMorph gene in the content was quantified from the extract by SYBR green qPCR in order to determine how much IgMorph gene the standard solution contained. The gene content of the internal standard was compared to a known amount of the same gene in the plasmid dilution series. A work flow of the preparation and characterisation of the internal standard is presented in Figure 7.

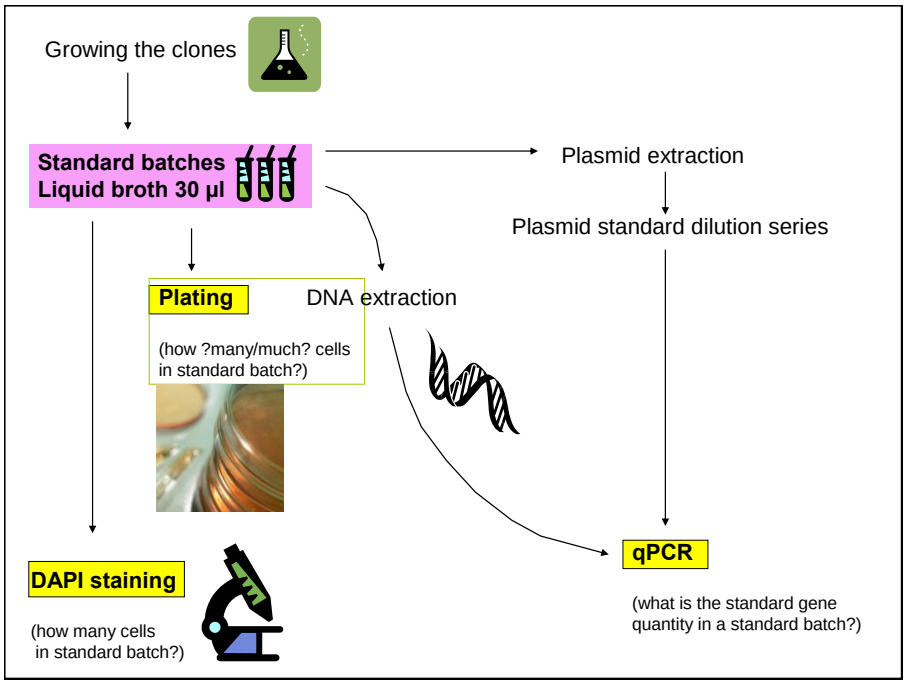


Figure 7. *Procedure used for quantifying the IgMorph gene copy number in the internal standard liquid batch (30 µl) consisting of E. coli cells.*

The feasibility of the internal standard with the groundwater sample was tested by filtering the water, as earlier described, on a sterile 0.2 µm filter to concentrate the microbial cells. The filter was then cut into two halves: the internal standard batch was added to one half, and the DNA was extracted from both halves. The filter halves and the IqMorph content were quantitated by qPCR.

DNA loss + PCR inhibition in extraction procedures = EXT_{sample} /EXT

where EXT = the IgMorph content of a native DNA extraction standard

EXT_{sample} = the IgMorph content of the DNA extraction standard co-extracted with the sample

This calculation gives the percentage of IgMorph gene recovered from the sample after the DNA extraction and PCR steps. The same percentage affects all the genes in the sample and therefore, for example, when SRB genes are examined in the same sample this percentage must be taken into account for the results to be accurate.

4 RESULTS

4.1 Physico- chemical characterization of groundwater samples

4.1.1 The results of the groundwater analysis are presented in Appendix 1.

The pH of the samples was alkaline and varied from 7.5 to 8.6. Electrical conductivity of the sample OL-KR8 /556.1-561 m was 10 times higher than in the other samples. The temperature was between 9.0-10.8^{o}C in all the samples. A low temperature generally slows down microbial processes.

There was some variation between the inorganic carbon concentrations of the samples. The dissolved inorganic carbon (DIC) concentration was the highest (53.2 mg/l) in ONKALO sample ONK-PVA3/83 m. Shallow groundwater typically has high DIC concentrations, which indicate higher microbial activity during infiltration through the organic soil zone. As the shallow groundwater is mixed with more saline groundwater during flow, the sample may also contain microbial populations typical of overburden and deep bedrock conditions in the vicinity of the research tunnel. In contrast, DIC was even below the detection limit in the OL-KR8/556.1-561 m drillhole. This may be due to a high Ca concentration (4500 mg/l), which buffers the DIC concentration to a low level due to the precipitation of DIC as calcite in order to maintain equilibrium in the groundwater system.

Dissolved organic carbon (DOC) is an important parameter when studying micro-organisms, because it is an energy source as well as source of nitrogen compounds. A suitable carbon to nitrogen (C/N) ratio for microbial growth is generally considered to be between 10 and 40. However, nitrogen fixation may occur through the action of micro-organisms capable of performing autotrophic metabolic processes, and of assimilating dissolved inorganic carbon into organic compounds.

Oxygen was detected in small quantities in the OL-KR42/175-179 m and OL-KR43/96-102 m drillholes, but in very large quantities in OL-KR8/556.1-561 m. Oxygen is the first electron acceptor used in metabolic processes, followed by nitrate and nitrite after the oxygen has been depleted. Nitrite was below the detection level. The redox values were very low, clearly indicating anaerobic conditions.
OL-KR42/175-179 m contained 545 mg/l SO_4 but no detectable amounts of sulphide, while OL-KR8/556.1-561 m contained 0.11 mg/l sulphide and the SO_4 concentration was below the detection limit. Comparison between these results and the redox values show that the oxygen measurement must be an error caused by contamination during sampling. In addition, the measurements indicate that microbial sulphate reduction may also be taking place in the sampling section of drillhole OL-KR8/556.1-561 m.

In the future, these data should be compared to the microbial data based on *dsrB* quantitation in order to confirm that the geochemical processes have been identified correctly.

4.2 Number of micro-organisms in the groundwater samples

The number of micro-organisms based on staining and microscopy in the Palmottu samples was around 1.15-4.6 x10^5 cells/ml, with a viability from 40% to 73%.

The microbial density in the Olkiluoto groundwater samples was very similar (varying from 0.9-7 x 10^5), but according to the viability kit the number of viable cells was much lower (Table 2).

Table 2. *The viability of micro-organisms in Olkiluoto samples was as follows:*

	viable/dead cells
ONK-PVA3/83 m	36/64
OL-KR8/556.1-561 m	53/47
OL-KR42/175-179 m	23/77
OL-KR43/96-102 m	36/64

4.3 Development of the qPCR method for SRBs

4.3.1 External standard in evaluation of the quantity of sulphate reducing bacteria

An external standard is needed in order to obtain an absolute value for an unknown concentration of target DNA. For the quantification to be accurate, the amplified standard sequence should be homologous to the target sequence. The ideal control for this purpose would be a genomic DNA of any sulphate-reducing micro-organism containing the *dsrAB* operon. However, as they are anaerobic micro-organisms, it is very time-consuming to maintain SRBs and to culture them for DNA isolation. In contrast, the plasmid DNA can be easily purified in large quantities. In this work the plasmid pK5dsrAB (Figure 6) was constructed. It contains the qPCR target sequence, *dsrB*, within a l.9 kb portion of the whole *dsrAB* operon. The standards and samples were amplified in separate capillaries, but within the same qPCR run on each occasion.

The standard curve generated in this study is shown in Figure 8. The relationship between the crossing point values and the log amount of target DNA was linear over five orders of magnitude, ranging from 3.7x10^2 gene copies to 3.7x10^6 gene copies of target gene fragment. The no-template control sample (ntc) gave a fluorescence signal only after 37 cycles of amplification.

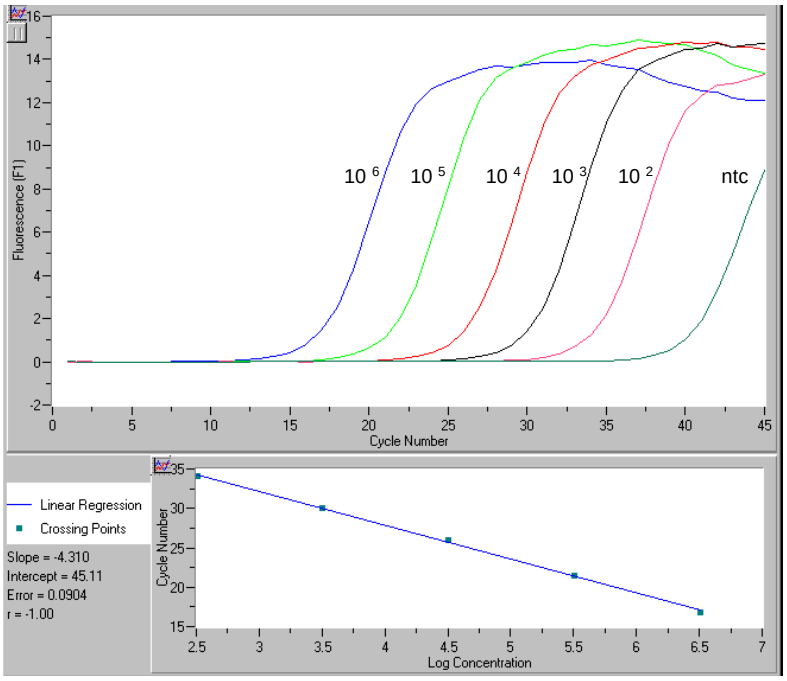


Figure 8. *Standard curve generated from a dilution series of the plasmid pK5dsrAB. Crossing cycle numbers (crossing points) are plotted versus the logarithm of the concentration of each sample. The slope of the curve is related to the efficiency of the PCR reaction. The qPCR amplification curves are shown above. (ntc = no template control).*

A number of qPCR runs were first carried out in order to identify the final analysis conditions. The optimal crossing point value (Cp) for DNA working template dilutions should be 10-30. The annealing temperature and sample dilutions mainly were tested (results not shown). This was obtained by using the undiluted or 1:10 diluted samples. Non-inhibiting reaction conditions for PCR were assumed when the amplification curves of the samples had the same shape as the standards and, when diluted, the DNA extract led to a proportional increase in the Cp.

4.3.2 qPCR method for *dsrB* detection

A quantitative PCR method has been published for both *dsrA* (Chin et al., 2007) and *dsrB* fragments (Foti et al., 2007), which detect the *dsrAB* gene from different ends.
The fluorescent dye SYBR Green I binds to any double-stranded DNA, which means that the specific product, non-specific products and primer-dimers are detected equally well. Primer dimers are the product of non-specific annealing and primer elongation events, and this may lead to reduced amplification efficiency and a less successful PCR. Melting curve analysis is one way to check the specificity of an amplified qPCR product, and the analysis is typically included in the analysis software of qPCR instruments. The melting curve analysis was first performed on the amplification products of the SRB pure cultures (Figure 9). A characteristic melting temperature peak corresponds to each SRB sample, and they are distinct from the primer-dimer peak which is formed only in the ntc-control sample. The primer-dimer fraction is already denatured at temperatures above 80^{o}C, whereas the lowest melting temperature of the

SRB sample, *Desulfobacterium autotrophicum*, is 85.9°C. A single distinct melting peak was also generated when the mixture of the four DNAs was used as a PCR template.

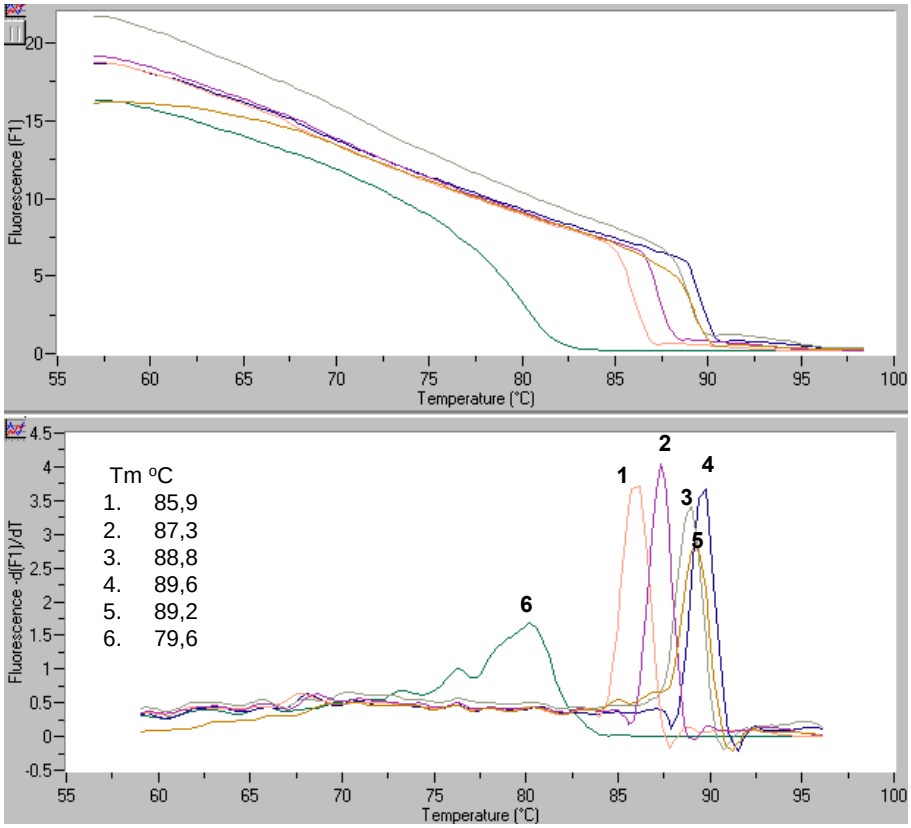


Figure 9. *Melting analysis of qPCR products amplified from total DNAs isolated from the SRB pure cultures, 1. Desulfobacterium autotrophicum 2. Desulfobulbus propionicus 3. Desulfosarcina variabilis 4. Desulfovibrio vulgaris 5. Mixture of the four DNAs. 6. No template control (ntc). The melting temperature for each sample is shown.*

A similar melting analysis was also performed on the end products of the environmental samples (Figures 10, 11. and 12.). In contrast to the SRB pure cultures, the decrease in fluorescence in the environmental samples was not as sharp. This is probably caused by the different melting points of the individual sequences within the PCR product. A specific melting peak was obtained for five samples (Palmottu 832, 833, 834, 835 and 842, see Table 1, page 18) corresponding to a melting temperature of approximately 90°C (Figure 10). This temperature is similar to the melting temperatures obtained for the SRB pure culture DNAs (Figure 9). The melting curves of Palmottu samples 843, 844 (Figure 10) and Olkiluoto sample 865 (ONK-PVA3/83 m) did not show a distinct peak (Figure 14). Mispriming or priming at a secondary site cannot be excluded, even though only one main product was seen in the agarose gel (Figure 13). The analysis shows that the fluorescence signal obtained from Palmottu samples 1 and 2 is unspecific. The melting curves showed no sharp change and, thus, no distinct melting temperature was observed. Primer-dimer formation was observed in sample 2. The importance of melting analysis is emphasized in the case of samples 2 and 834 that gave a similar quantification result (Table 3.), but had entirely different melting curves.

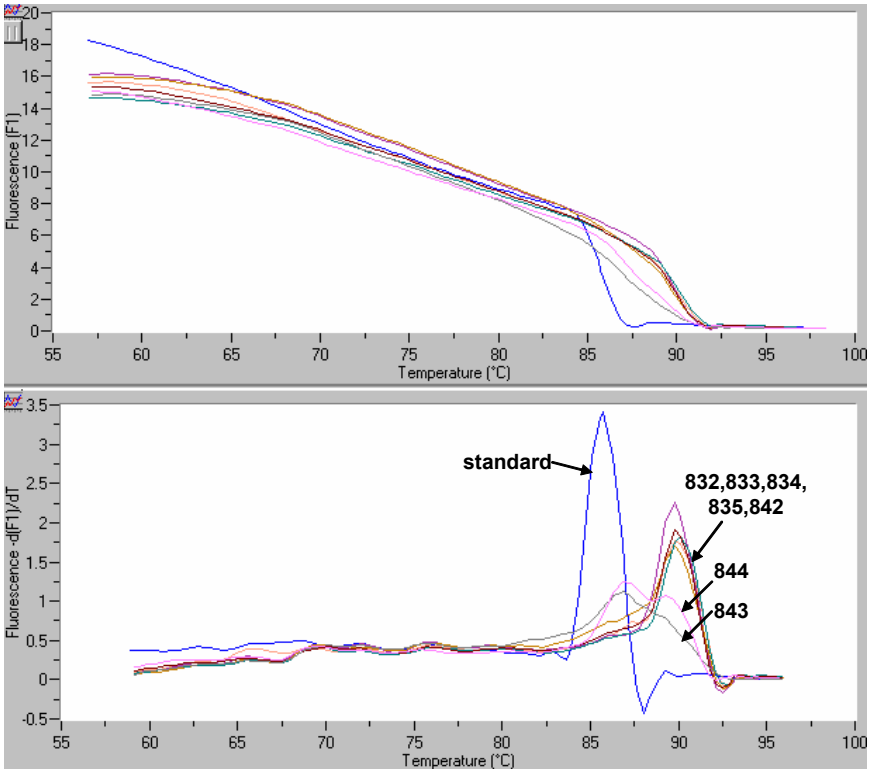


Figure 10. *Results of the melting curve analysis on the Palmottu samples. See Table 1, page 18 for details of the samples.*

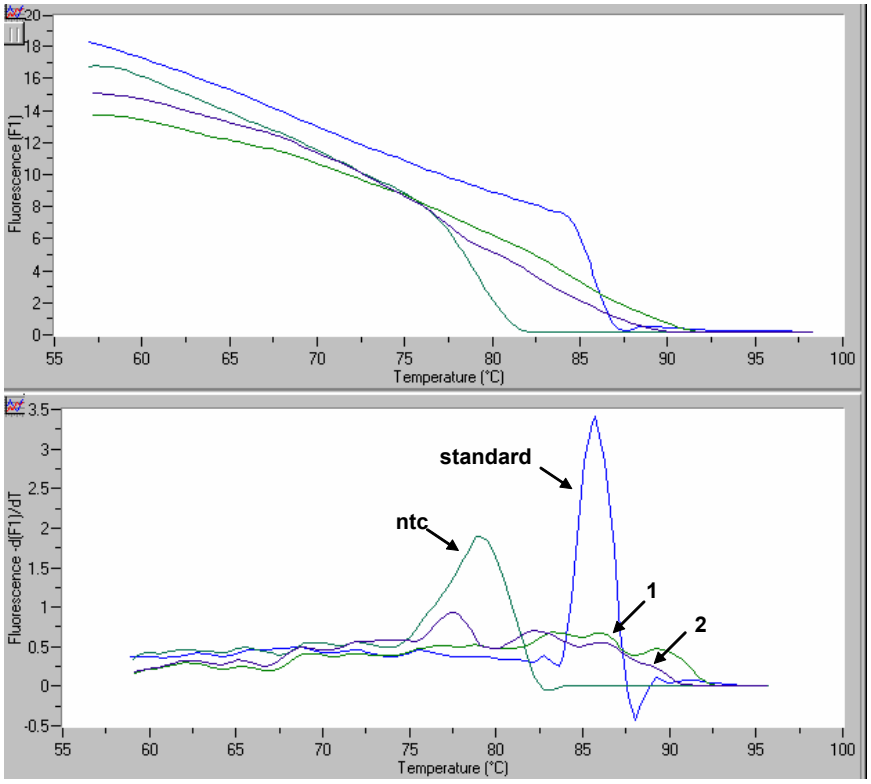


Figure 11. *Results of the melting analysis on Palmottu contamination samples 1 and 2. See Table 1, page 18 for details of the samples.*

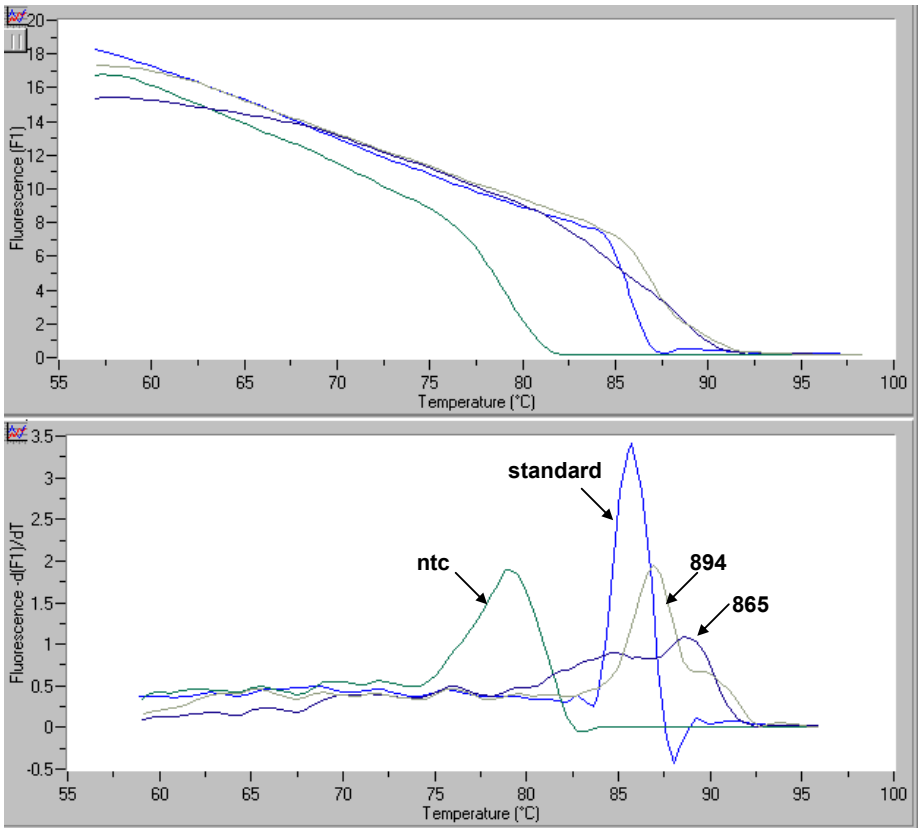


Figure 12. *Results of the melting curve analysis on the Olkiluoto samples. 865 = ONK-PVA3/83 m, 894 = OL-KR8/556.1-561 m.*

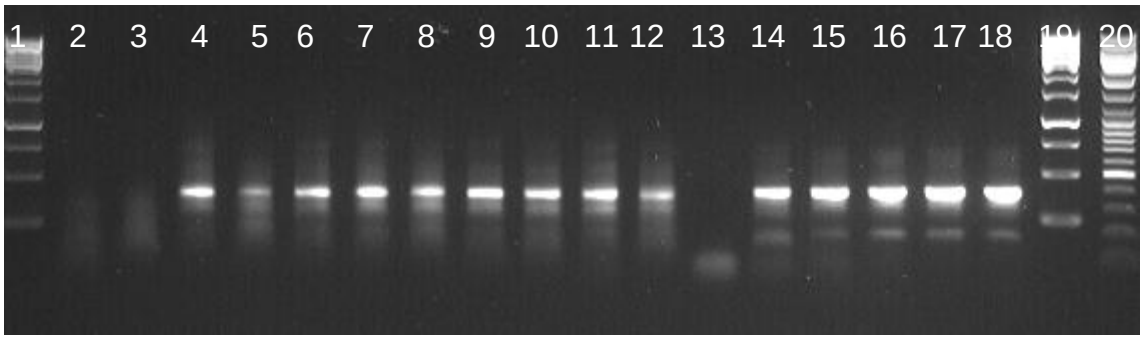


Figure 13. *Agarose gel electrophoresis of qPCR end products. Lanes 1, 19 and 20: molecular weight markers; lanes 2-12: Palmottu samples 2, 1, 844, 843, 842, 835, 834, 833, 832, Olkiluoto samples OL-KR8/556.1-561 m (894), ONK-PVA3/83 m (865); lane 13: no template control; lanes 14-18: standards 102, 103, 104, 105, 106.*

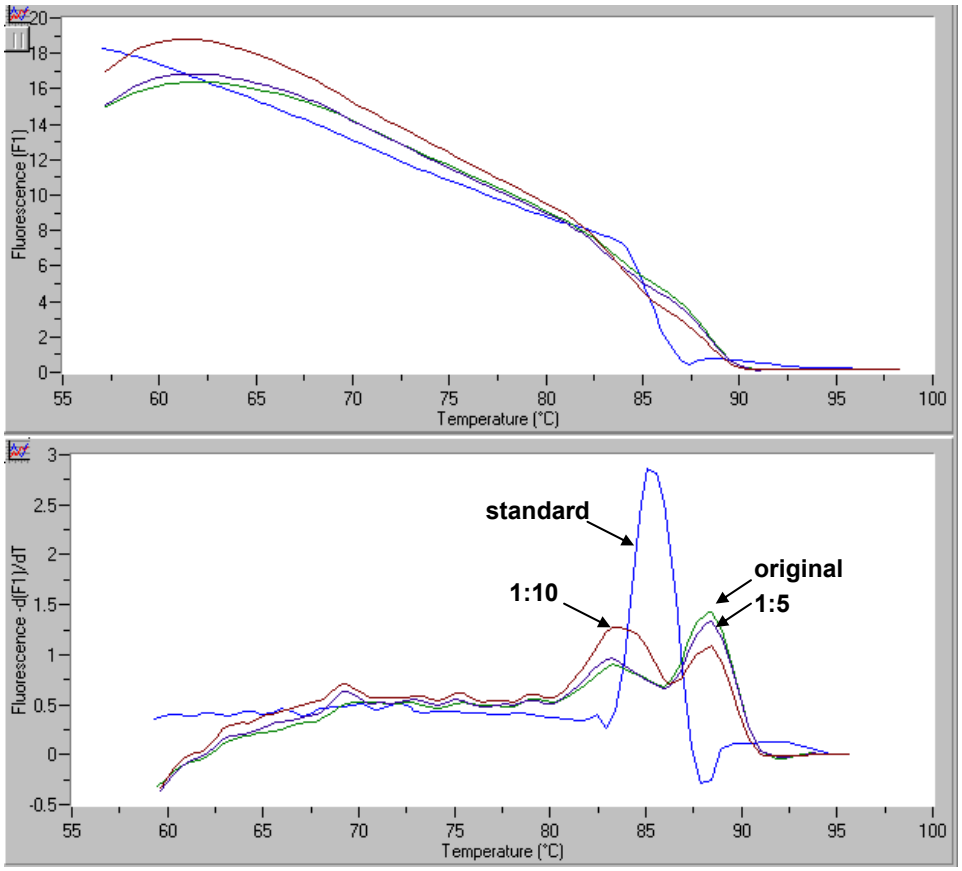


Figure 14. *A melting curve analysis of the Olkiluoto sample ONK-PVA3/83 m (865).*

4.3.3 Sulphate reducers in the groundwater samples

In order to get comparative information about the presence of sulphate reducers in the groundwaters the samples from Palmottu site was investigated. The pumping water from the Palmottu Nummi-Pusula drillhole 837 (samples 832-835) contained a large number of sulphate-reducing micro-organisms (Table 3). The reason for the abundance of sulphate reducers in the Palmottu borehole water is probably due to contamination by surface water containing large amounts of organic matter. The Palmottu site has been extensively investigated in several EU projects, and geophysical, geochemical and geomicrobiological studies have been carried out earlier. Based on measurements performed in the field, the environmental conditions have changed completely since the time of the earlier measurements.

Table 3. *Quantification of the sulphate-reducing micro-organisms. Samples 832-844 represent water samples from the Palmottu site: 832 = 20-26m, 833 = packer sample 20-26 m, 834-842 = pumping water, 843 = 110 m pumping water, 844 = 170 m packer sample, Samples: 1 = drillhole opening, 2 = river water, 4 = sampling tube, 7 = sampling tube. Olkiluoto groundwater samples 865 = ONK-PVA3/83 m, 894 =OL-KR8/556.1-561 m, 914=OL-KR42/175-179 m.*

sample		volume ml	consentration method	Cp	Gene copies /ml	Tm ºC
Palmottu	832	10	centifugation	25,5	1,8 x 105	89,8
	833	10	centifugation	27,2	7 x 104	89,7
	834	10	centifugation	29,2	1,6 x 104	89,1
	834	50	filtration	28,1	8,9 x 103	89,6
	835	10	centifugation	27,1	4,7 x 104	89,8
	835	50	filtration	26,4	2,1 x 104	90,1
	842	100	filtration	23,8	4,4 x 104	89,8
	843	100	filtration	20,3	4,1 x 103	86,7
	844	100	filtration	22,7	7,8 x 104	87,6
	1	-	swab	29,5	4,2 x 103	-
	2	10	filtration	30,0	1,6 x 104	-
	4	-	swab	-	no result	-
	7	-	swab	-	no result	-
Olkiluoto	ONK-PVA3/ 83 m	200	filtration	27,1	3,8 x 103	88,3
	OL-KR8/556.1-561 m	35	filtration	30,6	2,5 x 103	86,9
	OL-KR42/175-179 m	100	filtration	26,5	2 x 102	89,8

In order to evaluate the possibility of contamination during sampling the drillholes, several samples were taken to investigate the presence of microbial communities in environmental samples taken near to the drillhole. The river located in the vicinity of the drillhole contained considerably higher numbers of sulphate reducers (1.6×10^4 gene copies/ml) than the samples taken from the surface of the sampling tube. The drillhole opening also contained small numbers of SRBs.

All the Olkiluoto samples ONK-PVA3/83 m, OL-KR8/556.1-561 m, OL-KR42/175-179 m, OL-KR43/96-102m contained a very small number of SRBs, which was

demonstrated as the low copy numbers 2 x 10^3 to 3.8 x 10^3 *dsrB* gene involved in sulphate reduction.

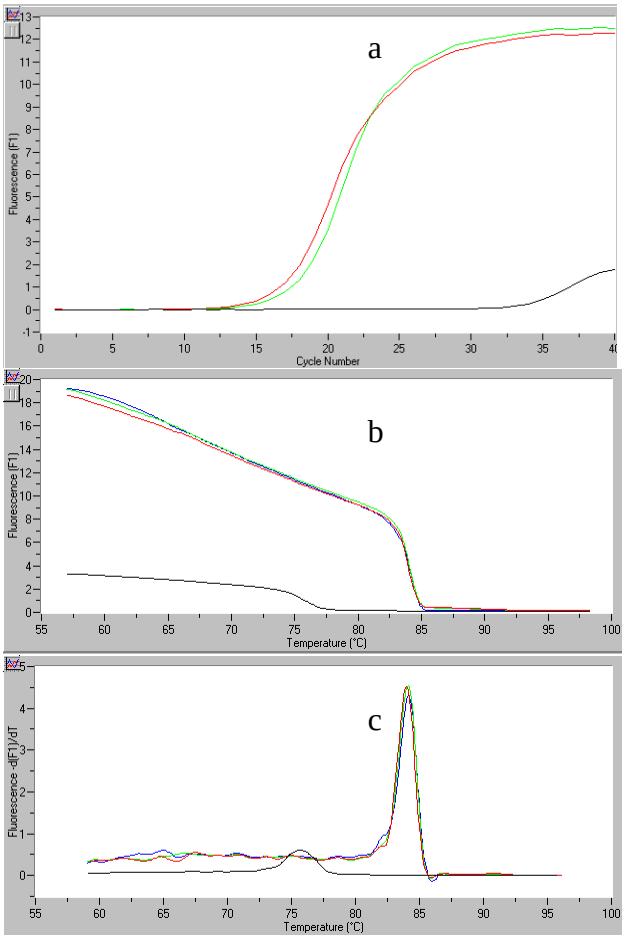
The crossing point values obtained showed that the runs were reproducible. However, most of the water samples studied in the present work have been treated in a different way because they were taken from Palmottu studies performed earlier. Some of the samples were filtered, or centrifuged and different amount of water was concentrated for DNA extraction (Table 3.). The sample volumes varied from 10 ml to 200 ml, which may affect the final result due to the variation in DNA extraction efficiency. Therefore the quantitative results cannot be compared reliably to each other.

In future studies it will be important to standardize the amount of water used for qPCR and the DNA extraction efficiency in order to obtain reproducible results. The use of different pre-treatments for concentrating the biomass probably impairs the efficiency of DNA –extraction, and thus also the accuracy of the final quantification, e.g. sample 834 and 835 (DNA extractions from larger sample volumes gave smaller quantifications).

4.3.4 Development of internal standard to study DNA extraction efficiency and PCR inhibition

The internal standard solution (*E. coli* cells containing IgMorph gene) was developed in this project to validate the DNA extraction efficiency and to estimate qPCR inhibition which might affect the final quantitative results. The internal standard is a batch of *E. coli* cells cloned with IgMorph gene. During standardization, one internal standard batch is added to the sample prior to DNA extraction and the standard gene quantity (IgMorph) is studied by qPCR after DNA extraction. Comparison between the standard IgMorph gene number and the IgMorph number after DNA extraction and PCR steps makes it possible to evaluate the loss of DNA during extraction and PCR inhibition.

Prior to standardization, qPCR was used to determine the IgMorph gene number in an individual internal standard batch (Figure 17). The IgMorph gene number in one standard batch was 1.87 x 10^8 copies. According to the DAPI staining method, the cell concentration in a single standard batch was 2.37 x 10^7 cells/ml. This means that there were approximately five copies of the standard gene IgMorph in one *E. coli* cell.



Sample	Copy number
qPCR copy number standard	4,34*106
IgMorph gene copies from internal standard in 2 µl sample	7,446*106
No Template Control	4,457*102

Figure 15. *Real-time PCR quantification of the Igmorph gene content in the internal standard batch. The red line illustrates the DNA extracted from the internal standard batch. The green line is a plasmid dilution containing 4.34 x 10^6 copies of the gene IgMorph, and was used as one of the quantification references in qPCR. The black line is the negative control. a) Amplification curves. b-c) Melting curve analysis.*

The internal standard was then tested with a groundwater sample. A batch of the internal standard was added to one half of sample ONK-PVA3/83 m. After DNA extraction from both the ONK-PVA3/83 m + standard and ONK-PVA3/83 m, the IgMorph gene content of both the standard-added and "water only" samples was quantified by SYBR Green qPCR (Figure 16).

a b

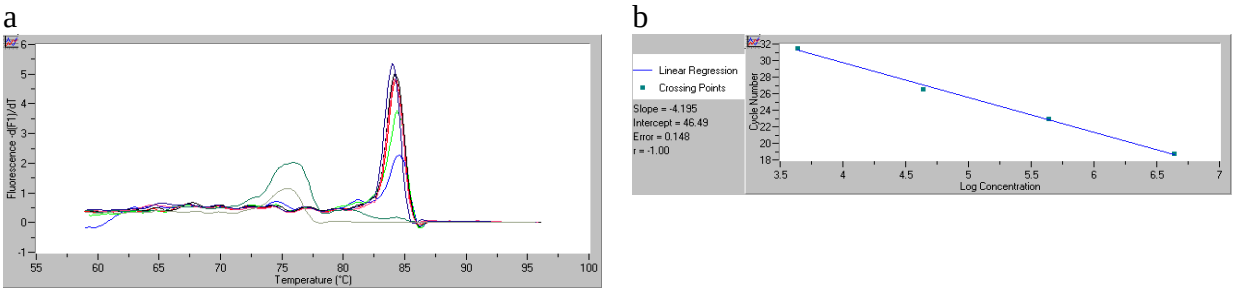


Figure 16. *qPCR quantification of Igmorph gene in the ONK-PVA3/83 m + standard (dark green line) and ONK-PVA3/83 m(dark blue line) samples. No sample was added to the negative control (grey line). The Igmorph gene content was quantified based on: a dilution series of the internal standard (other lines). a) amplification curves and standard curve and b) melting curve analysis.*

The filtered groundwater sample ONK-PVA3/83 m + internal standard contained 6.02 x 10^6 copies of IgMorph in 2 µl, which means that 7.9 x 10^8 copies of the gene were recovered from the internal standard. Because the calculated IgMorph gene number in the standard batch was 5.75 x 10^6, it can be concluded that there was no loss of DNA during DNA isolation and no PCR inhibition in the sample.

Figure 15b illustrates the melting analysis of qPCR products. There is a characteristic melting temperature peak for IgMorph gene at +84ºC in the ONK-PVA3/83 m + standard sample and no specific peak in ONK-PVA3/83 m where no standard was added, demonstrating that the test run for the internal standard was successful. However, the negative control (grey line) and ONK-PVA3/83 m without added standard (dark green line) have another peak at +75ºC. +75ºC is the temperature at which the primers used in the reaction melt which indicates that the quantification result that is obtained from these samples (1.66 x 10^4 gene copies) is a product of primer dimer formation and not contamination. In order to minimize the effect of primer dimer formation on quantification, the PCR reaction will be optimized for groundwater samples in the near future. When the quantification results are not interfered by primer dimer formation, the internal standard can be used to calculate the efficiency of DNA extraction and PCR inhibition.

It is very important to estimate the loss of DNA and PCR inhibition when conducting quantitative analyses, because different environmental samples behave differently in the PCR reaction. When quantitative analyses are conducted, it is necessary to differentiate true differences between the samples from false results produced by different efficiencies in the extraction methods. The standard is also used to make different samples comparable with each other. When the DNA loss and inhibition rates for each separate sample are known, there are no false results concerning the amount of gene numbers, because the differences brought on by different conditions that result in PCR inhibition can be corrected.

5 CONCLUDING DISCUSSION

The subsurface geochemical and microbiological characterization of the drillholes has been ongoing since 1996 at Olkiluoto (Pitkänen et al., 2004, Pedersen, 2008)

The earlier sampling campaigns have demonstrated the presence of a significant amount of dissolved gases and variable amounts of water in the PAVE pressurized sampling device used in sampling microbes from deep groundwater (Gascoyne, 2000). In the present work the PAVE devices contained variable amounts of water and gases. When designing experiments for qPCR, the sample amounts have to be adjusted to the same volume before filtration for quantitative analysis in order to avoid errors when extracting DNA. There may be variation in the DNA extraction efficiency and this should be taken into account.

Contamination is also of special concern because, in the qPCR studies based on DNA, even very small amounts of DNA originating from dead cells can be replicated. Pedersen tested the sterilizing efficiency of the pumps and tubing by ATP measurements, and detected only very small numbers of microorganims (Pedersen, 2008). ATP is a valid method for analyzing cultivable micro-organisms in aerobic conditions. In PCR work, however, this method does not evaluate the DNA left in the tubings. Therefore, if possible, a zero sample should be taken from the sampling device before installing it in the drillhole. This sample should then be subjected to the same DNA extraction method on the same volume as the sample taken from the drillhole.

The qPCR method allows the detection and quantification of sulphate-reducing micro-organisms based on sulphite reductase gene detection and quantification. Optimally, even rare sequences can be increased to assayable concentrations by the polymerase chain reaction. However, the high sensitivity of qPCR assays may be compromised in one or more of the following steps: sampling, sample preparation and storage, nucleic acid isolation, target amplification, detection of amplicon, and data analysis.

Accurate quantification of specific microbial populations is strongly dependent on the quality and yield of DNA extracted from the environmental sample. Therefore careful collection and handling of the samples is of fundamental importance. To avoid the degradation of nucleic acids the samples should be kept in the cold, and the extraction performed as soon as possible after sampling. Sample volumes should be as equal as possible in order to normalize the DNA extraction procedure between samples.
Commercially available kits optimised for the isolation of genomic DNA from environmental samples are desirable due to the high throughput and elimination of contaminants that can inhibit the enzyme reactions used in PCR. A standardized extraction method will further reduce the differences in cell lysis, DNA binding to the purification matrix and DNA elution, which may lead to variation between samples.

Standardisation of DNA extraction efficiency is critical when working with quantitative methods. The PCR efficiency of the samples can be determined by preparing a dilution series of the sample DNA. However, this may not be possible due to the low DNA concentrations. One option to control the variability between samples is to use a heterologous marker gene.

At its best

1. The genome of the recombinant bacterial strain does not contain the qPCR target gene.
2. The marker gene is not inherently contained in the genomes of the recombinant strain and the strains in the environmental sample.
3. The cell wall structure of the recombinant strain is similar as possible to the qPCR target organisms in the environmental sample

The environmental samples can be spiked with a recombinant bacterial strain harbouring the marker gene that can be detected in PCR by using specific primers. The reproducibility of the DNA extraction between samples could be checked by following the behaviour of the marker gene during the extraction procedure. Parallel samples can be reliably compared, since the step prone to produce the most variation can be reliably evaluated.

In the present project we developed a marker gene based method, which we called the internal standard. The method is based on the Igmorph gene which was transferred into an *E. coli* plasmid. This internal standard method will be used for this purpose when comparing deep subsurface samples taken from different sites and depths and at different times. While the test run has shown that our method is easy to use and fully functional, some further testing will have to be done to overcome primer dimer formation. The primer concentrations and sample volumes will be optimised to produce reliable results. The gene content of the internal standard will also be measured with another gene quantification method in order to reaffirm the qPCR results.

As no single method can fully describe a microbial community, the qPCR results must be combined with other data obtained from the environment, e.g. geochemical information.

Both active and inactive micro-organisms contain DNA, but active ones contain proportionally more RNA. In the future it is desirable to apply the qPCR technique to community RNA, as well as to community DNA, in order to address the active members of the microbial community. Unlike RNA, DNA derived from dead organisms can persist extracellularly for a long time due to the absorption of DNA on clay minerals. This RT-qPCR technique is, however, much more challenging compared to the DNA-based qPCR, and the viability of micro-organisms is a prerequisite for the success of RNA-based assays.

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APPENDICES

Appendix 1. Physico-chemical analysis of the groundwater samples.

Date	17.1.2008	3.12.2007	2.10.2007	19.6.2007
Sample nr Depth m	OL-KR43 96-102	OL-KR42 175-179	OL-KR8/ 556.1-561	ONK- PVA3/83
sampling date	25.2.2008	27.11.2007	24.9.2007	4.6.2007
TDS mg/l	860	7862	21992	1259
Alcalinity meq/l	3.94	1.17	0.11	4.64
Oxygen PPb	0.7	0.1	11.10	
pH	8.6	7.48	8.10	8
mS/cm	1.29	13.07	35.310	1.98
EmvAu	-285	-381	-335	
EmvPT	-351	-386	-342	
temperatu-re ^{o}C	9.6	10.8	9.0	8.1
DIC mg C/l	41.3	13.7	<0.3	53.2
DOC mg C/l	6.3	5.50	13.1	9.5
S_2 mg/l	0.03	<0.01	0.11	< 0.1
$S0_4$ mg/l	69	545	<0.1	86.8
PO_4		<0.1	<0.1	< 0.1
NO_2, N03	<0.1	<0.03	<0.1	< 0.1
tot N mg/l	0.18	0.39	0.55	0.35
NH_4 mg/l	0.063	0.075	0.02	0.335
Cl	260	4390	13600	323
Fe_2+mg/l	0.05	0.18	0.02	0.04
Na mg/l	250	1920	3700	276
K mg/l	6.47	11	9.3	8.83
Ca mg/l	15.3	690	4500	31.3
Mg mg/l	5.11	200	22	12.1
Mn mg/l	0.035	0.55	0.23	0.07
CH_4 ml/l	0.127	x	230	x