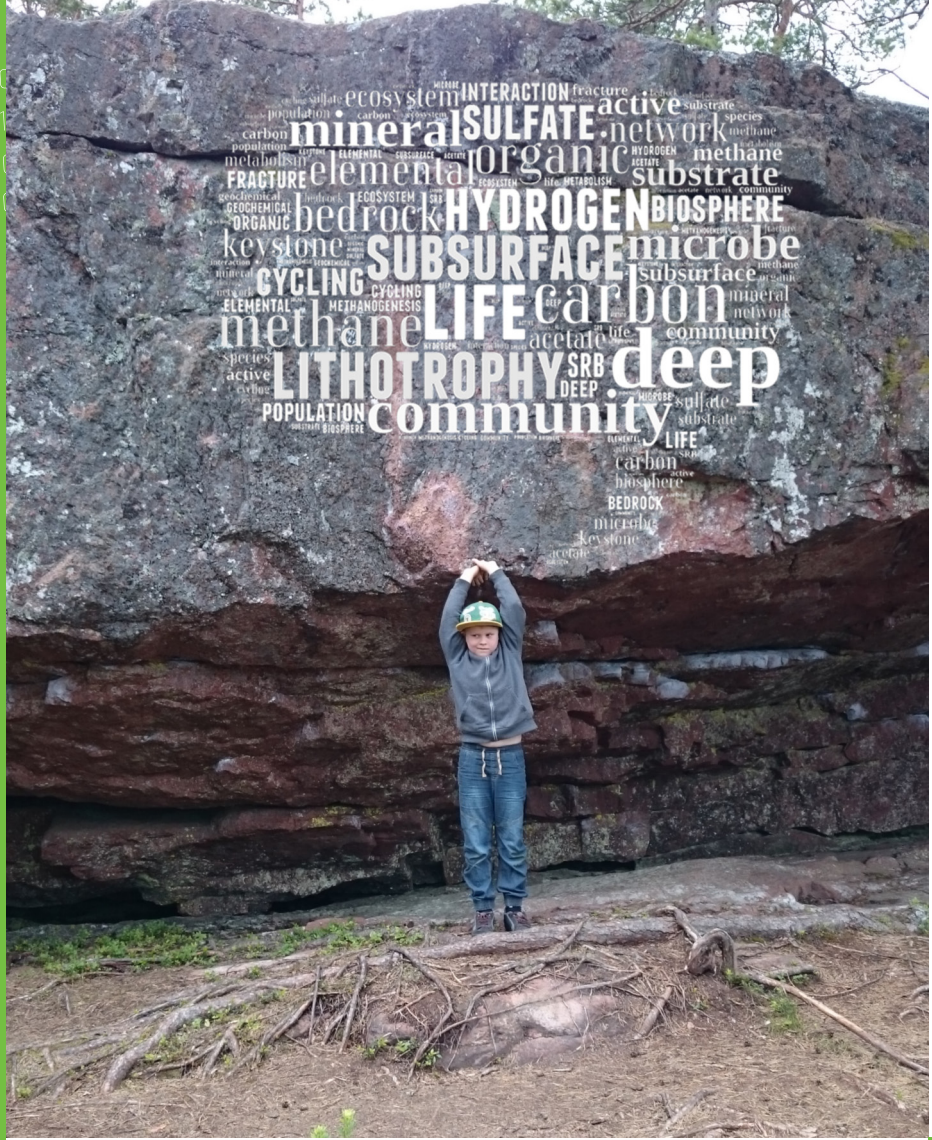

SCIENCE • TECHNOLOGY • RESEARCH HIGHLIGHTS • VISIONS

Dissertation
116

Microbial ecology and functionality in deep Fennoscandian crystalline bedrock biosphere

Lotta Purkamo

VTT SCIENCE 116

Microbial ecology and functionality in deep Fennoscandian crystalline bedrock biosphere

Lotta Purkamo

VTT Technical Research Centre of Finland Ltd

Thesis for the degree of Doctor of Science to be presented with due permission of the Faculty of Agriculture and Forestry of the University of Helsinki, for public examination and criticism in Auditorium 2041 at Viikki Biocenter 2 on the 18th of December 2015, at 12 noon.

ISBN 978-951-38-8370-6 (Soft back ed.)
ISBN 978-951-38-8371-3 (URL: http://www.vttresearch.com/impact/publications)

VTT Science 116

ISSN-L 2242-119X
ISSN 2242-119X (Print)
ISSN 2242-1203 (Online)
http://urn.fi/URN:ISBN:978-951-38-8371-3

JULKAISIJA – UTGIVARE – PUBLISHER

Teknologian tutkimuskeskus VTT Oy
PL 1000 (Tekniikantie 4 A, Espoo)
02044 VTT
Puh. 020 722 111, faksi 020 722 7001

Teknologiska forskningscentralen VTT Ab
PB 1000 (Teknikvägen 4 A, Esbo)
FI-02044 VTT
Tfn +358 20 722 111, telefax +358 20 722 7001

VTT Technical Research Centre of Finland Ltd
P.O. Box 1000 (Tekniikantie 4 A, Espoo)
FI-02044 VTT, Finland
Tel. +358 20 722 111, fax +358 20 722 7001

Cover image: Front cover photo and word cloud art by Lotta Purkamo and Niina Saari

Juvenes Print, Tampere 2015

Preface

This thesis consists of studies conducted at the VTT Technical Research Centre of Finland Ltd during 2009–2015. The research projects were funded by the Academy of Finland, Finnish Research Programme for Nuclear Waste Management (KYT), Foundation for Research of Natural Resources in Finland and Kone Foundation. University of Helsinki's Dissertation completion grant enabled the final effort in composing the thesis.

I cordially thank Professor Anu Kaukovirta-Norja and Dr. Tuulamari Helaja for providing excellent research facilities at VTT, and our team leader Päivi Kinnunen for support and encouragement during the years.

I thank my whole advisory group, Merja Itävaara, Ilmo Kukkonen, Lasse Ahonen, Malin Bomberg and Mari Nyyssönen for introducing me to the world of science and for the excellent scientific collaboration. I sincerely thank my primary supervisor, Docent Merja Itävaara for the opportunity to contribute to this intriguing field of research and the autonomy in planning, carrying out and reporting my research. My genuine appreciation goes to Dr. Ilmo Kukkonen, who has encouraged me and believed in my skills during the whole process. I warmly thank Dr. Lasse Ahonen for the excellent collaboration and conversations during the years that we have worked together, both in the field and in the office. My warmest gratitude goes to Dr. Malin Bomberg, who tirelessly pushed me towards this goal. Sharing her insights, expertise, knowledge and time unselfishly with me is invaluable. Her kindness and friendship during these years have helped me to believe in myself and accomplish this stage in my scientific career. I warmly thank Dr. Mari Nyyssönen for the time and effort she has provided in discussing, encouraging and especially reviewing the articles and the thesis.

I thank Prof. Kaarina Sivonen for her positive encouragement, patience and guidance during the preparation of the thesis. I'm grateful for Prof. Martin Romantschuk and Prof. Jaak Truu for particularly fast review process and their valuable comments for improving the quality of this thesis.

All co-authors and collaborators are warmly acknowledged for their contribution. A special thanks my peers Riikka Kietäväinen, with whom I have had pleasure to learn, write and travel together, Maija Nuppunen-Puputti, friend, neighbor and a fellow PhD student in joy and struggle and Elina Sohlberg for always helpful attitude and keeping calm in all situations. I also would like to acknowledge Mirva

Pyrhönen for her efficient and skillful technical work that she does in the laboratory. I warmly thank and acknowledge all the past and present co-workers in VTT: Pauliina, Hanna, Minna, Heikki, Anna, Leea, Tiina, Heini, Ilkka, Vertti among others. I would also like to thank my peers in other institutes, such as Paula Niinikoski, Stiina Rasimus, Aura Nousiainen, Anna Reunamo and Kirsti Leinonen with whom I have shared this period of pursuing the PhD.

I'm indebted to my friends in the neighborhood and farther away for showing interest on my research, taking care of the kids, sharing the time at the sandbox and playground, emptying my mind from science-related matters and dragging me along to do physical exercise once in a while. Special thanks goes to Niina the ultimate Word-wizard, Regine for the language correction and Saara for just being awesome.

I'm deeply grateful to my family: my dearest mum Sisko and dad Markku, you have transferred your interest in nature and appreciation of science early on to me. Heli and Elli, I have the best sisters in the world. You bring happiness, laughter, comfort and support to my life. Last but not least, my dearest and most loving thanks to my guys. Sami, my soulmate, my best friend, thanks for walking this journey of life side by side with me. I highly appreciate your ability to keep this circus running and thank you for your (almost) endless patience with my mishaps. Vili and Masi, world is full of wonders and you are among those. I thank you for your unconditional love and just for being who you are.

As the note from fictional icelandic alchemist Arne Saknussem said in Jules Verne's book *A journey to the Centre of the Earth*: "Descend, bold traveller, to the centre of the earth. I did it."

Kirkkonummi, November 2015
Lotta Purkamo

Academic dissertation

Supervisors	Merja Itävaara VTT Technical Research Centre of Finland Ltd
	Ilmo Kukkonen Department of Physics, University of Helsinki
	Malin Blomberg VTT Technical Research Centre of Finland Ltd
	Lasse Ahonen Geological Survey of Finland
	Mari Nyyssönen VTT Technical Research Centre of Finland Ltd
Custos	Prof. Kaarina Sivonen The Department of Food and Environmental Sciences, University of Helsinki
Reviewers	Professor Martin Romantschuk The Department of Environmental Sciences, University of Helsinki
	Professor Jaak Truu Department of Geography, University of Tartu
Opponent	Professor Karsten Pedersen Microbial Analytics Sweden

List of publications

This thesis is based on the following publications, which are referred in the text as Article I, Article II and Article III.

I Purkamo L, Bomberg M, Nyyssönen M, Kukkonen I, Ahonen L, Kietäväinen R, Itävaara M. Dissecting the deep biosphere: retrieving authentic microbial communities from packer-isolated deep crystalline bedrock fracture zones. *FEMS Microbiol Ecol.* 2013, 85(2):324–37. doi: 10.1111/1574-6941.12126.

II Purkamo L, Bomberg M, Nyyssönen M, Kukkonen I, Ahonen L, Itävaara M. Heterotrophic communities supplied by ancient organic carbon predominate in deep Fennoscandian bedrock fluids. *Microb Ecol*. 2015, 69(2):319–32. doi:10.1007/s00248-014-0490-6.

III Purkamo L, Bomberg M, Kietäväinen R, Salavirta H, Nyyssönen M, Nuppunen-Puputti M, Ahonen L, Kukkonen I, Itävaara M. The keystone species of Precambrian deep bedrock biosphere belong to *Burkholderiales* and *Clostridiales.* Accepted to *Biogeosciences Discussions*.

Author's contributions

I Lotta Purkamo took part in planning of the experiments and collecting the samples. She carried out molecular biological work and took part in microscopy analyses. She did the cluster analysis of DGGE and sequence analyses, interpreted the results, wrote the paper and is the corresponding author.

II Lotta Purkamo took part in planning the experiments and participated in the molecular biological analyses. She carried out sequence and statistical analyses, interpreted the results and wrote the paper. She is the corresponding author.

III Lotta Purkamo planned the experiment, took part in sampling and laboratory work, carried out the community analyses, bioinformatics and network analysis. She interpreted the results, wrote the paper and is the corresponding author.

Supporting publications

Kietäväinen, R and Purkamo L. The origin, source, and cycling of methane in deep crystalline rock biosphere. *Front. Microbiol.* 2015. (6) 725. doi.org/10.3389/fmicb. 2015.00725

Contents

List of abbreviations

ANME	anaerobic methane oxidizing archaea
ATP	adenosine triphosphate
CCA	canonical correspondence analysis
CRB	Columbia River Basalts
DAPI	4'-6-diamidino-2-phenylindole
DGGE	denaturing gradient gel electrophoresis
DNA	deoxyribonucleic acid
FID	flame ionization detector
FTT	Fischer-Tropsch type
Ga	Giga-annuum
HTP	high throughput (sequencing)
ICP-MS	inductively coupled plasma mass spectrometry
ICP-OES	inductively coupled plasma optical emission spectrometry
MPN	most probable number
MQ	Milli–Q (ultrapure water)
NSTI	nearest sequenced taxon index
PCR	polymerase chain reaction
PLFA	phospholipid fatty acid
qPCR	quantitative polymerase chain reaction
RNA	ribonucleic acid
RO	reverse osmosis
rRNA	ribosomal ribonucleic acid

SLiME	subsurface lithotrophic microbial ecosystem
SMTZ	sulfate-methane transition zone
SRB	sulfate reducing bacteria
TCD	thermal conductivity detector
TDS	total dissolved solids
TEA	terminal electron acceptor
WGA	whole genome amplification

1. Introduction

1.1 The deep biosphere

The deep biosphere can be defined as **an ecosystem of organisms and their living space** extending from several tens of meters to several kilometers in the Earth's crust (Hoehler and Jørgensen 2013). Thomas Gold (1992) suggested that if the estimated pore space of the land areas down to 5 km depth of the Earth's crust would be filled with water and 1% of this volume would be microbial biomass, this biomass would be sufficient to cover Earth's land surface with a 1.5 m thick layer. The anthropogenic use of deep terrestrial subsurface as a source of material, energy, or storage space has triggered the motivation to study the deep biosphere of these environments. These operations include oil and hydrocarbon recovery and storing of the crude or refined materials of these processes, CO_2 sequestration, traditional mining activities as well as modern biohydrometallurgical processes, geothermal heat and energy facilities and nuclear waste repositories (e.g. Pedersen 1996, Stroes-Gascoine and West 1997, Head et al. 2003, Mitchell et al. 2008, Das et al. 2011, Nyyssönen et al. 2012, Gniese et al. 2014, Wouters et al. 2013). Microbiological risks for both the financial profitability of these operations and for the environmental safety where such operations are conducted are present. Petroleum reservoirs are susceptible for microbial biodegradation of crude oil, resulting in alteration of the hydrocarbon composition of the reservoir and excess methane production (Jones et al. 2008). A major risk for many operations is microbially induced corrosion (Javaherdashti 2011, Gniese et al. 2014, Lerm et al. 2013). Sulfate-reducing bacteria are key organisms acting in microbially induced corrosion processes, and thus have been under study also in many deep subsurface studies that are aiming to characterize microbial risks of for example underground storage of gas and nuclear wastes (e.g. Pedersen et al. 2008, Pedersen 2012a,b, Nyyssönen et al. 2012, Gniese et al. 2014, Rajala et al. 2015). Another severe challenge for example in geothermal energy operations is scaling of the equipment surfaces induced by thick corrosion products or insoluble minerals. Usually scaling becomes more severe as temperature decreases in the system (Valdez et al. 2009, Lerm et al. 2013).

However, some technologies may harness the microbial power for economical profit and restoration of contaminated environments. The adverse effect of micro-

bial biodegradation of for example crude oil can be useful for bioremediation purposes (eg. Scow and Hicks 2005, Meckenstock et al. 2015), it may also provide a way of recovering energy from oil fields with environmentally less harmful technologies, in form of methane (Jones et al. 2008). Uranium or heavy metal - contaminated mining sites can be bioremediated by the help of sulfate reducers by precipitation of metal sulfides through sulfate reduction (Lloyd and Lovley 2001). The in-situ mining of low-grade ores exploiting the known microbial processes in controlled conditions can be utilized to extract valuable metals (Das et al. 2011).

Beyond the anthropogenic point of view of economical utilization of the deep subsurface and understanding of the microbial risks, deep biosphere studies will provide a window to the past and even to the other worlds. The deep subsurface may have been the only refuge for life during the early history of Earth when meteoric impacts have sterilized the surface regularly (Cockell et al. 2012). In addition, understanding diversity, richness and functionality of the microbial communities in life-sustaining environments on Earth facilitate our ability to interpreting the possibility of life on other planetary bodies (Amend and Teske 2005).

The first attempt to evaluate the magnitude of the biomass in Earth's deep biosphere was made almost two decades ago by Whitman et al. (1998). They suggested that the deep subsurface would host 3.8×10^{30} microbial cells or 5×10^{17} g of carbon in microbial biomass. The recent reassessment of the amount of biomass in deep continental biosphere supported the early estimates. The biomass of the deep continental biosphere can be up to 19% of the total biomass on Earth, and is equal or even somewhat greater than the biomass of the deep marine subsurface (Hinrichs and Inagaki 2012, Kallmeyer et al. 2012, McMahon and Parnell 2014).

1.2 Environmental conditions affecting the existence of life in deep subsurface

Ultimately four factors must be met for microbial colonization of a certain environment: space, water, suitable temperature and material for energy and cellular building blocks. Habitat volume for organisms in the deep biosphere depends on the geological history of the subsurface. Sedimentary rocks are considered more porous than igneous rocks. However, igneous or metamorphic crystalline rocks can be fractured and shattered, thus rubble and fracture zones provide substantial living space for deep subsurface microbial communities (Stevens 1997).

Water is commonly present in the subsurface in pores, fissures and in larger fracture zones, and is not limiting microbial life. However, the very large solid rock vs. water ratio is limiting the living space of microbes in deep biosphere (Pedersen 2000). In shallower depths, water circulates slowly and mixing with the meteoric water is likely to happen, but with decreasing hydraulic conductivity deeper in a rock formation, water becomes stagnant resulting in very long residence times (Holland et al. 2013, Kietäväinen et al. 2014).

While water availability is not the limiting factor for life in the deep subsurface, the temperature of the environment may be. The current estimate for temperature limit of life is 122 °C and the record holder is a hyperthermophilic archaeon *Methanopyrus kandleri* (Takai et al. 2008). The depth range, where temperature exceeds this limit, is however very broad. It can vary from the surface of the sea floor at the tectonic plate boundaries or hydrothermal fields to depth of 10 km and even deeper in stable sedimentary rock formations (Pedersen 2000). To date, the deepest continental site where bacteria have been detected is in Gravberg, Sweden, where glucose-fermenting thermophilic bacteria originating from a depth of over 5.2 km were isolated (Szewzyk et al. 1994).

CHNOPS – Carbon, hydrogen, nitrogen, oxygen, phosphorus and sulfur are the elements of life, the Lego blocks that all living organisms are made of. Carbon forms the chemical backbone of all organic compounds and the versatility of forming single, double or triple bonds, long chains, flexible ring structures and stable complex molecules makes carbon so special. Three vital components of a cell are based on carbon chains: DNA, membranes and proteins (Cockell and Nixon, 2013). Deep bedrock environments are commonly oligotrophic due to their spatial isolation and the usually nonexistent connection to meteoric water cycle (Lovley and Chapelle 1995). If deep subsurface fractures are connected with water cycle on the surface and shallow groundwater systems, the dissolved organic carbon is typically consumed already in the shallow depths leaving only the most recalcitrant material left for the deep ecosystems (Kotelnikova 2002). Therefore, life in the deep subsurface is defined by the scarcity of photosynthetically produced energy and carbon and other environmental factors, such as low concentration or absence of oxygen, pH extremes, high salinity and pressure.

In addition to above mentioned, life in the deep subsurface has to be adapted to other extreme conditions. Extremophiles, i.e. microbes living in extreme environments, have developed numerous mechanisms coping with nutrient deprivation and limited energy availability in addition to high pressure, temperature, extreme alkaline or acidic conditions and even metal toxicity and radioactivity (Pikuta et al. 2007).

1.3 Energetics in the deep biosphere

The primary energy source in the deep biosphere is proposed to be geochemical. This is obvious, because several deep subsurface systems appear to be completely detached from the ultimate source of energy on the surface, namely sunlight (Gold 1992, Amend and Teske 2005, Colwell and D'Hondt 2013). The concept of the thermodynamic tower or ladder, where electron accepting-processes are arranged hierarchically from most energy yielding towards less energetic processes has been used in many environmental studies to describe the microbial zonation of for example pristine and contaminated aquifers (Christensen et al. 1994, Lovley and Chapelle 1995, Meckenstock et al. 2015). However, ecological and physiological factors control the distribution of microbial life in the deep sub-

surface in addition the thermodynamic ladder (Bethke et al. 2011). Aerobic respiration, in which photosynthetically produced oxygen is the terminal electron acceptor (TEA), is the most energy-yielding process. Although oxygen can diffuse to some extent to the subsurface, it is most likely consumed quickly as it enters the subsurface (Lovley and Chapelle 1995). Yet, oxygen may be produced in deep crustal surroundings during radiolysis of H_2O in addition to H_2 and thus may facilitate aerobic growth of microbes in deep systems (Pedersen 2000, Lin et al. 2005a,b). Nevertheless, the deep biosphere is mostly dependent on other terminal electron acceptors in lower rungs of the thermodynamic ladder, including nitrate, iron, manganese, sulfate and carbon compounds.

Energy acquisition in deep, anoxygenic subsurface can be based on anaerobic respiration and fermentation. In anaerobic respiration, an organism uses inorganic or organic compounds other than oxygen as electron acceptors and ATP is produced via proton motive force in a process called oxidative phosphorylation. This is called chemotrophy as the electron donors and acceptors are chemical, in contrast to phototrophy, where light is the source of energy. Chemotrophs are divided further into two groups. Chemoorganotrophs use organic compounds as electron donors and can utilize the same material for biosynthesis, while chemolithotrophs can only use inorganic compounds as their electron donors and thus, have to gain their carbon for biosynthesis elsewhere. Autotrophic carbon fixation is characteristic to chemolithotrophs, meaning that carbon is assimilated in the form of CO_2 and thus these organisms are sometimes referred as chemolithoautotrophs (Kim and Gadd 2008).

Fermentation is another anaerobic process, where cells use organic material for production of energy and biosynthesis. Substrate variety for fermentation is wide, ranging from carbohydrates to organic acids, amino acids and nucleic acid bases. ATP is produced via substrate-level phosphorylation in contrast to anaerobic respiration. Fermentation is energetically in the lower rungs of the thermodynamic ladder as it occurs at very low redox potentials (e.g. Amend and Teske 2005). Organic matter fermenters enable the growth of other microbial groups by providing acetate or CO_2 to chemoorganotrophs and chemolithotrophs, respectively. H_2 is produced in many fermentation processes and is a prominent electron donor for chemolithotrophs in the deep subsurface. However, if the partial pressure of H_2 exceeds a certain level, fermentation will be inhibited. Thus, in subsurface, radiolytically formed hydrogen (see next paragraph) can inhibit fermentative growth (Lin et al. 2005b).

Because organic compounds for fermentation and anaerobic respiration are scarcely available in deep crystalline bedrock environments, chemolithotrophy is traditionally considered to be the prevailing metabolism and CO_2 the predominant source of carbon in deep bedrock (Fredrickson et al. 1997, Fredrickson and Balkwill 2006, McCollom and Amend 2005). In chemolithoautotrophy, both the energy source and the electron sink i.e. the TEA is inorganic and biomass is produced from inorganic carbon (e.g. Stevens 1997). As mentioned, a useful source of reducing power for chemotrophic microbes is H_2, which is abundantly available in many crystalline bedrock systems (e.g. Pedersen 1997, 2000, Nealson et al. 2005,

Sherwood Lollar et al. 2014, Kietäväinen et al. 2014). Thus, H_2 is considered the sole energy source for photosynthesis-independent hydrogen-driven lithotrophic microbial ecosystems (SLiMEs) in deep subsurface (Amend and Teske 2005, McCollom and Amend 2005, Nealson et al. 2005). Geochemical hydrogen can be produced in deep terrestrial bedrock via abiotic water-mineral interactions, such as serpentinization occurring in ultramafic rock systems or by dissociation of water molecules with the energy released in radioactive decay, or can be derived from deeper, hotter parts of the crust via gas flux (e.g. Pedersen 2000, Nealson et al. 2005, Lin et al. 2005a,b, McCollom and Bach 2009). In addition, some microbes are able to mediate H_2 production from minerals, thus securing a continuous supply of energy for themselves (Parkes et al. 2011).

However, it should be noted that the theoretic energetic potential is not usually applicable as in natural systems. Therefore it would be useful to weigh the energetics in reference to the availability of reactants in order to provide a more realistic view of the potential energy metabolisms in the deep biosphere (Osburn et al. 2014).

Finally, the growth rates in deep subsurface are extremely low. Due to the extremely low flux of energy and nutrients, estimates on generation time reach 1000 years (Jørgensen and D'Hondt 2006). Thus, microbial communities in deep subsurface are likely adapted to the low energy flux and prefer to use such mechanisms in their cell metabolism that save energy (Hoehler and Jørgensen, 2013). Recently, a minimum flux of energy, i.e. power requirement for a cell to remain viable was estimated to be 1×10^{-15} μW $cell^{-1}$ (LaRowe and Amend 2015).

1.4 Geochemical sources of carbon

On the surface of the Earth, most of the primary production relies on photosynthesis, in which carbon dioxide from the atmosphere is converted into sugars by photoautotrophs, such as green plants. After the postulation of the so-called hydrogen-driven deep biosphere (Gold 1992), several studies have suggested that autotrophic H_2-utilizing microbes are responsible for primary production in the deep subsurface (Stevens and McKinley 1995, Pedersen 1997, 2000). These autotrophic chemolithotrophs are using carbon dioxide found usually in dissolved form as HCO_3^- in deep groundwater (Frape et al. 2013) (**Table 1**). Carbon dioxide is formed in the mantle or in lower crust from where it is outgassed to shallower depths of the crust during various geological processes, such as metamorphism, mountain building and volcanic activities (Kerrick 2001) (**Figure 1**).

However, primary production in deep biosphere has also been proposed to rely on organic molecules synthetized abiotically in geochemical processes from CO_2 and H_2 (Amend and Teske 2005, Schrenk et al. 2013). During serpentinization of ophiolitic rocks, water interacts with ferrous iron-rich minerals of ultramafic rocks and H_2 is produced (McCollom and Bach 2009). Serpentinization provides a continuous flux of H_2 resulting in a highly reducing environment where methane, C_2+ hydrocarbons and minor amounts of organic acids are formed from CO_2

(McCollom and Seewald 2001, Lang et al. 2010, Russell et al. 2010, Szponar et al. 2013) (**Figure 1**). While many chemolithotrophs can use H_2 as an energy source, H_2 produced during serpentinization enhances the reduction of CO_2 to methane and other organic molecules. Therefore, serpentinization may enable also chemoorganotrophic microbial life (Russell et al. 2010, Schrenk et al. 2013).

In addition, small alkanes and alkenes are typically formed in laboratory experiments of Fischer-Tropsch type (FTT) synthesis (Taran et al. 2007, McCollom et al. 2010, Zhang et al. 2013). Proskurowski et al. (2008) demonstrated that synthesis of small hydrocarbons is possible in FTT reactions *in situ* in natural hydrothermal environment supplied by ultramafic crust. Moreover, methane and other light hydrocarbons can be produced abiotically in either high temperature (> 500 °C) reactions in the mantle as well as in gas-water-rock reactions in the crust, at lower temperatures (25–500 °C) (Etiope and Sherwood Lollar 2013, McCollom 2013). These organic carbon molecules are prominent carbon and energy source for heterotrophs, which have been discovered in many deep crystalline rock sites (see Section 1.7).

Microbial communities in rock formations can also be fuelled by ancient photosynthetically produced organic carbon preserved into minerals such as black shale (Krumholz et al. 1997, Petsch et al. 2001). Interlayers of metamorphosed black schist can contain refractory organic material also in Outokumpu (Taran et al. 2011), and thus provide one plausible carbon source for the deep bedrock biosphere.

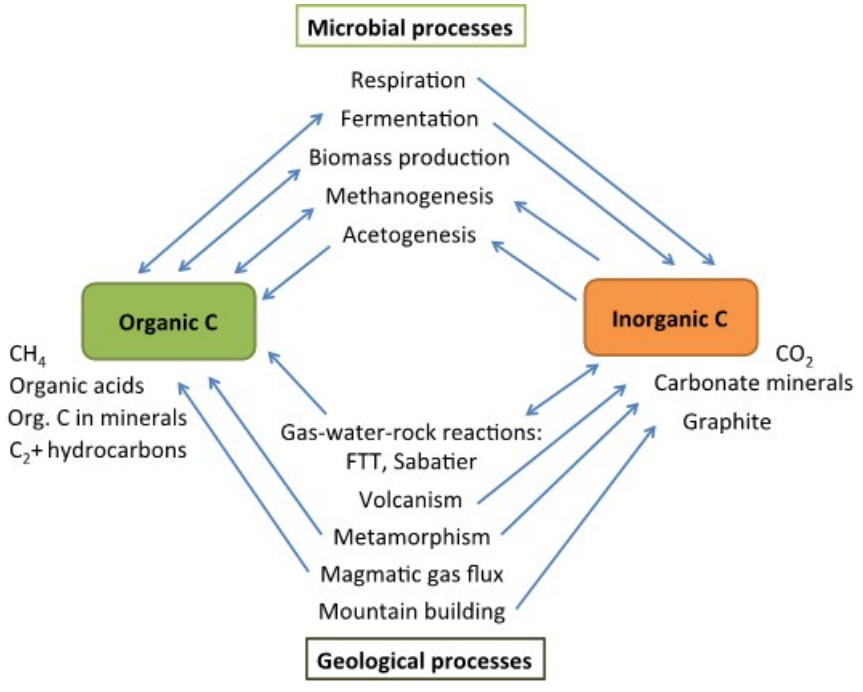

Figure 1. The geological and biological sources and cycling of carbon in deep crystalline bedrock.

1.5 Microbial ecology in the deep subsurface

As the amount of microbial biomass in the subsurface is significant, these microbes likely have functional potential beyond our current knowledge. Therefore, microbial ecology studies are needed for understanding and possible subsequent harnessing of this functionality to our benefit. Additionally, these studies enable the microbial risk assessment of many technological applications using the deep bedrock. Moreover, the microbial biomass in the deep subsurface presumably has a significant role in global elemental cycling.

Microbial ecology research focuses on diversity, interactions and activity of microbes in their living environment. The diversity of microbial communities in deep terrestrial subsurface varies from single or few-species ecosystems to communities with significant diversity (Tyson et al. 2004, Chivian et al. 2008, Wouters et al. 2013, Nyyssönen et al. 2014). The diversity of a microbial community is measured by the number of unique operational taxonomic units (OTUs) corresponding to a kind of organisms or species, found in individual samples (Sogin et al. 2006). This is called the α-diversity. One commonly used diversity measure is the Shannon index (H'), describing both richness and evenness of the taxa in the community. Richness is the number of species or different taxa in the sample. Evenness de-

scribes how close are the total numbers of different species in the environment. In addition to richness and evenness estimates, rarefaction analyses also describe the community's diversity, particularly how well the diversity of each habitat has been captured (Sogin et al. 2006). Multivariate statistics measurements such as analysis of principal coordinates are used to characterize the β-diversity, i.e. the diversity between different communities and abiotic factors affecting the microbial community structures (Griffiths et al. 2011, Barberán et al. 2012).

The β-diversity links to biogeography, i.e. a concept of placing microbes on the map (Martiny et al. 2006). It is stated that the global spatial distribution of free-living microbes depends on the environmental factors determining the selection pressures. Biogeography of deep-dwelling microbial lineages has been under study recently in hydrothermal vent systems (Anderson et al. 2015). The origin of microbes in deep subsurface is also linked to biogeography. Microbes colonizing the deep subsurface are most likely transferred from surface environments concurrently with slow movement of water or have been trapped and isolated between depositional events (Amy et al. 1992). For example in Äspö, Sweden, and in Olkiluoto, Finland, deep aquifers contain groundwater mixed from different sources, such as glacial melt, old Litorina Sea and recent Baltic Sea in addition to some very old saline water (Laaksoharju et al. 1999, Posiva 2012).

Changing gears from global to minute scales, it is likely that microbial processes in deep subsurface occur in spatially isolated microenvironments. This is true for example in soil aggregates (Mummey et al. 2006). Such microenvironments are often formed on interfaces of solid material and water, forming a miniature microbial ecosystem called a biofilm. Biofilms form also in deep subsurface (Anderson et al. 2006, Wanger et al. 2006, MacLean et al. 2007). However, these are mostly monolayers or irregular patches of exopolysaccharides and sporadically distributed microbes (Anderson et al. 2006, Wanger et al. 2006). Consequently, most of the microbial ecology studies of the deep subsurface to date have concentrated on planktonic communities.

1.5.1 Culture-dependent methods

Microbial ecology has been studied traditionally with cultivation–based methods, such as plate counting and most probable number methods. These have also been used to describe the microbial communities in deep subsurface environments (Pedersen et al. 2008, Hallbeck and Pedersen 2008a, 2012). The advantage of these methods is that once a certain microbial strain is purified and growing in the laboratory conditions, the physiology of the strain can be characterized, the strain can be taxonomically classified and material for further studies can be continuously collected. However, the major setback is that only 0.01–0.1% of the total microbial cells in the aquatic environments are cultivable or produce visible colonies with standard plating methods (Pedersen et al. 2008). However, with careful development of the methods for example for anaerobic groundwater, much higher yields in microbial numbers can be obtained (Pedersen et al. 2008).

1.5.2 Marker genes as molecular biological tools

Identification of microbes

The 16S subunit of ribosomal RNA gene, i.e. 16S rRNA gene provides the basis for culture-independent microbial diversity investigations. The 16S rRNA gene has been the golden standard for defining evolutionary relationships between microbes since Woese et al. divided life on Earth into the three domains; *Bacteria*, *Archaea* and *Eucarya* (Woese 1987, Woese et al. 1990). The 16S rRNA gene occurs in all prokaryotic life forms and it has a relatively slow mutation rate. Due to these features, it is a good molecular chronometer that allows determination of phylogenetic relationships of microbes and identification based on comparison to databases. The sequence information stored in several databases is growing rapidly as high-throughput sequencing and metagenomic analyses combined with bioinformatics tools are providing increasing amount of novel information (e.g. Rinke et al. 2013, Petitjean et al. 2015). For example, Ribosomal Database Project's (RDP) current release 11.4 from May 2015 consists of over 3.2 million aligned and annotated 16S rRNA sequences[1].

Targeting microbes with specific functionality

Since the demonstration of congruence of phylogenetic trees of bacterial 16S rRNA and the gene coding for the ATP-synthase (Amann et al. 1988), a range of functional genes have been used as (phylogenetic) markers for metabolic processes, many of these in deep marine subsurface (Blazejak and Schippers 2011, Lever et al. 2013, Lever 2013, Nercessian et al. 2005, Newberry et al. 2004). An ideal functional marker gene must fulfill certain requirements (Lever 2013). First of all, the enzyme that the marker gene encodes is only used for one specific reaction. A database containing enough gene sequences has to be available for the identification of the functional gene sequences acquired from environmental samples. The marker gene must be conserved so that general or group-specific molecular probes can be designed to match the gene sequence. Lastly, the evolutionary history of the gene must be known and possible lateral gene transfer should be recognized.

The contribution of deep subsurface microbial communities to the global elemental cycling has been studied with a variety of functional marker gene assays (Lever 2013). The most studied functions are sulfate reduction and methanogenesis (Teske and Biddle 2008). The dissimilatory sulfite reductase (*dsrAB*) and adenosine 5′-phosphosulfate reductase (*aprA*) genes have been used to widely describe the sulfate reducing microbial communities in the deep biosphere (e.g. Baker et al. 2003, Blazejak and Schippers 2011, Bomberg et al. 2015a, Itävaara et al. 2011a,b, Lever et al. 2013, Meyer and Kuever 2007, Moser et al. 2005, Nakagawa et al. 2002, Nyyssönen et al. 2012, Tiago and Veríssimo 2013). Methano-

[1] http://rdp.cme.msu.edu/misc/rel10info.jsp

gens have been detected using the methyl coenzyme M reductase gene (*mcrA*) (e.g. Bomberg et al. 2015a, Fry et al. 2009, Dhillon et al. 2005, Moser et al. 2005, Nyyssönen et al. 2012). However, many functional genes used as marker genes do not completely meet these characteristics. For example, the dissimilatory sulfite reductase enzyme, coded by the gene *dsrAB* is also operating in the opposite direction, i.e. for sulfide oxidation (Loy et al. 2009). However, these functions can be separated because sulfate reducers generally fall to different genetic clusters than the sulfide oxidizers. Similarly, the methyl coenzyme M reductase coded by the *mcrA* gene may work in both directions depending on whether it is used by anaerobic methane oxidizers or methanogens (Hallam et al. 2004). However, thus far the *mcrA* of methanogenic archaea and anaerobic methanotrophic archaea (ANME) fall into different phylogenetic branches so that the function of the methyl coenzyme M reductase can be deduced from the nearest neighbors in the phylogenetic tree (Knittel and Boetius 2009).

Other functional genes involved in carbon cycling pathways such as aerobic methanotrophy and methylotrophy, acetogenesis and autotrophic carbon fixation have been analyzed from deep subsurface environments (e.g. Lever et al. 2010, Rajala et al. 2015, Tiago and Veríssimo 2013). Marker genes used for the detection of methanotrophy and methylotrophy are the particulate methane monooxygenase gene (*pmoA*) and the methanol dehydrogenase gene (*mxaF*), respectively. Lever et al. (2010) used the formyl tetrahydrofolate synthetase gene (*fsh*) as a functional marker gene for acetogenesis in deep subseafloor sediments. The ribulose-1,5-bisphosphate carboxylase/oxygenase, shortly RuBisCO enzyme is involved in the autotrophic carbon fixation via the Calvin-Bassham-Benson cycle. Genes coding for two forms of this enzyme (*cbbL* and *cbbM*) have been used for characterization of chemolithoautotrophic organisms in deep hydrothermal vents (Campbell and Cary 2004, Takai et al. 2005). Takai et al. (2005) also used genes coding key enzymes of another type of autotrophic carbon fixation pathway, the reductive tricarboxylic acid cycle (ATP citrate lyase beta subunit gene *aclB*, pyruvate:ferredoxin oxidoreductase gene *porAB*, 2-oxoglutarate: ferredoxin oxidoreductase gene *oorAB*). In addition to these genes, Tiago and Veríssimo (2013) also used the *accC* gene coding the acetyl coenzyme A carboxylase:biotin carboxylase, in order to reveal the dominant carbon fixation pathway in a deep subsurface aquifer. The key microbial players in nitrogen cycling in the deep biosphere have been identified with functional genes for nitrogen fixation (H-subunit of nitrogenase gene complex, *nifH*) and nitrate reduction (nitrate reductase gene *narG*) (Lau et al. 2014, Orsi et al. 2013, Rajala et al. 2015).

1.6 Deep biosphere studies

Although some natural environments and pre-existing manmade infrastructure can allow sampling from the deep biosphere, in many cases drilling technologies are required to reach greater depths (Sahl et al. 2010, Moser et al. 2003, Onstott et al. 2009, Wilkins et al. 2014). Even though the drilling operations are expensive and

rigorous, there are several locations around the globe where such operations have been and currently are conducted (Wilkins et al. 2014, ICDP web page[2]). The deep terrestrial biosphere has been studied in many of these sites (**Table 1**). These locations differ greatly in lithology, age, geochemical composition and microbiology. The deep biosphere of the Kalahari Shield in South Africa and the Canadian Shield in North America represent the most ancient environments mainly composed of metamorphosed sedimentary rocks (e.g. Stotler et al. 2009, Onstott et al. 2006). The formation of the Fennoscandian Shield also dates to the Precambrian and the dominant rock types in deep bedrock of the Fennocandian Shield are granites and high metamorphic grade gneisses (e.g. Haveman et al. 1999, Haveman and Pedersen 2002a, Pedersen et al. 2008, Itävaara et al. 2011b). The youngest rocks hosting a deep terrestrial biosphere are reported from Japan (Fukuda et al. 2010, Shimizu et al. 2006, Mills et al. 2010). The temperature range spans over sixty degrees of °C and pH varies from mildly acidic to very alkaline (eg. Sahl et al. 2008, Stevens 1993). Most saline fluids are detected from Driefontein (total dissolved solids, (TDS) 103 g l^{-1}, Onstott et al. 2006, Katz et al. 2011), Olkiluoto (TDS 125 g l^{-1}, Ahokas et al. 2014) and Outokumpu (TDS 68.9 g l^{-1}, Kietäväinen et al. 2013). Microbial presence in deep terrestrial subsurface has been confirmed in many locations around Earth, which are discussed in the next chapters. In fact, only few studies, where microbial presence has been searched for, have been unsuccessful in detecting prokaryotic life in deep subsurface. However, the very low cell counts in deep subsurface and/or the inability to obtain enough representative sample material might have affected to these results (Colwell and D'Hondt 2013).

Studies show that deep terrestrial subsurface environments host microbial communities with structural similarities. These include the frequent detection and domination of the microbial communities by Proteobacteria and Firmicutes (e.g. Zhang et al. 2006, Gihring et al. 2006, Lin et al. 2006a,b, Shimizu et al. 2006, Fukuda et al. 2010, Rastogi et al. 2010, Itävaara et al. 2011a, b, Nyyssönen et al. 2014, Articles I and III). These bacterial phyla also frequently dominate the microbial communities of deep subsurface rock samples (Zhang et al. 2005, Sahl et al. 2008). Sulfate reducing microbes and methanogens have been discovered from several deep subsurface sites around the globe (**Table 1**). Sulfate reduction is a significant energy-yielding process in many of these sites and the numbers of sulfate reducers can be high (Moser et al. 2005, Stotler et al. 2011, Hallbeck and Pedersen 2012). On the other hand, the abundance of methanogens in the microbial communities in deep terrestrial subsurface environments is often low (e.g. Fry et al. 1997, Hallbeck and Pedersen 2012, Nyyssönen et al. 2014). Nevertheless, methanogen community composition appears to be depth-related in bedrock, as many aceticlastic methanogens are relatively more abundant in shallower depths, whereas autotrophic methanogens are more frequent in deeper fluids (Kietäväinen and Purkamo 2015).

[2] http://www.icdp-online.org/projects/

Table 1. Characteristics of several deep terrestrial subsurface sites where microbes have been detected.

Site		Main rock types	Age (rock)[1] Ma	Depth[1] mbs	T[1] °C	pH	Salinity[1] TDS g L^{-1}	DIC mM	TOC mM	DOC mM	Detected microbes[4]	References[5]
Canada	Lupin	Metagraywacke, slate, banded iron formation	2600	1130	11	7.9 … 9.2	40	0.088 … 0.20	0.12 … 0.33	0.15 … 0.36	B, SRB	37, 53, 54
China	Donghai	Granitic gneiss, paragneiss, eclogite, garnet peridotite	240	3350	87	9.2 … 9.4					A, B	57
Finland	Hästholmen	Rapakivi granite	1630	985		6.8 … 8.5	31.8	0.38 … 2.22	0.30 … 0.34	0.08 … 0.82	SRB, IRB, AG	15, 16, 27
	Kivetty	Granite, granodiorite	1880	855		7.8 … 9.0	0.23	0.99 … 2.15	0.18	0.14 … 1.27	SRB, IRB, AG	3, 15, 16, 43
	Olkiluoto	Migmatitic gneiss, mica gneiss, granite	1850	960	19	7.8 … 8.3	125	0.04 … 5.9	0.12 … 0.14		SRB, IRB, AG, MG, MT, ANME	1, 5, 15, 16, 34, 41
	Outokumpu	Mica schist, black schist, granodiorite, serpentinite	1900	2480	40	8.4 … 10.1	68.9				SRB, MG, MT	17, 22, 35, 44, 45, 46, 47
	Palmottu	Garnet-cordierite gneiss, granite	1900	417		6.2 … 9.4	1.6	0.5 … 3[2]			SRB, IRB, AG	2, 18, 40
	Romuvaara	Tonalite gneiss	2700	566		8.4	0.17	1.85	1.05		SRB, IRB, AG	15, 16, 42
Japan	Horonobe	Mudstones, sandstone	23	625	36	6.8		450		31	SRB, MG	51
	Mizunami	Granite	75	1169	22	8.2 … 8.6		0.12 … 0.18		0.10 … 0.13	B	10
	Tono	Granite	68		25	7 … 10		0.08 … 4		170 … 1190	MT	28
	Toyoha	Pyrite, chalcopyrite		550	71						A, SRB	32
South Afric	Beatrix	Conglomerate, quartzite	2900	1390	40	7.7 … 7.9		0.34	0.14 … 0.18	0.14	B, MG	6, 11, 25, 56
	Driefontein	Andesite, quartzite	2700	3300	43	6.0 … 7.4	103	0.41 … 0.57	0.40 … 1.83	0.67	SRB, MG, ANME	4, 6, 11, 20, 25, 30, 31, 50
	Evander	Conglomerate, quartzite	2900	2230	45	7.2 … 8.6			0.22 … 0.61		SRB, MG, ANME	8, 11, 25, 56
	Kloof	Andesite	2700	3400	59	7.6 … 8.5	16.5	0.02[3]	0.46 … 1.67		A, B	11, 20, 21, 25, 50, 56
	Mponeng	Metabasalt	2700	3300	52	9.3	11.8		0.43		B, MG	11, 20, 25, 26, 50
Sweden	Forsmark	Meta-granite	1900	1002		7.3 … 8.3	15	0.04 … 1.5[2]		0.12 … 0.13	IRB, SRB, AG, MG, MT	6, 12, 14
	Laxemar	Granite, quartz monzodiorite	1800	922	19	7.5 … 8.4	18	0.13 … 5.4[2]	0.12 … 1.67	0.12 … 1.75	IRB, SRB, AG, MG	12, 13, 14, 39
	Äspö	Granite, granodiorite	1800	860	19	6.8 … 7.8	16	0.16 … 0.34[2]	0.11 … 0.57	0.025 … 1.5	IRB, SRB,N, AG, MG, MT	7, 12, 19, 23, 24, 39
USA	Columbia River Basalt	Basalt	23	1270	18	9.9	0.7	1.07 ... 2.84		0.12 … 0.44	IRB, SRB, MG	3, 9, 52
	Henderson mine	Granite	2500	1044	40	5.8 … 6.3		15.7 … 32		0.03 … 0.05	N, A	49, 55
	Homestake, SURF	Metasediments	1900	1478	33	6.6 … 8.5					SRB, IRB, N, MG, MT	29, 38, 48
	Snake River Plain	Basalt	10	235	15	7 … 8.3				0.08 … 0.22	A, MT	33, 36

[1]Maximum values are given for age, depth, temperature (T) and salinity

[2]HCO_3^- concentration

[3]TIC

[4]A=archaea, B=bacteria, SRB=sulphate-reducing bacteria, IRB=iron-reducing bacteria, N=N-cycling microbes, AG=acetogens MG = methanogens, MT= aerobic methanotrophs, ANME = anaerobic methanotrophs

[5]1) Ahokas et al. (2014), 2) Ahonen et al. (2004), 3) Anttila et al. (1999), 4) Baker et al. (2003), 5) Bomberg et al. (2015a), 6) Borgonie et al. (2011), 7) Chi Fru (2008), 8) Davidson et al. (2011), 9) Fry et al. (1997), 10) Fukuda et al. (2010), 11) Gihring et al. (2006), 12) Hallbeck and Pedersen (2008a), 13) Hallbeck and Pedersen (2008b), 14) Hallbeck and Pedersen (2012), 15) Haveman et al. (1999), 16) Havemann and Pedersen (2002b), 17) Itävaara et al. (2011a), 18) Kaija et al. (1998), 19) Kalyuzhnaya et al. (1999), 20) Katz et al. 2011 21) Kieft et al. (2005), 22) Kietäväinen et al. (2013), 23) Kotelnikova and Pedersen (1997), 24) Kotelnikova and Pedersen (1998), 25) Lin et al. (2005a), 26) Lin et al. (2006b), 27) Luukkonen et al. (1999), 28) Mills et al. (2010), 29) Morelli et al. (2010) and references therein, 30) Moser et al. (2003), 31) Moser et al. (2005), 32) Nakagawa et al. (2002), 33) Newby et al. (2004), 34) Nyyssönen et al. (2012), 35) Nyyssönen et al. (2014), 36) O'Connell et al. (2003), 37) Onstott et al. (2009), 38) Osburn et al. (2014), 39) Pedersen and Ekendahl (1990), 40) Pedersen and Haveman (1999), 41) Pitkänen and Partamies (2007), 42) Pitkänen et al. (1996), 43) Pitkänen et al. (1998), 44) Purkamo et al. (2013)(ArticleI), 45) Purkamo et al. (2015)(ArticleII), 46) Purkamo et al. (2015)(ArticleIII), 47) Rajala et al. (2015), 48) Rastogi et al. (2009), 49) Sahl et al. (2008), 50) SherwoodLollar et al. (2006), 51) Shimizu et al. (2006), 52) Stevens (1993), 53) Stotler et al. (2009), 54) Stotler et al. (2012), 55) Swanner et al. (2011), 56) Ward et al. (2004), 57) Zhang et al. (2006)

1.6.1 South Africa

Gold mines in South Africa have provided relatively easy access to the deep subsurface. Thus, microbial ecology of the deep biosphere of Kalahari Shield has been extensively characterized during the last 15 years (e.g. Takai et al. 2001, Gihring et al. 2006, Silver et al. 2010, Davidson et al. 2011, Lau et al. 2014) (**Table 1**). The diversity of microbial communities was reported to be low likely due to the scarcity of nutrients and electron acceptors in the borehole and bedrock fracture fluids (Gihring et al. 2006). However, many species of SRB and methanogenic archaea, in addition to an archaeal candidate division (SAGMEG) without any cultured representatives to date have been discovered from the deep biosphere of Witwatersrand Basin (Takai et al. 2001, Moser et al. 2003, 2005, Lin et al. 2006a, Gihring et al. 2006, Blanco et al. 2014). Furthermore, some intriguing findings from these deep bedrock sites have been made. For example, a unique single-species ecosystem was discovered from a bedrock fracture zone from Mponeng mine. Candidatus *Desulforudis audaxviator* as a sole member of the fracture fluid community is an apparent self-sufficient organism capable of surviving in this environment over a long period of time by using geologically produced sulfate and hydrogen (Chivian et al. 2008). In addition, Borgonie et al. (2011, 2015) found nematodes in the deep bedrock fracture fluids and from the stalactites growing from the ceilings of the mines. One entirely new species of nematode, *Halicephalobus mephisto* was described from the Beatrix mine from a depth of 1300 m below ground level (Borgonie et al. 2011).

1.6.2 Asian locations

Among the deepest drilling projects where microbial ecology has been studied is the Chinese Continental Scientific Drilling Project in Donghai, China. The drill hole is located in the Dabie-Sulu ultra-high pressure metamorphic belt, formed during the Triassic, in a convergent plate boundary in Eastern China. Microbial communities were characterized from rock cores and drilling fluids from a depth range of 0.5–3.3 km (Zhang et al. 2005, 2006). Proteobacteria dominated the clone libraries derived from the rock cores. α-proteobacterial sequences were only derived from the rock sample from the shallowest depth at 529 m, while β-proteobacteria were prominent in the rock core from 730 m depth. Furthermore, *Pseudomonas*-related clone sequences became more abundant with depth (Zhang et al. 2005). Similar proteobacteria also dominated the drilling fluids above 2 km, but below this depth Firmicutes predominated in Dabie-Sulu (Zhang et al. 2006).

In Japan, deep terrestrial biosphere studies have been linked to reliable and safe geological disposal of nuclear waste. Two different sites, Mizunami Underground Research Laboratory[3] and Horonobe Underground Research Center[4] have been engaged in this research (Niibori 2015). Mizunami, located in central Japan

[3] http://www.jaea.go.jp/04/tono/miu_e/index.html
[4] http://www.jaea.go.jp/english/04/horonobe/

offers access to Cretaceous granitic crystalline bedrock groundwater, where β-proteobacteria dominated the microbial community at 1.1 km depth (Fukuda et al. 2010). In Horonobe, northern Japan, on the other hand, several experimental boreholes have been drilled into the Wakkanai formation. This formation is comprised of sandstones with high porosity but low permeability and is divided by a major fault zone. The Omagari fault divides the area to a northeastern and a southwestern side, and the microbial communities at these sites differed significantly from each other (Shimizu et al. 2006). β-proteobacteria dominated the microbial community and methanogens were not detected in the northeastern side of the fault at the depth of 458 m. At the southwestern side of the fault zone at 374 m depth, a possible sulfate-methane transition zone (SMTZ) with sulfate reducing Firmicutes and δ-proteobacteria living together with methanogenic archaea was described. Interestingly, the archaeal sequences were all affiliating closely with a single archaeal species, *Methanoculleus shikugoensis* (Shimizu et al. 2006).

Near Mizunami lies Tono uranium mine, where Mills et al. (2010) detected two different microbial ecosystems from a borehole intersecting Toki lignite-bearing and granite formations at depths of 160-200 m. Microbial cell membrane phospholipid fatty acid (PLFA) signatures suggested that the microbial community in lignite-bearing formation could utilize ancient recalcitrant organic matter originating from lignite. It was hypothesized that fermentative heterotrophs may be responsible for the initial breakdown of this material, possibly in a syntrophic relationship with methanogens. The granitic bedrock on the contrary hosted chemolithoautotrophic microbes using dissolved inorganic carbon and possibly H_2 produced in radiolysis of water in the uranium rich rocks of the formation for primary production. In addition, specific signatures of aerobic methanotrophs were detected from these samples (Mills et al. 2010).

1.6.3 North America

The deep biosphere of the Canadian Shield has been mostly studied with emphasis on hydrogeochemistry (e.g. Sherwood Lollar et al. 1993, 2006, Stotler et al. 2012, Holland et al. 2013). However, microbial communities and microbial sulfate reduction activity have been detected from groundwater samples from the continuous permafrost area of the Lupin gold mine and High Lake, respectively (Onstott et al. 2009, Stotler et al. 2011). The studies demonstrated that sulfate reduction was the dominant microbial process in these environments reaching depths over 500 m. Recently, Holland et al. (2013) reported record-breaking 1.5 Ga residence times of fracture fluids from Timmins mine in Canadian Precambrian Shield.

In USA, deep biosphere has also been studied through mines. Sahl et al. (2008) described the microbial communities of deep fractures and a rock core sample from Precambrian granitic bedrock of the Henderson molybdenum mine. Fluid samples taken from two different packer-isolated fractures represented similar microbial communities, dominated by bacterial phylotypes closely affiliating to each other. These phylotypes formed an intrinsic candidate division Henderson Group 1 that had no close phylogenetic relationship with any described bacterial

species. However, these microbes might play a role in nitrogen fixation in deep biosphere in the Henderson mine deep subsurface (Swanner and Templeton 2011). The rock core microbial community was dominated by the β-proteobacterial family *Ralstoniaceae* and Firmicutes.

After the closure of the mining activities at Homestake gold mine in 2001, The Sanford Underground Research Laboratory (SURF) was established to provide scientific opportunities for direct exploration of the deep subsurface in this deepest mine in North America reaching the depth of 2.4 km. Microbial ecology of the mine was studied by Rastogi et al. (2009, 2010) and Osburn et al. (2014). Proteobacteria dominated both the clone libraries and the PhyloChip biosignatures from soil samples and high-throughput sequences from borehole fluids (Rastogi et al. 2009, 2010, Osburn et al. 2014). Many of the proteobacterial species reported from Homestake mine have been previously detected from gold mines in Japan and South Africa (Rastogi et al. 2010).

In situ -evidence from Columbia River Basalts (CRB) supports the hypothesis of hydrogen-driven deep biosphere (Gold 1992, Stevens and McKinley 1995, Pedersen 1997) to some extent. Stevens et al. (1993) reported that viable bacterial populations reflect the groundwater chemistry of Grande Ronde and Priest Rapids aquifers in Columbia River Basalts at depths of 1270 and 316 m, respectively. Subsequently, Stevens and McKinley (1995) provided evidence for the existence of subsurface lithotrophic microbial ecosystem (SLiME) in CRB. Enrichment of the heavier stable carbon isotope, ^{13}C, below 200 m depth suggests preferential removal of ^{12}C by methanogens. However, bacterial species, including active hydrogen-oxidizing sulfate reducers and homoacetogens are more abundant than methanogens that comprise only a small fraction of the total microbial community in CRB (Fry et al. 1997).

Even though methanogenic Archaea have been suggested to be the primary producers in photosynthesis-independent deep terrestrial biosphere (Gold 1992, Pedersen 1997), as in CRB and other sites, the numbers of methanogens are low. However, Chapelle et al. (2002) described a subsurface microbial community composed of over 90% methanogens relying on hydrogen and CO_2 from Lidy Hot Springs, Idaho. This community differed from all other previously described deep terrestrial subsurface microbial ecosystems and filled most of the criteria proposed to be designated as a true SLiME, i.e. that chemolithotrophic organisms are present and metabolically active using solely geological energy source and electron acceptors in this ecosystem (Nealson et al. 2005).

1.6.4 Fennoscandia

Microbial ecology of Precambrian Fennoscandian Shield has been studied for more than two decades (e.g. Pedersen and Ekendahl 1990, 1992, Pedersen 1997, Kotelnikova and Pedersen 1998, Haveman et al. 1999, Haveman and Pedersen 2002, Hallbeck and Pedersen 2008a,b, 2012, Itävaara et al. 2011a,b, Nyyssönen et al. 2012, Pedersen 2012a,b, Nyyssönen et al. 2014, Bomberg et al. 2014, 2015a,b, Articles I–III). The most extensive studies on microbial ecology in granitic

bedrock have probably been done in Äspö Hard Rock Laboratory (HRL)[5] that has been running since 1995. Other important study sites include planned nuclear waste repository sites of Olkiluoto in Finland and Laxemar-Simpevarp and Forsmark in Sweden. This thesis is based on studies conducted in Outokumpu Deep Scientific Drill Hole in Finland (see the following section).

Microbial communities have been characterized by resolving the dominant taxa and enumeration of different physiological groups from both Finnish and Swedish sites (Pedersen et al. 1996, Haveman et al. 1999, Haveman and Pedersen 2002, Pedersen et al. 2008, Hallbeck and Pedersen 2008a,b, 2012, Nyyssönen et al. 2012) (**Table 1**). These studies have relied on traditional most probable number (MPN) cultivation methods and molecular biological methods, such as clone library sequencing and community fingerprinting methods. Metabolically diverse communities comprised of autotrophic and heterotrophic acetogens, nitrate, iron, manganese and sulfate reducing microbes and methanogens have been identified in these studies. Interestingly, the microbial communities differ from site to site and from one drill hole to another (Haveman and Pedersen 2002, Pedersen et al. 2008, Hallbeck and Pedersen 2008a, 2012, Nyyssönen et al. 2012, Itävaara et al. 2011a,b). Nevertheless, these studies conclusively propose that nitrate reduction, sulfate reduction and acetogenesis are significant energy-yielding processes for deep subsurface microbes at these sites. In addition, aerobic methane-oxidizing microbes have been detected from Äspö, Forsmark and Olkiluoto fluids from shallow depths (Pedersen et al. 2008, Chi Fru 2008). In Outokumpu, evidence for aerobic methane oxidation capacity has been demonstrated in the 500 m fracture (Rajala et al. 2015). Methanogens on the other hand are present especially at greater depths in these environments, representing only a minority of the total microbial community (Pedersen et al. 2008, Hallbeck and Pedersen 2012, Nyyssönen et al. 2012). High-throughput sequencing analyses have revealed more precisely the microbial community composition at different depths of Olkiluoto deep biosphere. β-, γ- and δ-proteobacteria are the most dominant classes whereas α- and ε-proteobacteria represent lower relative abundance in various fractures at depths above 200 m and below 385 m (Bomberg et al. 2014). A sulfate-methane transition zone lies in between these depths in Olkiluoto, and at these depths, ε-proteobacteria are dominating the communities (Bomberg et al. 2015a). This zone hosts typical indicator organisms for anaerobic methane oxidization, such as ANME-1 archaea in addition to ANME-2D archaea (Pedersen 2013, Bomberg et al. 2015a). Thus, the detection of ANME archaea supports the previously proposed possibility of anaerobic methane oxidation in Olkiluoto deep bedrock based on the detection of *mcr*A genes affiliating with those of ANME-1 archaea (Nyyssönen et al. 2012). In addition to ANME archaea, the other dominating archaeal groups in Olkiluoto fractures are *Methanobacteriales* and *Thermoplasmatales* -related representatives (Bomberg et al. 2014, Bomberg et al. 2015a). In addition to prokaryotic life, fungal communities have been characterized from Äspö and Olkiluoto sites (Pedersen et al. 1996, Sohlberg et al. 2015).

[5] http://www.skb.com/research-and-technology/laboratories/the-aspo-hard-rock-laboratory/

1.7 The Outokumpu Deep Drill Hole

This thesis is based on studies executed in the Outokumpu Deep Scientific Drill Hole, located in Eastern Finland (62.72°N, 29.07°E). The vertical drill hole reaches the depth of 2516 m in Palaeoproterozoic crystalline bedrock in Fennoscandian Shield (**Figure 2**). The 22 cm in diameter drill hole is cased only to the depth of 39 m, thus providing access to the crystalline bedrock *in situ* below this depth. The Outokumpu district is characterized as a classical ore province in Finland with its copper, cobalt and zinc sulfide deposits. Drilling was conducted during the years 2004–2005 by a Russian company NEDRA in collaboration with The Geological Survey of Finland (Kukkonen 2011). The core drilling technique used steel-tooth drilling bits, and municipal tap water was used as the primary drilling fluid. Drilling fluid conditioners were used to stabilize the drill hole wall only when necessary, mostly above 1000 m. The drilling fluid was labeled with fluorescein dye to estimate the mixing of the intrinsic formation water and drilling fluid. When drilling was finished, the drill hole was flushed with fresh tap water. Gradual replacement and mixing of this fresh water with saline formation fluids in the drill hole has been observed over several years of hydrogeochemical monitoring in the Outokumpu site (Ahonen et al. 2011, Kietäväinen et al. 2013).

The drill core reveals a cross section of the lithology of the Outokumpu bedrock, comprising of metasedimentary, igneous and ophiolite-related rocks, such as mica schist, pegmatitic granite and serpentinite and skarn, respectively. Numerous thin black schist veins are detected throughout the drill hole lithology (Västi 2011). The groundwater in the area is typical for shield brines affected by long-term water-rock interactions. Sodium, calcium and chloride ions build up salinity in Outokumpu groundwater. In addition to characteristic salinity, fluids contain ample amount of dissolved gas of which CH_4 is the major component above 2000 m (Kietäväinen et al. 2013). Five different water types have been detected in Outokumpu Deep Drill Hole, based on the geochemistry and isotopic composition of the water stable isotopes, each emanating from different fracture zone of the bedrock (Kietäväinen et al. 2013). According to the noble gas stable isotope composition and concentration, the age of the formation fluids is estimated to be at least several tens of millions of years (Kietäväinen et al. 2014).

The low-porosity bedrock of continental crust in Outokumpu provides a unique environment to study photosynthesis-independent, nutrient-poor but still very alive deep biosphere. The microbial communities in the drill hole water column of Outokumpu bedrock have previously been characterized (Itävaara et al. 2011a,b, Nyyssönen et al. 2014), and recently Rajala et al. (2015) described methanotrophic subpopulations from a fracture fluid at 500 m depth. Proteobacteria and Clostridia dominated in the drill hole water column, although the bacterial community structure varied at different depths (Itävaara et al. 2011a,b, Nyyssönen et al. 2014). At 200 and 600 m depths, *Comamonadaceae* and *Acholeplasmataceae* were the most abundant phyla, while the highest diversity was detected between depths from 1000–1500 m. Here, the proportion of clostridial phylotypes was exceptionally high compared to other depths (Nyyssönen et al. 2014). *Methanobac-*

terium has been shown to be the most common archaeal genus throughout the drill hole water column (Itävaara et al. 2011a, Nyssönen et al. 2014) with the exception of the depth 1100 m, where *Methanolobus* dominated (Nyyssönen et al. 2014). Methanogens represent only a fraction of the total microbial community in the Outokumpu deep biosphere determined by the low copy numbers of the methanogenesis marker gene, *mcrA*, compared to the total amount of microbes (Itävaara et al. 2011a). Metagenomic analysis of microbial communities at 600, 1500 and 2300 m depths of the drill hole revealed metabolic potential for different types of autotrophic carbon fixation, but also for fermentation of organic acids at 1500 m, possibly due to the high proportion on fermentative clostridia detected at this depth (Nyyssönen et al. 2014). Although these microbial communities in Outokumpu are probably fairly inactive, some microbial groups can readily respond to environmental changes. This was demonstrated in a recent study, in which the transcription of marker genes for methane oxidation, sulfate reduction and nitrate reduction was rapidly stimulated when the microbial communities derived from the 500 m fracture in Outokumpu were supplied with methane and SO_4^{2-} (Rajala et al. 2015).

However, the intrinsic microbial communities of the fractures from where the ancient fluids are emanating to the Outokumpu drill hole have not been characterized. In addition, only a little is known about the functionality of these microbial communities, and even less about the connections between these microbes and the ecosystem functions.

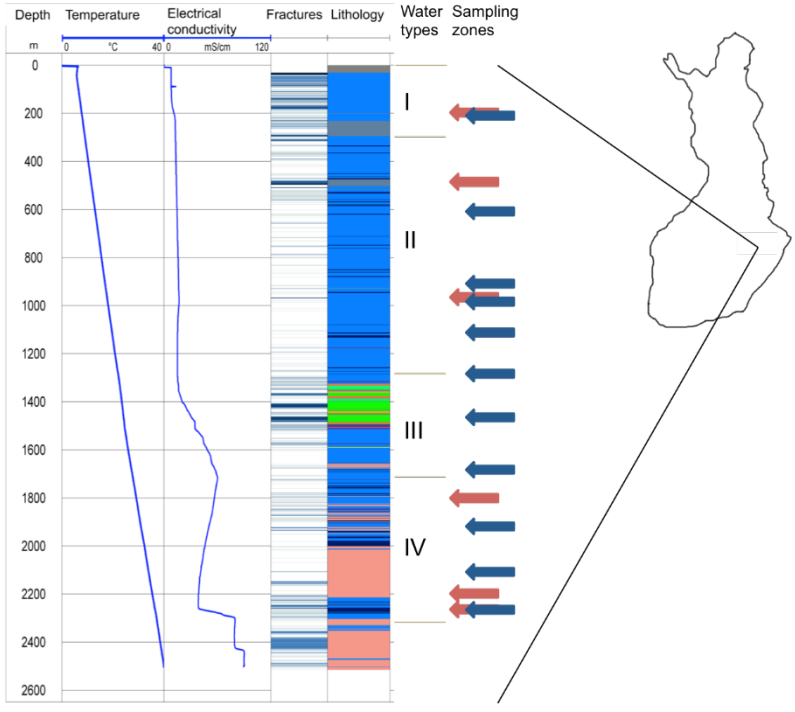

Figure 2. Schematic of in situ temperature, electrical conductivity, fracturing and lithology, water types and sampling depths of the bedrock in Outokumpu Deep Drill Hole in Finland (Kukkonen et al. 2011a,b, Västi 2011, Kietäväinen et al. 2013). Fractures are indicated with dark lines. Lithology is described as follows: grey and blue indicate metasediments, green and orange indicate ophiolite-derived rock types and pink pegmatitic granite. Water types are marked with roman numerals, studied fracture depths are indicated with red arrows and studied depths of the drill hole water column with blue arrows. (Modified from Article I.)

2. Aims of this thesis

The objective of this work was to characterize the microbial communities living in the deep Fennoscandian bedrock biosphere in Outokumpu, in addition to estimate the functionality of the archaeal and bacterial communities in this habitat. Moreover, this work targeted on identification of the core microbial community and the key players of the communities in addition to the description of species interactions.

Modern molecular biological methods based on the genetic properties of microbes were used to resolve the structure and the metabolic properties of microbial communities. This work concentrates on the intrinsic microbial communities dwelling in the pristine fractures of the deep crystalline bedrock in addition to the functionality and carbon cycling in the water column of the Outokumpu Deep Scientific Drill Hole.

- Developing methods for microbiological sampling of the Outokumpu bedrock fractures and assessing the feasibility of these sampling methods for collection of indigenous fracture fluids from deep crystalline bedrock (Article I). Hypothesis: the fracture zones in Outokumpu host endemic microbial communities that differ from the drill hole water column communities. The sampling method must be verified in order to ensure that the samples are derived from the actual fracture fluids.
- Characterization of the microbial communities present and functioning in the Outokumpu deep subsurface (Article I and III). Hypothesis: Diverse microbial communities are detected from Outokumpu deep crystalline bedrock biosphere. Microbial communities are similar to those found in the deep subsurface in other Fennoscandian Shield sites.
- Characterization and enumeration of microbes with key metabolic properties important in risk assessment of industrial utilization of deep subsurface (sulfate reduction and methanogenesis) (Article I, II and III). Hypothesis: Sulfate reduction and methanogenesis are significant functions in the anaerobic deep subsurface.
- Determination of the dominant carbon fixation metabolism of microbial communities of Outokumpu Deep Drill Hole (Article II and III). Hypothesis: Autotrophy is the dominant type of metabolism, as organic carbon is scarce in Outokumpu deep bedrock.

- Discovering the links between the microbial community composition, functionality of the community and the geochemical factors in deep crystalline bedrock biosphere (Article III). Hypothesis: The chemical properties of the fracture fluids and the prevailing lithology at each fracture shape the microbial community structure, for example by determining the electron acceptors, carbon and/or nutrient sources or favoring specialized extremophiles.

3. Materials and methods

This chapter shortly describes the materials and methods used in this study. More detailed information and references are presented in the original publications and in **Table 2**.

3.1 Sampling of the fracture fluids and drill hole water column

Fracture fluid samples were collected from fracture zones located at six different depths (**Figure 2**). Two techniques were applied for collection of intrinsic fracture fluids: I) pumping the fluid from the fracture isolated from the rest of the drill hole water with the expandable rubber and stainless steel packers (Lapela Oy, Finland) placed above and below each zone (Ahonen et al. 2011) (**Figure 3**) and II) a slow continuous pumping for several weeks from the level of each fracture zone allowing the fluids to discharge from the fracture (Article I, Article III). The fluid was pumped through a sterile PA tube initially filled with mQ or RO water. In both cases stable levels of pH, electrical conductivity and oxygen detected with continuous monitoring during the pumping ensured that indigenous water was obtained.

Drill hole water column was sampled using a 2300 m long sterile polyamide tube with 50 m long sections connected together by sterilized tube fittings or ball valves and a backpressure valve at the lower end (Nurmi and Kukkonen 1986). The tube was lowered into the drill hole and allowed to slowly fill up with drill hole fluid. The tube was then lifted whereupon the backpressure valve closed, and each ball valve was closed immediately as it emerged from the drill hole. Each 2 × 50 m section of the tube was treated as one sample representing a 100-m interval of the drill hole (Nyyssönen et al. 2014). Microbiological analyses were made from 11 depths ranging from 200–2300 m (**Figure 2**.) (Article II.)

3.2 Hydrogeochemical measurements

Electrical conductivity, pH, redox potential, temperature, and oxygen levels were continuously monitored and recorded during the pumping of the fractures. Cation and anion composition, alkalinity and gas composition of the fluids were analyzed

as described in Ahonen et al. (2011), Nyyssönen et al. (2014) and in Article I. Water for cation analysis was filtered and acidified with ultrapure HNO_3. Analysis was done with ICP-MS or ICP-OES. Anions were determined with ion chromatography. Alkalinity was titrimetrically measured, and gas composition was analyzed with gas chromatography with TCD and FID detectors. Due to the high salinity in the drill hole, analysis of many chemical components of the fluid was problematic.

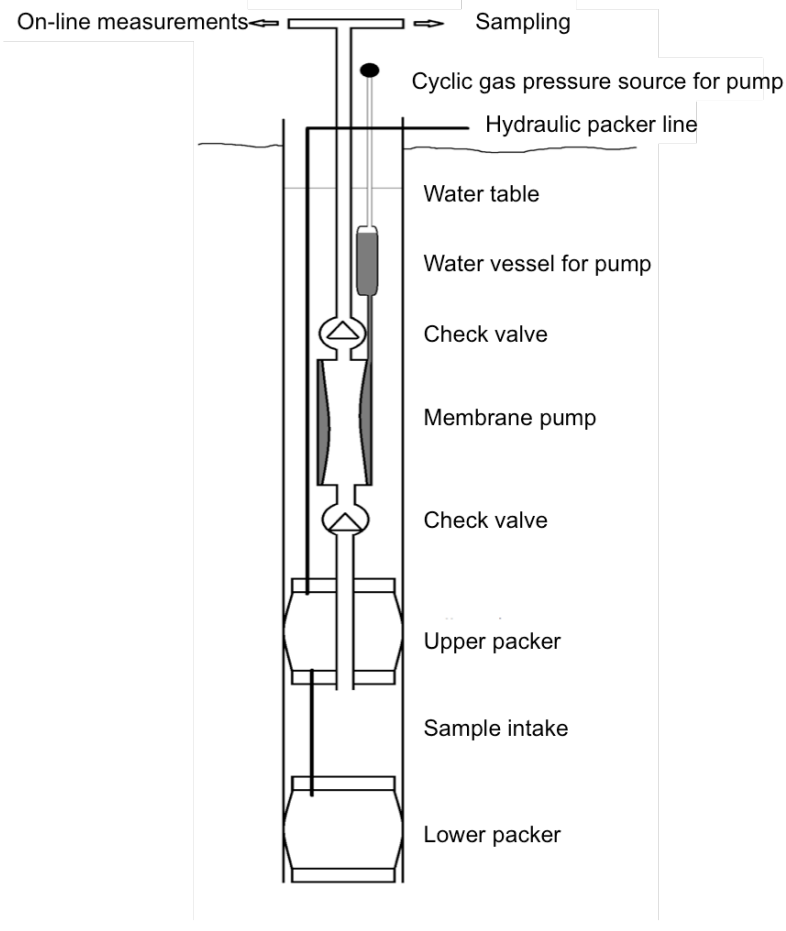

Figure 3. Schematic drawing of the packer system used in Outokumpu (courtesy of L. Ahonen).

3.3 Enumeration of microbes

Enumeration of microbial cells was conducted with microscopy or by molecular biological methods. Samples for microscopy were placed into acid-washed, sterile headspace glass flasks in the anaerobic chamber and closed with butyl rubber stoppers and aluminum crimp caps. Samples were kept chilled prior to staining with fluorescence stains (Article I, II, III, Itävaara et al. 2011b, Nyyssönen et al. 2014). Either Live/Dead or DAPI fluorescent stains were used to visualize the cells in the samples. The number of viable and dead cells was determined with the BacLight Bacterial Viability Kit (Molecular Probes, Invitrogen Corp., USA) (Article I, II) as described in Itävaara et al. (2011b). DAPI-staining was conducted as described in Article I.

Quantitative PCR (qPCR) was used in enumeration of the copy numbers of several marker genes in the samples. The copy number of 16S rRNA gene was used as an approximation of bacterial and archaeal cell numbers in fractures (Article III). The copy numbers were determined from DNA with qPCR targeted either to bacterial or archaeal 16S rRNA gene using Roche LightCycler technology and commercial qPCR mastermix (**Table 2**) as described in Article III.

The abundance of functional marker genes used as a proxy for key anaerobic respiration processes, sulfate reduction and methanogenesis was calculated with qPCR. Amplification protocols are described in detail in Articles I and III, commercial kits and used primers are shown in **Table 2** and **Table 3**. The numbers of SRB and methanogens were estimated by comparing the amplification result to a standard dilution of *Desulfobulbus propionicus* DSM 2554 *dsrB* gene and *Methanothermobacter thermoautotrophicus* DSM 1053 *mcrA* gene, respectively.

Table 2. Microbiological and molecular biological methods used in this thesis.

Assay	Method	Target gene[1]	Details[2]	Article	Reference[3]
Cell density	DAPI staining			I,III	2
Cell wall integrity, amount of living vs. dead cells	Live/Dead staining		LIVE/DEAD Bacterial Viability Kit (BacLight), Molecular Probes, Invitrogen	I,III	3
DNA extraction	Bead beating		PowerSoil DNA isolation kit, MoBio	I, II, III	
RNA extraction	Bead beating		PowerWater RNA isolation kit, MoBio	I, II, III	
Purification of RNA	DNase treatment		RQ1 Rnase-Free Dnase, Promega	I,III	
Production of cDNA from RNA	reverse transcription		SuperscriptIII, Invitrogen	I, III	
Increasing DNA yield	WGA		Illustra GenomiPhi V2, GE Healthcare	II	
Bacterial community fingerprint analysis	DGGE	16S rRNA		I	5
Bacterial community identification	HTP sequencing	16S rRNA		III	4
Archaeal community fingerprint analysis	DGGE	16S rRNA		I	5
Archaeal community identification	HTP sequencing	16S rRNA		III	4
Sulphate reducing community fingerprint analysis	DGGE	*dsrB*		I, II	5
Sufate reducing community identification	HTP sequencing	*dsrB*		III	4
Methanogen community analysis	Cloning	*mcrA*	TOPO TA Cloning Kit, Invitrogen	II	6
Methanogen community identification	HTP sequencing	*mcrA*		III	4
Carbon assimilation via chemoorganotrophy	DGGE	*accC*		II	6
Carbon assimilation via Calvin cycle	Cloning	*rbcL*, *cbbM*	TOPO TA Cloning Kit, Invitrogen	II	6
Carbon assimilation via Wood-Ljungdahl pathway	Cloning	*acsB*	TOPO TA Cloning Kit, Invitrogen	II	6
Methanotrophic community analysis	Cloning	*pmoA*	TOPO TA Cloning Kit, Invitrogen	II	6
Nitrate reducing community analysis	Cloning	*narG*	TOPO TA Cloning Kit, Invitrogen	II	6
Ammonia-oxidizing community fingerprint analysis	DGGE	*amoA*		II	5
Quantification of bacterial 16S rRNA gene copy number	qPCR	16S rRNA	KAPA SYBR® Fast 2× Master mix, Kapa Biosystems, Roche LightCycler 480	III	1
Quantification of archaeal 16S rRNA gene copy number	qPCR	16S rRNA	KAPA SYBR® Fast 2× Master mix, Kapa Biosystems, Roche LightCycler 480	III	1
Quantification of methyl-coenzyme M reductase gene copy number	qPCR	*mcrA*	KAPA SYBR® Fast 2× Master mix, Kapa Biosystems, Roche LightCycler 480	III	1
Quantification of dissimilatory sulphate reduction gene copy number	qPCR	*dsrB*	KAPA SYBR® Fast 2× Master mix, Kapa Biosystems, Roche LightCycler 480	I,III	1

[1]see details: Table 3

[2]used commercial kits

[3]of the original method, 1) Higuchi et al. (1993), 2) Kepner and Pratt (1994), 3) Lloyd and Hayes (1995), 4) Margulies et al. (2005), 5) Muyzer et al. (1993), 6) Pace et al. (1986)

3.4 Biomass collection, nucleic acids extraction and subsequent analyses

Biomass from the fracture fluids was collected in the field laboratory in a portable anaerobic chamber using filtration (Article I, III). Sampling fluid was pumped directly into the anaerobic chamber via the sampling tube and biomass was collected from a defined amount of sample fluid (1 L or 500 mL) on sterile cellulose acetate filters of 0.2 µm pore size (**Figure 4**). Biomass was stored at -80 °C prior to DNA and RNA extraction.

Figure 4. Biomass filtration in the anaerobic cabinet of the field laboratory.

Biomass from the drill hole fluids was collected from 11 depths. Sampling was performed directly from the tube section through a flame-sterilized, pressure-tight valve. Water samples for nucleic acid analyses (500 ml each) were filtered directly with Sterivex filter units and immediately frozen on dry ice. Prior to DNA extraction, filters were cut from the filter units and sliced into smaller pieces before placing them on the extraction kit's bead tube.

DNA and RNA were extracted from the biomass with commercial extraction kits (**Table 2**) according to manufacturer's instructions. RNA was transcribed to cDNA as described in Articles I and III, whole genome amplification of drill hole water samples was done according to manufacturer's instructions and described in Article II and in Nyyssönen et al. (2014). The PCR amplification procedures for subsequent molecular biological analyses of fracture and drill hole fluid samples were conducted as described in Articles I–III.

3.5 Microbial community characterization

Microbial community studies were conducted either by denaturing gradient gel electrophoresis (DGGE) (Article I) or high-throughput sequencing methods (Article III). DGGE and cloning in addition to Sanger sequencing were applied for functional marker gene studies (Article II). PCR primer details used in molecular biological characterization and assessment of functionality of the microbial communities are presented in **Table 3**.

DGGE profiling of bacterial, archaeal, SRB and dark carbon fixating communities

PCR products containing a GC clamp at the 5′-end of the forward primer were run on denaturing gradient gel electrophoresis (DGGE) with gene-specific gradient, voltage and run time described in Articles I (16S rRNA gene of bacteria and archaea, *dsrB*) and II (*accC*, *dsrB*).

Prominent bands were excised from the DGGE gels and DNA from the gel fragment was allowed to suspend to 20 µl of molecular-grade water overnight at +4 °C. DNA from each band was reamplified using the same PCR protocols as in original PCR and sequenced at Macrogen, Inc. (South Korea).

Cloning of marker genes for methanogenesis, methanotrophy and nitrate reduction

Amplified PCR products were purified from agarose gel slices using commercial gel extraction kits and ligated with a plasmid vector overnight at +12 °C. Transformation reaction was performed according to the manufacturer's instructions using chemically competent *Escherichia coli* cells. The transformants were grown on Luria-Bertani agar plates containing kanamycin as a selective agent at 37 °C overnight. Clones were checked for insert with colony PCR. PCR products of clones containing an insert of the expected size as determined by agarose gel electrophoresis were sequenced at Macrogen, Inc., Korea. Detailed information and reaction conditions are described in Article II.

High throughput sequencing of bacterial, archaeal, SRB and methanogenic communities

Barcoded primers were used to produce amplicon libraries of bacterial and archaeal 16S rRNA, *dsrB* and *mcrA* genes. The composition of the reaction mixes and run conditions are described in Article III and the used primers in **Table 3**. PCR products were verified with agarose gel electrophoresis and successful reactions from replicate reactions were pooled prior to sequencing. The sequencing was performed at the Research and Testing Laboratory, Texas, USA (180 m sam-

ples) and at the Institute of Biotechnology, Helsinki, Finland (all other samples) using the 454 FLX Titanium platform.

Statistical methods

DGGE gel images were normalized, Dice's coefficient of similarity was calculated and UPGMA cluster dendrograms were constructed in order to compare the similarity of the DGGE fingerprint profiles using the Bionumerics software. (Articles I and II).

The PAST software (Hammer et al. 2001) was used to test the normality and perform a canonical correspondence analysis on presence/absense data of phylotypes of different functional genes in connection to geochemistry (Article II) or microbial community structure and geochemical variables of the fractures (Article III).

Table 3. Primers used in molecular biological characterization of Outokumpu deep biosphere.

Target gene	Gene name	Method	Primer names forward / reverse	Forward primer sequence 5'->3'	Reverse primer sequence 5'->3'	Size of the product,	Article	Reference[*]
Ribosomal gene coding the 16S subunit of rRNA in bacteria	16S rRNA	PCR-DGGE	P2 / P3	ATTACCGCGGCTGCTGG	CCTACGGGAGGCAGCAG[1]	193	I,III	11
		qPCR	P1 / P2	CCTACGGGAGGCAGCAG	ATTACCGCGGCTGCTGG	193	I,III	11
		HTP sequencing	8f / P2	AGAGTTTGATCCTGGCTCAG	ATTACCGCGGCTGCTGG	500	I, II, III	4, 11
Ribosomal gene coding the 16S subunit of rRNA in archaea	16S rRNA	PCR-DGGE	A344f / 519rP	ACGGGGCGCAGCAGGCGCGA	TTACCGCGGCKGCTG[2]		I, II, III	2, 18
		nested PCR 1. step	A109f / A915r	ACKGCTCAGTAACACGT	GTGCTCCCCCGCCAATTCCT	800	I,III	7, 16
		qPCR	A109f / A744r	ACKGCTCAGTAACACGT	CCCGGGTATCTAATCC		I, III	7, 10
		HTP sequencing	A344f / A744r	ACGGGGCGCAGCAGGCGCGA	CCCGGGTATCTAATCC	430	II	2, 10
Dissimilatory sulfite reductase gene, β-subunit	*dsrB*	PCR-DGGE	Dsr2060f+GC / Dsr4r	CAACATCGTYCAYACCCAGGG	GTGTAGCAGTTACCGCA[1]	370	III	6, 17
		qPCR	Dsr2060f / Dsr4f	CAACATCGTYCAYACCCAGGG	GTGTAGCAGTTACCGCA		I	6, 17
		HTP sequencing	Dsr2060f / Dsr4f	CAACATCGTYCAYACCCAGGG	GTGTAGCAGTTACCGCA	370	III	6, 17
Methyl coenzyme M reductase, α-subunit	*mcrA*	PCR-cloning	M1 / M2	GCMATGCARATHGGWATGTC	TCATKGCRTAGTTDGGRTAGT	760	I, II	8
		nested PCR 1. step for cloning	mcrA412f / mcr1615r	GAAGTHACHCCNGAAACVATCA	GGTGDCCNACGTTCATBGC		III	14
		qPCR	M1 / M3	GCMATGCARATHGGWATGTC	TGTGTGAAWCCKACDCCACC		II	8, 14
		nested PCR 1. step for HTP-seq	mcrA463f / mcrA1614r	GAAGTHACHCCNGAAACVATCA	GGTGDCCNACGTTCATBGC	1200	III	14
		HTP sequencing	M1 / M3	GCMATGCARATHGGWATGTC	TGTGTGAAWCCKACDCCACC		III	8, 14
Acetyl coenzyme A carboxylase, biotin carboxylase subunit	*accC*	PCR-DGGE	ACAC2254f-GC / ACAC720r	GCTGATGCTATACATCCWGGWTAYGG	GCTGGAGATGGAGCYTCYTCWATTA	460	II	1
Acetyl coenzyme A synthase, β-subunit	*acsB*	PCR-cloning	ACS_f / ACR_r	CTBTGYGGDGCIGTIWSMTGG	AARCAWCCRCADGADGTCATIGG	216	II	5
Ribulose-1,5-bisphosphate carboxylase/oxygenase, large subunit	*rbcL*	PCR-cloning	K2f / V2r	ACCAYCAAGGCCSAAGCTSGG	GCCTTCSAGCTTGCCSACCRC	490	II	12
Ribulose-1,5-bisphosphate carboxylase/oxygenase, form II	*cbbM*	PCR-cloning	RuIIF1 / RuIIR3	GGHAACAACCARGGYATGGGYGA	CGHAGIGCGTTCATGCCRCC	800	II	15
		PCR-cloning	RuIIF2 / RuIIR2	GGIACVATCATCAARCCVAA	TGRCCIGCICGRTGRTARTGCA	488	II	15
Particulate methane monooxygenase, α-subunit	*pmoA*	PCR-cloning	pmo1f / pmor	GGGGGAACTTCTGGGGITGGAC	GGGGGRCIACGTCITTACCGAA	330	III	3
Nitrate reductase, α-subunit	*narG*	PCR-cloning	1960m2f / 2050m2r	TAYGTSGGGCAGGARAAACTG	CGTAGAAGAAGCTGGTGCTGTT	110	III	9
Ammonia monooxidase, α-subunit	*amoA*	PCR-DGGE	amoA1f / amoA-2R+GC	GGGGTTTCTACTGGTGGT	CCCCTCGGCAAAGCCTTCTTC[3]	490	III	13

[*]GC-clamp:CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGG

[1]GC-clamp:CGCCCGCCGCGCCCGCGCCGTCCCGCCGCCCCCGCCC

[2]GC-clamp:CGCCGCGCGGCGGGCGGGGCGGGGGC

[*]1) Auguet et al. (2008), 2) Bano et al. (2004), 3) Cheng et al. (1999), 4) Edwards et al. (1989), 5) Gagen et al. (2010), 6) Geets et al. (2006), 7) Großkopf et al. (1998), 8) Hales et al. (1996), 9) López-Gutiérrez et al. (2004), 10) modified from Barns et al. (1994), 11) Muyzer et al. (1993), 12) Namba et al. (2004), 13) Nicolaisen and Ramsing (2002), 14) Nyyssönen et al. (2012), 15) Spiridonova et al. (2004), 16) Stahl and Amann (1991), 17) Wagner et al. (1998), 18) Øvreås et al. (1997)

Sequence analyses

Sequences from DGGE and clone libraries were manually checked, edited, aligned and phylogenetic trees were constructed with the Geneious Pro software (Article I and II). In addition, the functional gene sequences acquired were compared to previously published shotgun-sequenced metagenomic libraries from Outokumpu (Article II, Nyyssönen et al. 2014) using the Blast algorithm in Geneious Pro.

High-throughput sequenced amplicon libraries were analysed using mothur and QIIME programs (Schloss et al. 2009, Caporaso et al. 2010). An in-house developed QIIME-based pipeline was used with 16S rRNA gene sequences and mothur with the functional gene sequences. The setup for quality control for each gene is described in detail in Article III. 16S rRNA gene sequences were compared against Greengenes representative OTU set version gg_13_8 with 97% similarity and the taxonomy was assigned according to RDP (Wang et al. 2007). Functional gene sequences aligned with model alignments of *dsrB* and *mcrA* obtained from Fungene repository (Fish et al. 2013). Final taxonomy of the representative OTUs was obtained by comparing sequences to public sequence databases using Geneious Pro. α-diversity estimates (Shannon H', Chao1, ACE) were calculated in QIIME for 16S rRNA gene sequence data, using 97% species similarity from datasets normalized with random subsampling of sequences according to the sample with lowest number of sequence reads (bacteria 3030, archaea 270)(Article III).

All sequences retrieved from DGGE, clone libraries and HTP sequencing libraries were deposited in the European Nucleotide Archive[6], and the accession numbers are presented in **Table 4**.

Table 4. Accession numbers of the sequences retrieved in this study.

Gene	Method	Accession numbers	Article
bacterial 16S rRNA	DGGE	HF565417-HF565444	I
	HTP-sequencing	ERS846377-ERS846388	III
archaeal 16S rRNA	DGGE	HF565395-HF565416	I
	HTP-sequencing	ERS846389-ERS846397	III
*acc*C	DGGE	HG967562-HG967579	II
*dsr*B	DGGE	HF565370-HF565394	I
		HG967613-HG967637	II
	HTP-sequencing	ERS846399-ERS846407	III
*mcr*A	cloning	HG967593- HG967612	II
	HTP-sequencing	ERS846408-ERS846414	III
*nar*G	cloning	LN589977-LN589983	II
*pmo*A	cloning	HG967580-HG96759	II

[6] http://www.ebi.ac.uk/ena

3.6 Prediction of functionality and co-occurrence analysis

Article III describes in detail the estimation of the functional content of predicted metagenomes of Outokumpu microbial communities. Metagenomes were reconstructed from the 16S rRNA and rRNA gene sequence data with the PICRUSt program (Langille et al. 2013). OTUs without taxonomic reference were removed from the taxonomy data, which was subsequently uploaded to Galaxy pipeline (Goecks et al. 2010, Blankenberg et al. 2010, Giardine et al. 2005) for PICRUSt. Weighted nearest sequenced taxon indexes (NSTI) were calculated and metagenomes predicted from the normalized taxonomy data. Normalization was done by dividing the abundance of each organism by its predicted 16S rRNA gene copy number because of the variation between the copy numbers of 16S rRNA gene in different microbes. From the predicted metagenomes, the presence/absence and abundance of selected metabolic pathways of microbes was determined computationally using the HUMAnN program (Abubucker et al. 2012).

Correlation coefficients for OTUs present in the microbial communities were calculated with the otu.association command in mothur. The co-occurrence based on significant ($p < 0.01$) pairwise Pearson correlations between different OTUs was visualized with biological network analysis program Gephi (Bastian et al. 2009). With the network analysis the keystone genera and the connectivity of the microbial communities in the deep Outokumpu bedrock were determined.

4. Results

4.1 Obtaining the samples from the Outokumpu deep biosphere

During the years when hydrogeochemistry and microbiology have been under study in Outokumpu, besides the continuous development of the sampling techniques and equipment, also field instrumentation and working methods have been refined.

In 2008, water samples were collected from the drill hole using the tube sampling method. Altogether 11 samples were recovered from the drill hole water column for microbial analyses and 19 for chemical analyses (depths 200–2300 m). Generally, the bacterial communities at different depths of the drill hole resembled one another, although the relative abundance of the different groups varied between the depths. The most dominant phylotypes belonged to *Comamonadaceae*, *Acholeplasma* and *Clostridiales*. The archaeal communities changed towards higher abundance of *Methanobacter* in greater depths (Nyyssönen et al. 2014).

In 2009 isolation of the fracture zones with packers and continuous pumping methods were taken to use. These methods enabled the collection of greater amount of samples and obtaining the biomass in anaerobic conditions in the field laboratory. In order to ascertain that samples were attained from the indigenous fracture fluids, electrical conductivity, pH and concentration of oxygen of the water were continuously monitored throughout the time of pumping. After installation of the sampling gear and start of the pumping, water chemistry rapidly stabilized to that of the in-situ conditions regardless of the sampling method (either packer isolation or slow flow through PA tube, see Section 3.1). The microbial cell numbers decreased during the pumping period. At the end of the pumping period, the highest numbers of microbial cells were detected in shallow fractures and the lowest from the deeper fractures, thus the cell number was decreasing according to the depth. In addition, changes in microbial community structure were detected during the pumping, especially in fractures located deeper, where the microbial diversity decreased according to DGGE analysis (Article I). The bacterial communities of the fracture zones shared similarity with the drill hole communities especially in the beginning of the pumping. β-proteobacterial phylotypes dominated the microbial communities in 500 m and 967 m fracture zones after one hour of pump-

ing, as was the case with the bacterial community in 600 m depth in the drill hole. During the pumping, clostridial phylotypes became more dominant in microbial community at the 967 m fracture, therefore resembling more the communities in the drill hole water column at depths of 1100–1500 m. The bacterial community at 2260 m fracture with the dominant phylotype belonging to actinobacteria differed from the drill hole communities approximately at the same depth already in the beginning of the pumping. However, the bacterial communities of the fracture zones at these depths differ also from one another and characterization of the bacterial community structure in 2260 m with HTP sequencing verified the results of the community analysis made with DGGE (Article III).

The archaeal community structure changed less than the bacterial community during the pumping, and communities resembled the drill hole communities especially in 1000–2300 m depths. SAGMEG archaea dominated the community in the 967 m fracture of the fracture zone and were abundant also in the archaeal communities in the drill hole at depth range of 200–1000 m.

4.2 Microbial community structure

The microbial communities derived from RNA were used as a proxy of an active community and the data acquired from DNA as a representation of the total community present in the samples. Overall, the bacterial communities detected in the fractures were more diverse than the archaeal communities (Articles I and III). All studied fractures differed in their bacterial community composition, although in the fractures from shallower depths, the total and active bacterial communities did not differ as much as they did in the deeper ones. Most diverse total bacterial communities were detected in the fractures at 1820 and 2300 m depth (H' = 6.3) (**Table 5**a). β-proteobacterial *Comamonadaceae* were the dominant representatives of the bacterial communities at 180 and 500 m fractures (**Figure 5**). The DGGE- and pyrosequencing results were compatible with each other concerning the bacterial communities at 500 m fracture (**Table 6**) (Articles I and III). In the 967 m fracture, clostridial phylotypes *Dethiobacter* and *Syntrophobotulus* dominated the total and active communities, respectively. In the 1820 m fracture, the total bacterial community was characterized by high diversity (H' = 6.3), with γ-proteobacterium *Pseudomonas* being the most abundant genus (15% relative abundance). In contrast, Firmicutes dominated the active community with relative abundance of over half of the community. Noticeably less proteobacterial phylotypes were observed in the active community than in the total community in the 1820 m fracture (6% relative abundance). It was observed with both DGGE and HTP sequencing that actinobacteria dominated the total bacterial community in the 2260 m fracture (**Table 6**). Candidate phylum OPB41 comprised approximately half of the total community and *Comamonadaceae* was the second most abundant group (33%) in the 2260 m fracture. The active community differed significantly from the total community in this fracture, while most abundant group was α-proteobacterial *Bradyrhizobium* (20%). The majority of the phylotypes of the total community in

the 2300 m fracture belonged to β-proteobacterial *Burkholderiales* (*Comamonadaceae*, *Leptothrix*, *Janthinobacterium*) and γ-proteobacterial *Pseudomonas* and *Acinetobacter*. The active community of this fracture represented mostly unknown phylotypes that could be characterized only to kingdom or phylum level.

Table 5. HTP-sequence diversity, abundance and richness estimates of a) bacteria and b) archaea. The data were normalized with random subsampling according to the sample with lowest number of sequence reads in each dataset.

A) BACTERIA

	180 m		500 m		967 m		1820 m		2260 m		2300 m	
Diversity index	DNA	RNA	DNA	RNA	DNA	RNA	DNA	RNA	DNA	RNA	DNA	RNA
Shannon	2,9	2,6	3,4	4,9	5,3	5,1	6,3	3,5	2,7	5,1	6,3	1,8
Chao1	208	125	428	461	326	363	523	538	394	492	495	209
ACE	230	144	463	483	340	388	546	558	430	531	529	229
observed species	143	90	329	395	274	318	432	412	286	446	424	116

B) ARCHAEA

	180 m		500 m		967 m		1820 m		2260 m		2300 m	
Diversity index	DNA	RNA	DNA	RNA	DNA	RNA	DNA	RNA	DNA	RNA	DNA	RNA
Shannon	2,4	2,1	0,9	0,8	2,3	1,2	n.d	n.d	0,6	0,8	0,8	n.d
Chao1	45	32	3	3	58	32	n.d	n.d	7	8	5	n.d
observed species	44	29	3	3	58	30	n.d	n.d	7	8	4	n.d

n.d = not detected

Table 6. Most abundant bacterial phyla detected from the Outokumpu fracture communities with two different molecular biological methods.

Fracture depth	DGGE		HTP-sequencing	
m	DNA	RNA	DNA	RNA
180	n.a	n.a	*Comamonadaceae*	*Comamonadaceae*
500	*Rhodobacteraceae*	*Rhodobacteraceae*	*Comamonadaceae*	*Comamonadaceae*
	Comamonas	*Comamonas*	*Rhodobacter*	*Rhodobacter*
	Acholeplasma	*Acholeplasma*	*Acholoeplasma*	*Dietzia*
967	*Peptococcaceae*	*Peptococcaceae*	*Comamonadaceae*	*Syntrophobotulus*
	Uncl. Firmicutes	Uncl. Firmicutes	*Dethiobacter*	*Clostridia*
	Acholeplasma	*Acholeplasma*	*Acholeplasma*	
1820	n.a	n.a	*Pseudomonas*	Uncl. Bacteria
			Uncl. Firmicutes	Uncl. Firmicutes
			Dethiosulfatibacter	*Dethiosulfatibacter*
2260	*Arthrobacter*	*Dethiosulfatibacter*	OPB41 (Actinobacteria)	*Micrococcus*
			Comamonadaceae	*Bradyrhizobium*
				Uncl. Firmicutes
				Uncl. Bacteria
2300	n.a	n.a	Uncl. Firmicutes	Uncl. Firmicutes
			Comamonadaceae	Uncl. Bacteria
			Pseudomonas	
			Acinetobacter	

n.a = not analyzed

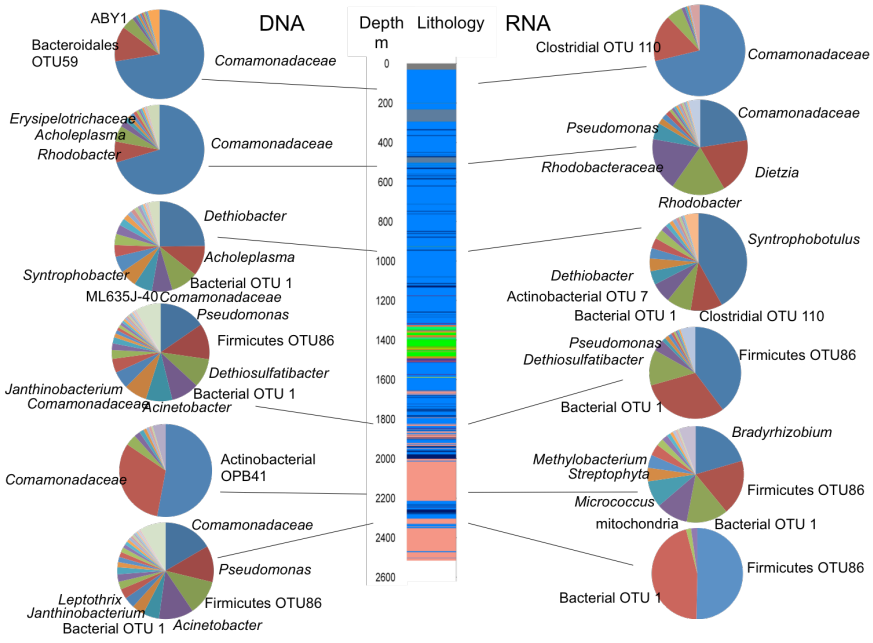

Figure 5. The bacterial community structure of the studied Outokumpu bedrock fractures at different depths. The bacterial groups with the highest relative abundance are named. The structures for the total communities are shown on the left and the active communities on the right of the Outokumpu lithology map in the middle. Explanation of the lithology can be found from **Figure 2**. The black line indicates the depth from which each sample originates (adapted from Article III).

The archaeal community structure revealed with DGGE matched with that obtained with the HTP sequencing (**Figure 6** and **Table 7**). *Methanobacteraceae*-like hydrogenotrophic methanogens dominated all archaeal communities except that of the fracture at 967 m depth. The total community of this fracture was dominated by archaea affiliating with candidate phylum SAGMEG-1. The active archaeal community at 967 m resembled the ones in the other fractures with *Methanobacteriaceae* being the most abundant group. The most diverse archaeal community was found in the fracture zone at 180 m (H' = 2.4), in which *Methanobacteraceae* dominated but also hydrogenotrophic *Candidatus Methanoregula* and methylotrophic *Methanolobus* archaea were abundant. The archaeal community at 967 m fracture had the highest amount of observed archaeal OTUs (58) (**Table 5**b).

Table 7. Most abundant archaeal phyla detected from the Outokumpu fractures with two different molecular biological methods.

Fracture depth	DGGE		HTP-sequencing	
m	DNA	RNA	DNA	RNA
180	n.a	n.a	*Methanobacteriaceae* Candid. *Methanoregula*	*Methanobacteriaceae* Candid. *Methanoregula* *Methanolobus*
500	*Methanobacteriaceae*	*Methanobacteriaceae*	*Methanobacteriaceae*	*Methanobacteriaceae*
967	SAGMEG-1	SAGMEG-1	SAGMEG-1	*Methanobacteriaceae*
1820	n.a	n.a	n.a	n.a
2260	*Methanobacteriaceae*	*Methanobacteriaceae*	*Methanobacteriaceae*	*Methanobacteriaceae*
2300	n.a	n.a	*Methanobacteriaceae*	*Methanobacteriaceae*

n.a = not analyzed

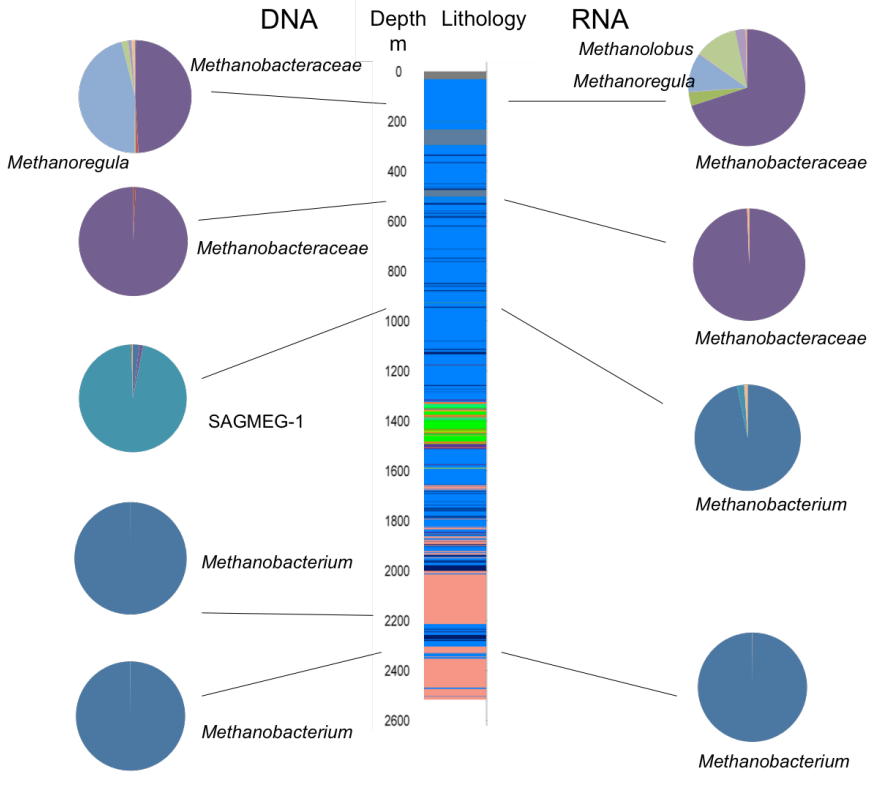

Figure 6. The archaeal community structure of the studied Outokumpu bedrock fractures at different depths. The phylotypes with the highest relative abundance are named. The structures for the total communities are shown on the left and the

active communities on the right of the Outokumpu lithology map in the middle. Explanation of the lithology can be found from **Figure 2**. The black line indicates the depth from which each sample originates (adapted from Article III).

4.3 The core microbial community

A phylotype is considered to belong to the core microbiome of Outokumpu deep biosphere if it is detected in all communities of Outokumpu fractures. Only a few proteobacterial and clostridial phylotypes constituted the core microbiome in Outokumpu deep bedrock (Article III) (**Table 8**). These phylotypes were detected in all fracture communities at different frequencies. When the total and active communities were observed separately, the core community of the total bacterial communities was composed of 14 phylotypes, of which *Comamonadaceae* were most abundant in addition to *Dethiobacter* and *Pseudomonas*. In contrast, only four phylotypes were detected in all active bacterial communities (**Table 8**). In addition, *Methanobacteraceae* -affiliating methanogenic archaea could be regarded as part of the core microbiome in Outokumpu, as *Methanobacterium* was detected in the archaeal communities at fracture depths of 967 m and below and *Methanobacteraceae* dominated the communities in 180 m and 500 m fractures. Many members of the core microbiome in Outokumpu deep subsurface represented only a minority in the communities.

Table 8. The core bacterial communities of the Outokumpu deep bedrock fractures.

	range of the relative abundance (%)			
	in the total community		community	
	min	max	min	max
Comamonadaceae	7	72		
Dethiobacter	0.04	25		
Pseudomonas	0.02	15	0.06	6
Firmicutes OTU86	0.1	12	0.03	50
Bacterial OTU2	1	10	1	46
Dethiosulfatibacter	0.05	10	0.07	12
Actinobacterial OTU7	0.09	4		
Leptothrix	0.2	4		
Legionella	0.07	4		
Geosporobacter	0.1	2		
Clostridia OTU110	0.01	1		
Burkholderiales OTU197	0.02	0.7		
Clostridiales OTU111	0.01	0.5		
Peptococcaceae	0.02	0.3		

4.4 Carbon cycling in the Outokumpu deep biosphere

Carbon assimilation

Three autotrophic carbon assimilation pathways, the reductive pentose phosphate cycle (Calvin cycle), the reductive acetyl-CoA pathway (Wood-Ljungdahl pathway) and the hydroxypropionate-hydroxybutyrate pathway were tested with marker gene assays to investigate whether carbon assimilation in Outokumpu deep biosphere occurs via an autotrophic pathway or are organic substrates a more prominent source of carbon. The acetyl-coA carboxylase:biotin carboxylase (*accC*) gene fragments used as a marker for the hydroxypropionate-hydroxybutyrate pathway were detected from all studied depths (200–2300 m). (**Table 9**.) *AccC* gene fingerprints were divided to four clusters corresponding to the depth from which they were retrieved (Article II). *Parabacteroides* and novel Outo II group *accC* sequences were detected in upper parts of the drill hole, while clostridial and *Methanobacterium* -type *accC* were detected below 1100 m. Neither of the common autotrophic carbon fixation pathways (Calvin-Bassham-Benson or Wood-Ljungdahl cycle) was detected from Outokumpu drill hole fluids (Article II). In addition, analysis of the predicted metagenomes showed that the relative abundance of the genes involved in these autotrophic carbon fixation pathways were low in the fracture communities (Article III).

Methanogenesis and methanotrophy

While copy numbers of the marker gene for methanogenesis, methyl-coenzyme M reductase (*mcrA*), were extremely low or below the detection limit throughout the drill hole and fracture fluids, many archaeal phylotypes detected in the drill hole samples and from the fractures were characteristically methanogens. Cloning of the *mcrA* gene revealed a distinct spatial distribution pattern. At shallower depths in the drill hole, *Methanosarcina* -affiliating *mcrA* sequences were more abundant, while *Methanolobus* -types of *mcrA* sequences were found at 1300 and 1500 m depths and *Methanobacterium* were exclusively found from the deepest samples at 1900 m and below. Methanogen communities of the fracture fluids were comparable to those in the drill hole fluid at approximately corresponding depths, with *Methanobacteriaceae* dominating the total communities in the fractures located at 2260 m and 2300 m depths (**Table 9**). *Methanosarcina* were abundant in the active methanogen communities in the 500 and 967 m fractures as in the drill hole fluids at depths of 600 and 900 m (Articles II and III). The detection of *Methanolobus mcrA* sequences at 1300–1500 m depths is in agreement with the archaeal community structure at these depths, where *Methanolobus* was present with approximately 10–15% relative abundance (Article II and Nyyssönen et al. 2014).

Methanotrophs were detected at 1500 m and above from the drill hole. All sequenced *pmoA* clones were similar to each other and affiliated with *Methylomonas pmoA* sequences (Article II) (**Table 9**).

Table 9. Comparison of microbial communities studied with different marker gene assays from the Outokumpu deep biosphere.

Depth m		Dark carbon assimilation	Methanogenesis			Methanotrophy	Nitrate reduction	Sulphate reduction				
		DGGE	cloning	HTP-sequencing		cloning	cloning	DGGE			HTP-sequencing	
Drill hole	Fracture	DNA	DNA	DNA	RNA	DNA	DNA	DNA	RNA	DNA	RNA
	180			Unclassified *mcr*A	n.d						*Desulfatirhabdum*	*Desulfotomaculum*
200		*Parabacteroides*						*Desulfotomaculum*				
		Outoll group						*Pelotomaculum*				
	500			*Methanobrevibacter*	*Methanosarcina*							
								Peptococcaceae	n.d	*Desulfotomaculum*	*Pelotomaculum*	
600		*Parabacteroides*	*Methanosarcina*			*Methylomonas*	*Methylibium*	*Desulfotomaculum*				
								Pelotomaculum				
900		*Parabacteroides*	*Methanosarcina*			*Methylomonas*		*Desulfotomaculum*				
								Pelotomaculum				
	967			n.d	*Methanosarcina*							
								Desulfobulbaceae	*Syntrophobacter*	*Desulfotomaculum*	*Desulfotomaculum*	
1000		*Parabacteroides*					*Pseudomonas*	*Desulfotomaculum*				
								Pelotomaculum				
1100								*Desulfotomaculum*				
1300		*Alkaliphilus*	*Methanolobus*				*Pseudomonas*	*Desulfotomaculum*				
			Methanosarcina				*Methylibium*	*Desulforudis*				
1500		*Alkaliphilus*	*Methanolobus*			*Methylomonas*		*Desulfotomaculum*				
		Outoll group						*Desulforudis*				
1700		*Alkaliphilus*						*Desulfotomaculum*				
		Methanobacterium						*Pelotomaculum*				
	1820			n.d	n.d					*Desulfovibrio*	*Desulfobulbus*	
											Desulfatirhabdum	
1900		*Alkaliphilus*	*Methanobacterium*					*Desulfotomaculum*				
		Methanobacterium						*Pelotomaculum*				
2100		*Alkaliphilus*	*Methanosarcina*					*Desulfotomaculum*				
		Methanobacterium						*Pelotomaculum*				
	2260			*Methanobacterium*	n.d					*Desulfotomaculum*	n.d	
								n.d	n.d	Cand. *Desulforudis*		
	2300	*Alkaliphilus*	*Methanobacterium*	*Methanobacterium*	n.d					n.d	n.d	
		Methanobacterium						*Desulfotomaculum*				
								Pelotomaculum				

n.d = not detected

4.5 Anaerobic respiration in the Outokumpu deep biosphere

The abundance of N_2 gas in the Outokumpu deep subsurface could be a result of microbial denitrification process. Even though the nitrate concentration was below the detection limit in the Outokumpu groundwater, nitrate reducing microbes were present in the drill hole water column. Two types of potential denitrifiers, *Pseudomonas* and *Methylibium* were detected from the depths of 600, 1000 and 1300 m (**Table 9**) (Article II).

Sulfate concentration was typically low, around 1–2 mg l^{-1} in Outokumpu groundwater (Kietäväinen et al. 2013). However, sulfate-reducing microbes were detected both in the fracture zones (Articles I and III) and the drill hole fluids (Article II). The copy number of the marker gene for sulfate reduction, the dissimilatory sulfite reductase *dsrB*, was used as a proxy for the total amount of sulfate reducers in Outokumpu deep bedrock. The *dsrB* copy numbers were in general 300-600 ml^{-1} in the fractures, but in the 500 m fracture the concentration of *dsrB* gene copies was over ten times higher (7.39×10^3 copies ml^{-1}) and in the 967 m fracture over ten times lower (15 copies) compared to the other fracture communities than the one at 500 m depth. Typical members of the sulfate reducing communities in both fracture and drill hole communities were peptococcal phylotypes, such as *Desulfotomaculum* and *Pelotomaculum* (**Table 9**).

4.6 Estimation of the functional potential of predicted archaeal and bacterial metagenomes

The predicted metagenomes of the communities in the different fractures were similar when examined at the top-level functionality. The most abundant genes of the predicted metagenomes were genes involved in metabolism. However, genes involved in amino acid and carbohydrate metabolisms were more abundant in bacteria, while in predicted archaeal metagenomes energy metabolism genes were the most common (Article III) (**Figure 7**). The 180 m fracture zone community differed from the others with lower abundance of genes involved in branched-chain amino acid degradation and fatty acid metabolism. The most abundant energy metabolism genes of the predicted metagenomes of the bacterial communities of the fractures were involved in oxidative phosphorylation. The predicted archaeal metagenomes reflected the pyrosequencing results, as the metagenome of the 967 m fracture differed from the other fractures with higher abundance of genes for amino acid metabolism and low number of genes for energy metabolism. Methane metabolism genes were most abundant in the predicted archaeal metagenomes. In addition, genes needed for biosynthesis of coenzyme M were

present in all predicted archaeal metagenomes. As the methanogenesis pathway from CO_2 and H_2 was present at all depths, methanogenesis from methylamines or methanol was likely operational only in the archaeal communities of the fracture at 180 m (Article III).

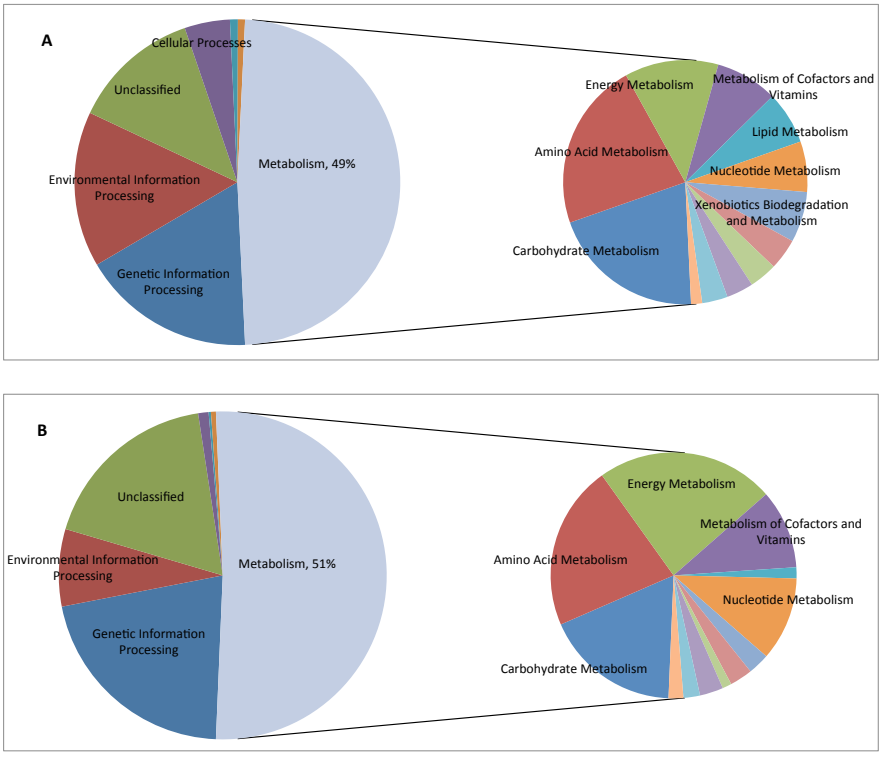

Figure 7. The average abundance of the genes of the predicted a) bacterial and b) archaeal metagenomes.

The nearest sequenced taxon index (NSTI) for predicted bacterial metagenomes ranged from 0.30 to 0.07, meaning that the OTUs of the communities shared on average 70–93% similarity with the reference genomes in the database, respectively. The archaeal NSTIs were lower indicating higher average similarity with reference genomes, except for the total archaeal community at 967 m depth with NSTI 0.29 (**Table 10**).

Table 10. Weighted nearest sequenced taxon indexes for bacterial and archaeal reconstructed metagenomes. The darker the background color of the cell, the higher NSTI value.

Depth	Bacteria		Archaea	
m	DNA	RNA	DNA	RNA
180	0,22	0,10	0,04	0,07
500	0,13	0,07	0,05	0,05
967	0,21	0,10	0,29	0,04
1820	0,07	0,07	n.d	n.d
2260	0,30	0,24	0,04	0,04
2300	0,07	0,12	0,04	n.d

n.d = not detected

4.7 Co-occurrence of OTUs and analysis of the keystone genera of the communities

Approximately 15% of all detected microbial OTUs in Outokumpu deep fracture zones showed significant correlation with each other. The network analysis of the significantly correlating OTUs of the total microbial community divided OTUs in 8 modules with number of nodes ranging from 4 to 41 (**Figure 8**A). The different OTUs in the network were highly connected. According to the high betweenness of centrality value, OTUs affiliating with *Burkholderiales* (*Comamonas*, *Herbaspirillum*, *Pelomonas* and other *Burkholderiaceae*) and with Clostridia (*Desulfitobacter*, other *Clostridiaceae*, *Dethiobacter*) were identified to be the keystone genera of the total microbial community in Outokumpu bedrock. The correlating OTUs of the active microbial community were divided in 8 clusters with number of nodes ranging from 2 to 64 in the network analysis (**Figure 8**B). This network was also highly connected and many of the keystone genera belonged to *Burkholderiales* (*Comamonas, Curvibacter*, other *Oxalobacteraceae* and *Herbaspirillum*).

A

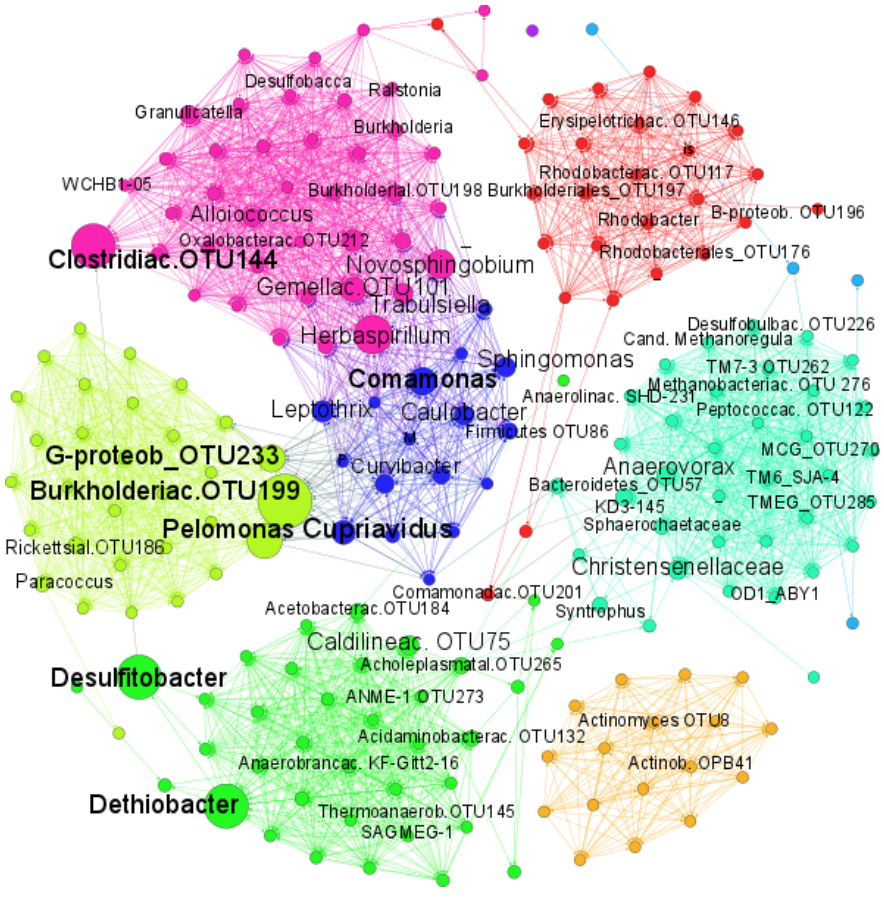

Figure 8. Co-occurrence network of a) the total microbial community and b) the active microbial community in Outokumpu bedrock. Size of the node corresponds to the betweenness of centrality value (Article III).

B

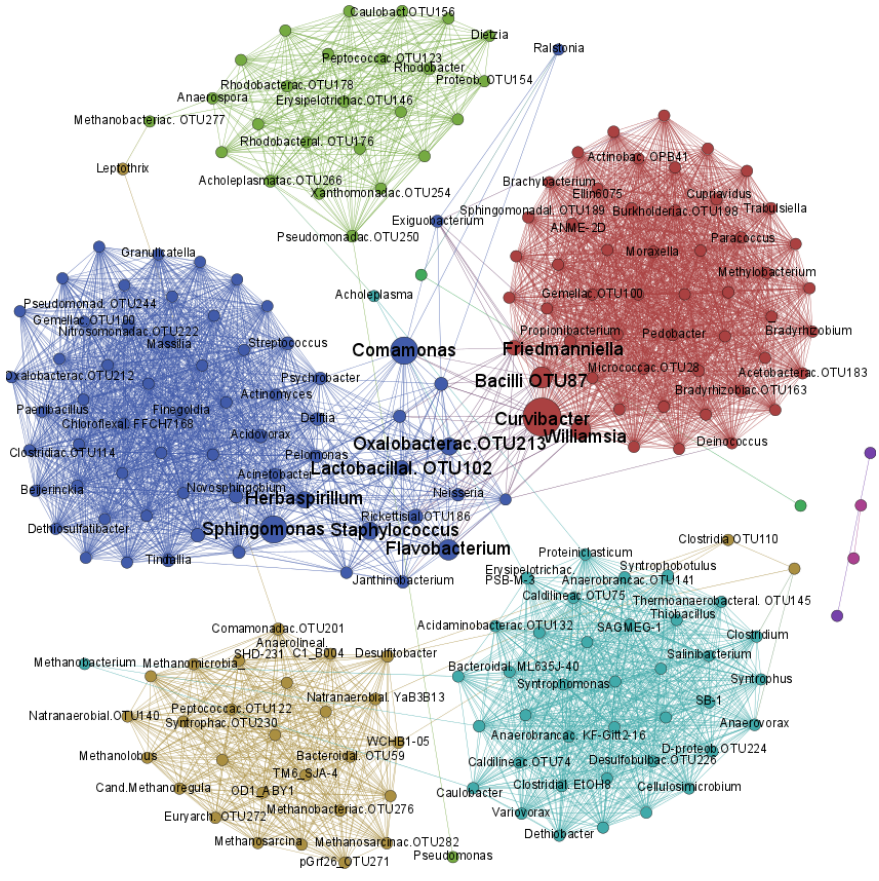

Figure 8. (continued)

5. Discussion

5.1 The microbial communities of the Outokumpu deep biosphere

Cell numbers are low but the diversity is high in Outokumpu deep biosphere. Generally, the most dominant group in the bacterial communities of both the fractures and the drill hole water column affiliate with *Comamonadaceae* (Article III, Itävaara et al. 2011b, Nyyssönen et al. 2014). Other abundant bacterial groups in belong to Clostridia that are commonly detected in deep terrestrial subsurface. Itävaara et al. (2011b) reported over 70% share of Clostridia in the microbial communities in Outokumpu drill hole at 900–1000 and 1400–1500 m. In addition, the highest proportion of clostridial phylotypes throughout the Outokumpu drill hole water column was detected from 1500 m depth (Nyyssönen et al. 2014). Thus, Clostridia are as abundant in fracture zones as they are in drill hole communities between depths of 900 and 1800 m in Outokumpu. Furthermore, the dominant sulfate reducers in Outokumpu fractures and drill hole water column affiliate with clostridial peptococci belonging to *Desulfotomaculum* and *Pelotomaculum* (Article II, III).

Archaea are vertically distributed in Outokumpu fracture zones and drill hole water column. Archaeal communities in the 180 m fracture were the most diverse (Article III). *Methanosarcina* and *Methanolobus* with versatile carbon metabolism in addition to SAGMEG archaea with unknown metabolic properties are more abundant above 1500 m depth in the archaeal communities in Outokumpu. Below this depth, the hydrogenotrophic *Methanobacterium* dominates the archaeal communities in both the fractures and the drill hole water column (Article III, Nyyssönen et al. 2014). Similar distribution is detected with methanogen communities (Article II).

The microbial community structure in the fractures does not reflect the water chemistry at Outokumpu. The bacterial communities in the 180 m and 500 m fractures were highly similar, although the prevalent water type is different. Additionally, the bacterial and archaeal communities in the 500 m fracture are different from the communities in the 967 m fracture albeit the same water type (II). Likewise, the communities in the 1820 m and the 2260 m fractures, both in the vicinity of the water type IV, are dissimilar. The total microbial communities in the 2300 m fracture share features with the microbial communities in the fractures located at shal-

lower depths despite the differences in the water chemistry (Article III, Kietäväinen et al. 2013).

The microbial communities of Outokumpu fractures represent numerous phylotypes with low abundance. These could be regarded as members of the rare biosphere with vast genetic potential to respond to possible environmental changes (Sogin et al. 2006, Article III).

5.2 Comparison of the microbial communities in Outokumpu and other deep terrestrial subsurface environments

The microbial communities in Outokumpu deep crystalline bedrock share common features with other deep ecosystems. The most striking resemblance in bacterial community structure is between Outokumpu fractures and serpentinization-driven environments. Bacteria affiliating with *Comamonadaceae* are dominant in bacterial communities and similar hydrogen-oxidizing clostridial species, such as *Dethiobacter* are detected from Outokumpu fractures, Lost City hydrothermal vents, alkaline spring fluids from Tablelands Ophiolite in USA and from subterrestrial aquifer in Portugal, (Article III, Brazelton et al. 2012, 2013, Tiago and Veríssimo 2013). The co-occurrence analysis revealed that OTUs belonging to *Burkholderiales*, *Clostridiaceae* and *Dethiobacter* were the keystone organisms of the microbial network in deep biosphere of Outokumpu. This further reinforces the importance of the discovery that Outokumpu deep biosphere resembles serpentinization-driven environments and might implicate that carbon and energy substrates formed in serpentinization reactions could sustain the microbial communities in this habitat. The spatial distribution of *Comamonadaceae* and clostridial hydrogenotrophs can be interpreted so that characteristically aerobic *Comamonadaceae* are using hydrogen oxidation in the shallower depths where trace amounts of oxygen can be available, while anaerobic *Dethiobacter* with capacity to reduce sulfur compounds coupled with H_2 oxidation has filled this ecological niche in 967 m fracture. While *Dethiobacter* is unable to reduce sulfate, this fracture also inhabits peptococcal sulfate reducers (*Desulfotomaculum*) in addition to fermenters (such as *Syntrophobotulus* in the active bacterial community).

The core bacterial community in Outokumpu is comprised of few phylotypes, which are also detected in fracture zones at the other Finnish deep bedrock site, Olkiluoto (*Comamonadaceae* and *Pseudomonadaceae*). *Comamonadaceae* are abundant in the bacterial community in the fracture at 600 m depth in Olkiluoto, but otherwise the bacterial communities in Olkiluoto fractures are different from those found from Outokumpu. Likewise, archaeal communities differ significantly in these two Finnish deep biosphere sites. In Outokumpu, archaeal communities are dominated by *Methanobacteriales* but in Olkiluoto, most of the dominant archaea belong to *Thermoplasmatales* and ANME-2D (Bomberg et al. 2014, 2015a,b). *Methanobacteriales* are strictly anaerobic, hydrogenotrophic methanogens, while *Thermoplasmatales* are mainly aerobic heterotrophs, except of the newly described H_2-oxidizing, methanol-reducing methanogens "*Methanoplasma-*

tales" (Paul et al. 2012). Based on the results in Article I and III, the archaeal communities of Outokumpu share features with those in the Witwatersrand deep subsurface (Takai et al. 2001, Moser et al. 2005, Gihring et al. 2006). Hydrogenotrophic CO_2-utilizing methanogens are found from deeper fractures (Takai et al. 2001, Moser et al. 2005, Gihring et al. 2006,) while archaea using more versatile carbon substrates inhabit depths above 1000 m (Lin et al. 2006a, Gihring et al. 2006). In addition, the archaeal community in 967 m fracture in Outokumpu is dominated by SAGMEG archaea, an archaeal candidate division first discovered from and abundant in South African gold mines (Takai et al. 2001). SAGMEG archaea have also been detected from Olkiluoto deep biosphere, from fracture fluids between 400 and 600 m depths (Bomberg et al. 2014, 2015).

Desulfotomaculum are abundant in sulfate reducing communities in Outokumpu and in several South African deep subsurface environments (Baker et al. 2003, Moser et al. 2003, 2005, Trimarco et al. 2006, Silver et al. 2010). This implies that the similar environmental conditions in these isolated deep subsurface environments favor especially sulfate reducers that are *Desulfotomaculum* -type of nonacetate oxidizers.

5.3 Inorganic energy substrates

In Outokumpu anaerobic deep subsurface, the possible energy conservation processes include nitrate, iron, manganese and sulfate reduction.

Potential for nitrate reduction was detected from drill hole water column from 1300 m and above (Article II). The *narG*-marker gene clones affiliated with *Pseudomonas*, which was one of the keystone genera in Outokumpu. *Pseudomonas* represents a typical denitrifier coupling organic carbon oxidation to nitrate reduction. In terrestrial deep bedrock biosphere of Äspö and other Swedish sites, nitrate reducers are the dominating physiological groups in addition to acetogens and sulfate reducers (Hallbeck and Pedersen 2008a, 2012). While some iron and manganese reducers have been detected in Äspö, very few or none of these taxa were detected in the community analyses in Outokumpu.

The traditional sulfate-reducing bacteria represent only a fraction of the total bacterial community. On the other hand, the total bacterial community structure suggests that thiosulfate reduction is a major metabolic trait in the fracture communities at 967 m and 1820 m depth with *Dethiobacter* and *Dethiosulfatibacter* as the dominating taxa. Neither of these thiosulfate-reducing bacteria can use sulfate as an electron acceptor (Sorokin et al. 2008, Takii et al. 2007). QPCR results confirm that in the 967 m fracture where *Dethiobacter* that does not possess *dsr*AB gene (Sorokin et al. 2008), was particularly abundant, the copy numbers of the *dsrB* gene were extremely low. Interestingly, *Syntrophobotulus* that was dominating the active bacterial community in the 967 m fracture is described to be a chemotrophic fermenter of glyoxylate but may also be the fermenting partner in a syntrophic relationship with methanogens or acetogens producing hydrogen and carbon dioxide for these microbial groups. The active archaeal and methanogen

communities in this fracture comprised of *Methanobacterium* and *Methanosarcina*, both of these can use hydrogen and carbon dioxide produced by *Syntrophobotulus*. The metagenomic data suggested that genes involved in acetyl-CoA fermentation and synthesis of acetone-ethanol-butanol at 1500 m depth in the drill hole (Nyyssönen et al. 2014). This depth also represented the highest proportion of clostridial phylotypes in the drill hole. The 1820 m fracture hosts the richest active sulfate reducing community and the copy numbers of *dsrB* gene transcripts were among the highest detected throughout the Outokumpu deep subsurface. Evidence for sulfate reduction can be sensed from the fluids especially at 1820 m depth, where the water has the distinctive odor of hydrogen sulphide.

5.4 Autotrophic potential vs. heterotrophy

The model for primary production in deep terrestrial biosphere is based on hydrogen and CO_2. Autotrophic organisms, such as acetogens or methanogens use the reducing power of hydrogen to produce acetate or methane from carbon dioxide (Pedersen 1997, 2000). However, organic compounds in deep subsurface can provide both carbon and energy source for heterotrophic microorganisms (Amend and Teske 2005, Colwell and D'Hondt 2013, Schrenk et al. 2013).

Many microbial groups abundant in Outokumpu fractures are able to use CO_2 as a carbon source (e.g. *Comamonadaceae*, *Dethiobacter*, *Desulfotomaculum*, *Methanobacterium*) (Article I, II, III). *Comamonadaceae* dominating the bacterial communities especially in the fracture zones at 180 and 500 m is a metabolically diverse group of organisms. *Comamonadaceae* are either chemoorganotrophs with a capacity to use a range of organic acid substrates or H_2-oxidizing aerobic chemolithotrophs (Willems et al. 1991). Clostridia were also frequently detected in microbial communities in Outokumpu (Articles I, II, III, Itävaara et al. 2011a,b, Nyyssönen et al. 2014). Many acetogens, autotrophic microbes producing acetate from CO_2 via autotrophic Wood-Ljungdahl pathway, belong to Clostridia. Acetogenesis has been demonstrated to have a significant role in the deep biosphere in Forsmark and Laxemar, Sweden, where acetogens were dominating the microbial communities (Hallbeck and Pedersen 2012). Bomberg et al. (2015b) detected genes coding the enzymes of Wood-Ljungdahl pathway in predicted bacterial metagenomes in Olkiluoto fractures in Finland, among other major autotrophic CO_2 fixation pathways. However, the specific marker gene for Wood-Ljungdahl pathway could not be detected with PCR from the Outokumpu drill hole (Article II) and the relative abundance of autotrophic CO_2 fixation mechanisms including the Wood-Ljungdahl pathway in the predicted bacterial metagenomes of the Outokumpu fractures was low (Article III). Conversely, in a metagenomic study of Outokumpu drill hole fluids, autotrophic reduction of CO_2 to acetate was evident (Nyyssönen et al. 2014). Nevertheless, as another autotrophic pathway, Calvin-Bassham-Benson cycle could not be verified either with the marker gene approach and *acc*C genes affiliating mainly with heterotrophic microbes were detected from all studied drill hole depths (Article II), it can be concluded that chemoor-

ganotrophs are major players in CO_2 assimilation in deep biosphere of Outokumpu (Article II). Incorporation of CO_2 via carboxylation reactions is important to heterotrophs for compensation of the metabolic imbalance created with utilization of carbon in biosynthesis and anaplerotic reactions. CO_2 incorporation by heterotrophs may be an important survival mechanism in oligotrophic, nutrient-depleted conditions (Alonso-Sáez et al. 2010) that also prevail in Outokumpu deep subsurface.

Some of the sulfate reducers detected in Outokumpu are facultative chemolithoautotrophs using Wood-Ljungdahl pathway for biosynthesis of organic compounds from CO_2, but many of these organisms can also use fermentation of organic compounds such as lactate, pyruvate and ethanol for energy and carbon source. *Desulfotomaculum*, *Desulfovibrio* and *Desulfobulbus* that were members of the sulfate reducing communities in Outokumpu fractures are characterized as nonacetate oxidizers, i.e. they excrete acetate as an end product (Madigan et al. 2008). Thus, these can provide a suitable carbon source for many heterotrophic microorganisms in Outokumpu deep biosphere.

Hydrogenotrophic methanogens are abundant members of the archaeal communities in Outokumpu, but as the number of archaeal cell number is only a fraction of bacteria (0.5% on average) based on 16S rRNA gene quantification (Article III), methanogens may not produce enough biomass to feed the heterotrophic bacterial communities. In addition, although marker genes of methane oxidation were detected in Outokumpu (Article II), known methane oxidizers were not among the most abundant taxa in the microbial communities.

Autotrophs are dependent on CO_2. However, the CO_2 concentration in Outokumpu deep bedrock is very low (Kietäväinen et al. 2013). This indicates that the CO_2 flux from other parts of the bedrock is either not very rapid and/or only a fraction of it enters the studied fractures while the gross of it has been consumed elsewhere. Since CO_2 is not abundantly available and autotrophic CO_2 fixation pathways could not be verified, the microbial communities in Outokumpu were presumed to use mainly organic carbon compounds. The classification of the bacterial community data on family level and determining the predominant metabolism of each family according to The Prokaryotes (Rosenberg et al. 2013) showed that organotrophy is the most common resolved metabolic trait in Outokumpu fracture zone communities (fracture zone communities (**Figure 9**) (Article III). However, in deeper fractures and especially in the active communities, several bacterial OTUs could not be classified to family level and thus their metabolic properties remain unclear.

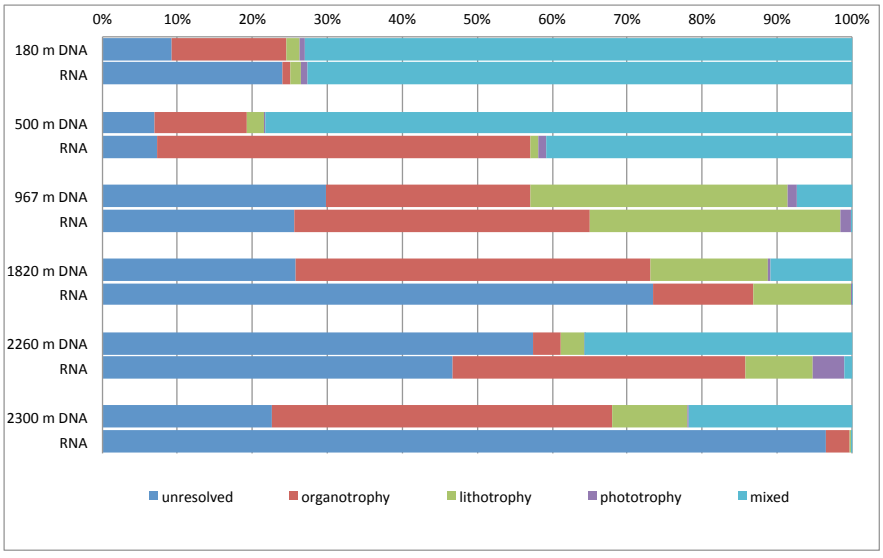

Figure 9. Bacterial physiotypes of OTUs in different fracture zone communities classified by their apparent metabolism (according to The Prokaryotes).

These results suggest that autotrophy may not be as common in Outokumpu as it is for example in Fennoscandian deep subsurface sites in Sweden (Pedersen 2000, Hallbeck and Pedersen 2012) and in Olkiluoto, Finland (Bomberg et al. 2015b).

5.5 Plausible geochemical organic carbon sources in Outokumpu

The drill hole pierces through the Outokumpu assemblage at the depth of 1300-1500 m (**Figure 2**). This assemblage contains ophiolitic rocks such as serpentinite, diopside and tremolite skarn (Västi 2011). This rock sequence can be the source of small organic carbon compounds that are known to be present in serpentinizing environments (McCollom and Seewald 2001, Lang et al. 2010, Lang et al. 2012). The seismic reflectors of the area indicate that this is not the only depth where ophiolite-derived rocks are located in Outokumpu area (Heinonen et al. 2011). For example, in the vicinity of the drill hole near 1000 m depth, a thin lens of ophiolitic rocks can be located in addition to the expansion of the main ore belt of the Outokumpu formation at depths above 200 m. Furthermore, size, volume and direction of the fracture zones are not known, thus some of these might reach these ophiolitic rock sequences. High microbial diversity in addition to high proportion of chemoorganotrophic clostridia at 1500 m depth in the drill hole (Nyyssönen et al. 2014) suggests that especially the small organic compounds formed in reducing conditions triggered by the serpentinization of ophiolitic rocks may provide

a prominent carbon source for the heterotrophic microbial communities. This might also be true for the microbial communities at 967 m and 1820 m fracture zones.

The lithology of Outokumpu Deep Drill Hole comprises of several black schist layers ranging from 0.1 to 13 m in thickness (Västi 2011). These were formed from organic matter deposited in anoxic seawater basins c. 1.9–2.0 Ga ago (Loukola-Ruskeeniemi 2011). Graphite in abundant black schist layers of mica gneiss in Outokumpu contains trace quantities of hydrocarbons (Taran et al. 2011). There are several reports on microbes using kerogen-containing black shale as sole carbon source, and these affiliate to *Clostridia* and *Pseudomonadales* (Petsch et al. 2001, 2005, Rosewarne et al. 2013). Both of these represent components of the Outokumpu core microbial community, although the abundance of these groups varies in the communities. In addition, clostridial phylotypes represent keystone species in Outokumpu deep biosphere. Fermenting clostridia could use the organic carbon derived from black schist in Outokumpu. In addition, some *Pseudomonas* species have the ability to degrade hydrocarbons in anaerobic conditions (Chayabutra and Ju 2000, Lalucat et al. 2006). By degrading complex organic materials, *Pseudomonas* in Outokumpu may provide more utilizable substrates for the rest of the microbial community. However, further proof is needed to demonstrate whether the microbial utilization of the recalcitrant, ancient carbon source of black schist graphite is possible in Outokumpu.

5.6 Evaluation of the used microbial community characterization methods

Microbial community changes can be tracked with fingerprinting methods such as DGGE. Thus, this method was chosen in Article I to evaluate the effect of the pumping period to the microbial community and also to get a first impression on the microbial community composition in the intrinsic fracture fluids in Outokumpu. This study demonstrated that an extensive pumping period was needed for attaining the intrinsic fracture fluid for microbial community analyses. DGGE revealed several bacterial, archaeal and sulfate reducer phylotypes. However, DGGE is limited in detecting only the dominant species of the microbial community, also in deep terrestrial biosphere (Muyzer et al. 1993, Kirk et al. 2004, Bomberg et al. 2014). The more detailed characterization of the microbial communities of the fractures was made with high-throughput sequencing (Article III). With this method, the rare biosphere and the co-occurrence network patterns of the microbial communities in deep terrestrial bedrock could be revealed. The number of bacterial phylotypes detected with DGGE in the fracture communities was almost a hundred times lower in most cases compared to the number of observed species from HTP data (**Table 11**). DGGE phylotypes of archaeal and sulfate reducing communities on the other hand covered more of the diversity. In the 500 m fracture, a double amount of archaeal phylotypes was detected with DGGE in comparison to HTP sequencing, probably because of the low amount of archaea (< 100 16S

rRNA gene copies ml^{-1}) in the sample affected to the amplification efficiency (Article III).

Table 11. Number of the detected phylotypes with DGGE and HTP sequencing in three fractures.

Fracture depth m		Characterization method			
		DGGE fingerprinting		HTP sequencing	
		DNA	RNA	DNA	RNA
500	bacteria	11	13	329	396
	archaea	7	8	3	3
	SRB	4	n.a	51	7
967	bacteria	10	15	274	318
	archaea	20	8	58	30
	SRB	7	5	25	12
2260	bacteria	6	4	286	446
	archaea	9	9	7	9
	SRB	n.a	n.a	37	n.a

n.a = not available

The DGGE analysis did perform well for rough detection of the dominating microbial groups, such as β-proteobacteria in 500 m fracture zone and *Clostridia* in deeper zones along with SAGMEG in 967 m fracture and *Methanobacteriales* dominating in other archaeal communities. Nevertheless, with over a hundred times more bacterial phylotypes detected by HTP sequencing in this study, the HTP sequencing method revealed the high diversity of the community more efficiently.

Functional communities were characterized with DGGE or clone libraries. Clone libraries based on nitrate reduction and methane oxidation marker genes revealed only few different phylotypes, while the *mcr*A clone library reflected fairly well the archaeal HTP sequencing results of the drill hole communities (Nyyssönen et al. 2014). Phylotypes affiliating with *Methanobacterium* were dominant in both clone libraries and pyrosequences at 1900 and 2300 m depth, while *Methanolobus*-, *Methanobacterium*- and *Methanosarcina* -affiliating clones were found at 1300 m depth in both studies. However, methanosarcinal phylotypes dominated the methanogen clone libraries at 900 m and above in the drill hole, but in the fracture zone communities, this group was represented a low relative abundance and was present only in the 180 m fracture. Hence, specific functional gene clone libraries may be sensitive enough to detect small but significant functional microbial groups.

6. Conclusions

Outokumpu fracture zones host diverse microbial communities depending mainly on chemoorganotrophic metabolism. These communities are intrinsic, i.e. they differ in structure from both the drill hole microbial communities and from fracture to fracture. Bacteria form the majority of these communities, but several archaeal taxa and different subcommunities based on functionality were detected. Most dominant phylotypes in the microbial communities belong to β-proteobacteria and *Clostridia*, particularly to *Comamonadaceae*, *Peptococcaceae* and *Anaerobrancaceae*. Representatives of these are the keystone genera of the Outokumpu fracture ecosystem. Many OTUs detected in this study were affiliated with phylotypes or species detected from serpentinization-driven ecosystems and other deep subsurface environments.

Hydrogenotrophic metabolism is characteristic to the most dominant microbial groups, but the organic carbon compounds usage infers that heterotrophy is important carbon assimilation strategy in deep subsurface on Outokumpu. In addition, autotrophy might play only a minor role in carbon fixation in deep subsurface of Outokumpu, as marker genes for common autotrophic carbon fixation pathways were not detected.

In summary, this thesis contributes to the biogeographic trend that different deep subsurface sites host microbial communities with structural similarities often related with depth. The geochemical properties of the deep subsurface appear to determine the microbial community structure to a certain level. The carbon and energy sources are likely the most important factors determining the community structure in Outokumpu.

7. Future outlook

Microbial communities have been characterized successfully from several terrestrial deep subsurface sites. With the aid of HTP sequencing methods and subsequent computational bioinformatics analyses and data mining methods, in addition to traditional enrichment or cultivation-based approaches, the functionality of the microbial communities is unveiling. However, there are several questions remaining that need to be addressed in future research of deep crystalline bedrock biosphere. For example, how deep can we still find life in the Earth's crust? How have microbes adapted to the long-term isolation and the environmental stress? How long have microbes existed in the depths? Distinguishing the environmental factors that hinder the existence of microbial life in depths of bedrock would allow us to determine the ultimate limits of life. In addition, answering these questions might bring us closer to understanding the origin of life on Earth and possibilities of life on other celestial bodies such as Mars.

Another question concerns the possible sources of carbon and other chemical elements imperative to life in deep bedrock biosphere. Geochemical processes yielding both soluble organic and inorganic carbon are relatively well known, but microbial contribution to these processes is not well understood in deep environments. In addition, microbial utilization of solid materials, such as rocks containing recalcitrant carbon compounds would need more research. Moreover, the occurrence and influence of serpentinization in Outokumpu remains unclear. On-going serpentinization is not reported in Outokumpu, even though ophiolitic rocks are abundant, so the hypothesis of serpentinization-driven microbial communities remain unsupported until the residence times of the products, the movements in the bedrock and flux from the origin of serpentinization are resolved.

Lastly, there are recent studies on eukaryotes inhabiting marine and terrestrial deep biosphere (Orsi et al. 2013, Sohlberg et al. 2015). These have not been characterized from Outokumpu, even though for example some fungi are known for their ability to degrade complex carbon compounds found in rocks. This capacity might be a key process for releasing the carbon from complex, recalcitrant material of black schist for the use of the rest of the microbial community.

References

Abubucker S, Segata N, Goll J et al. 2012. Metabolic reconstruction for metagenomic data and its application to the human microbiome. *PLoS Comput Biol*;**6**:e1002358

Ahokas H, Penttinen T, Pöllänen J. 2014. Quality Review of in situ TDS Data from the Olkiluoto Site – Drillholes OL-KR1 – KR53.

Ahonen L, Kaija J, Paananen M et al. 2004. Palmottu Natural Analogue: A Summary of the Studies. Geological Survey of Finland, Nuclear Waste Disposal Research, Report YST-121.

Ahonen L, Kietäväinen R, Kortelainen N et al. 2011. Hydrogeological characteristics of the Outokumpu deep drill hole. Kukkonen IT (Ed.). Geol Surv Finland, Spec Pap;51:151–68.

Anttila P, Ahokas H, Front K et al. 1999. Final Disposal of Spent Nuclear Fuel in Finnish Bedrock - Kivetty Site Report. Posiva report 99-09, Posiva Oy, Olkiluoto.

Amann R, Ludwig W, Schleifer KH. 1988. Beta-subunit of ATP-synthase: a useful marker for studying the phylogenetic relationship of eubacteria. J Gen Microbiol;134:2815–21.

Amend JP, Teske A. 2005. Expanding frontiers in deep subsurface microbiology. Palaeogeogr Palaeoclimatol Palaeoecol;219:131–55.

Amy PS, Haldeman DL, Ringelberg D et al. 1992. Comparison of identification systems for classification of bacteria isolated from water and endolithic habitats within the deep subsurface. Appl Environ Microbiol;58:3367–73.

Anderson C, Pedersen K, Jakobsson A-M. 2006. Autoradiographic comparisons of radionuclide adsorption between subsurface anaerobic biofilms and granitic host rocks. Geomicrobiol J;23:15–29.

Anderson RE, Sogin ML, Baross JA. 2015. Biogeography and ecology of the rare and abundant microbial lineages in deep-sea hydrothermal vents. FEMS Microbiol Ecol;91:1–11.

Auguet J-CC, Borrego CM, Bañeras L et al. 2008. Fingerprinting the genetic diversity of the biotin carboxylase gene (accC) in aquatic ecosystems as a potential marker for studies of carbon dioxide assimilation in the dark. *Environ Microbiol*;**10**:2527–36.

Baker BJ, Moser DP, MacGregor BJ et al. 2003. Related assemblages of sulphate-reducing bacteria associated with ultradeep gold mines of South Africa and deep basalt aquifers of Washington State. Environ Microbiol; 5:267–77.

Ball P. 2013. The importance of water. In: Smith IWM, Cockell CS, Leach S (Eds.) Astrochemistry and Astrobiology. Springer; 169-97

Bano N, Ruffin S, Ransom B et al. 2004. Phylogenetic composition of Arctic Ocean archaeal assemblages and comparison with Antarctic assemblages. Appl Environ Microbiol;**70**:781–9.

Barberán A, Bates ST, Casamayor EO et al. 2012. Using network analysis to explore co-occurrence patterns in soil microbial communities. ISME J; 6:343–51.

Barns SM, Fundyga RE, Jeffries MW et al. 1994. Remarkable archaeal diversity detected in a Yellowstone National Park hot spring environment. Proc Natl Acad Sci U S A;**91**:1609–13.

Bastian M, Heymann S, Jacomy M. 2009. Gephi: an open source software for explor-ing and manipulating networks. International AAAI Conference on Web-logs and Social Media.

Bethke CM, Sanford R a., Kirk MF et al. 2011. The thermodynamic ladder in geomicrobiology. Am J Sci;311:183–210.

Blanco Y, Rivas L a., García-Moyano A et al. 2014. Deciphering the prokaryotic community and metabolisms in South African deep-mine biofilms through antibody microarrays and graph theory. PLoS One;9:e114180.

Blankenberg D, Von Kuster G, Coraor N et al. 2010. Galaxy: a web-based genome analysis tool for experimentalists. Curr Protoc Mol Biol; Chapter 19: Unit 19.10.1–21

Blazejak A, Schippers A. 2011. Real-time PCR quantification and diversity analysis of the functional genes aprA and dsrA of sulfate-reducing prokaryotes in marine sediments of the Peru continental margin and the Black Sea. Front Microbiol;2:10.3389/fmicb.2011.00253.

Bomberg M, Nyyssönen M, Nousiainen A et al. 2014. Evaluation of Molecular Techniques in Characterization of Deep Terrestrial Biosphere. Open J Ecol;04:468–87.

Bomberg M, Nyyssönen M, Pitkänen P et al. 2015a. Active Microbial Communities Inhabit Sulphate-Methane Interphase in Deep Bedrock Fracture Fluids in Olkiluoto, Finland. Biomed Res Int.

Bomberg M, Lamminmäki T, Itävaara, M. 2015b. Estimation of microbial metabolism and co-occurrence patterns in fracture groundwaters of deep crystalline bedrock at Olkiluoto, Finland. Biogeosciences Discuss.;12, 13819–57

Borgonie G, García-Moyano a, Litthauer D et al. 2011. Nematoda from the terrestrial deep subsurface of South Africa. Nature;474:79–82.

Borgonie G, Linage-Alvarez B, Ojo A et al. 2015. Deep subsurface mine stalactites trap endemic fissure fluid Archaea, Bacteria, and Nematoda possibly originating from ancient seas. Front Microbiol;6.

Brazelton WJ, Nelson B, Schrenk MO. 2012. Metagenomic evidence for H2 oxidation and H2 production by serpentinite-hosted subsurface microbial communities. Front Microbiol;2:1–16.

Brazelton WJ, Morrill PL, Szponar N et al. 2013. Bacterial communities associated with subsurface geochemical processes in continental serpentinite springs. Appl Environ Microbiol;79:3906–16.

Campbell BJ, Cary SC. 2004. Abundance of Reverse Tricarboxylic Acid Cycle Genes in Free-LivingMicroorganisms at Deep-Sea Hydrothermal Vents. Appl Env Microbiol;70:6282–9.

Caporaso JG, Kuczynski J, Stombaugh J et al. 2010. QIIME allows analysis of high-throughput community sequencing data. Nature Methods;7:335-6

Chapelle FH, O'Neill K, Bradley PM et al. 2002. A hydrogen-based subsurface microbial community dominated by methanogens. Nature;415:312–5.

Chayabutra C, Ju LK. 2000. Degradation of n-hexadecane and its metabolites by Pseudomonas aeruginosa under microaerobic and anaerobic denitrifying conditions. Appl Environ Microbiol;66:493–8.

Cheng YS, Halsey JL, Fode KA et al. 1999. Detection of methanotrophs in groundwater by PCR. Appl Environ Microbiol;**65**:648–51.

Chi Fru E. 2008. Constraints in the colonization of natural and engineered subterranean igneous rock aquifers by aerobic methane-oxidizing bacteria inferred by culture analysis. Geobiology;6:365–75.

Chivian D, Brodie EL, Alm EJ et al. 2008. Environmental genomics reveals a single-species ecosystem deep within Earth. Science;322:275–8.

Christensen T, Kjeldsen P, Albrechtsen H-J et al. 1994. Attenuation of landfill leachate pollutants in aquifers. Crit Rev Environ Sci Technol;24:119–202.

Cockell CS, Voytek M a., Gronstal AL et al. 2012. Impact disruption and recovery of the deep subsurface biosphere. Astrobiology;12:231–46.

Cockell CS, Nixon S. 2013. The boundaries of life. In: Smith IWM, Cockell CS, Leach S (Eds.) Astrochemistry and Astrobiology. Springer; 211–41

Colwell FS, D'Hondt S. 2013. Nature and Extent of the Deep Biosphere. Rev Mineral Geochemistry;75:547–74.

Das AP, Sukla LB, Pradhan N et al. 2011. Manganese biomining: A review. Bioresour Technol;102:7381–7.

Davidson MM, Silver BJ, Onstott TC et al. 2011. Capture of planktonic microbial diversity in fractures by long-term monitoring of flowing boreholes, Evander Basin, South Africa. Geomicrobiol J;28:275–300.

Dhillon A, Lever M, Lloyd KG et al. 2005. Methanogen diversity evidenced by molecular characterization of methyl coenzyme M reductase A (mcrA) genes in hydrothermal sediments of the Guaymas Basin. Appl Environ Microbiol;71:4592–601.

Edwards U, Rogall T, Blöcker H et al. 1989. Isolation and direct complete nucleotide determination of entire genes. Characterization of a gene coding for 16S ribosomal RNA. Nucleic Acids Res;**17**:7843–53.

Etiope G, Sherwood Lollar B. 2013. Abiotic methane on Earth. Rev Geophys;51:276–99.

Fish JA, Chai B, Wang Q et al. 2013. FunGene: the functional gene pipeline and re-pository. Front Microbiol;4:291.

Frape SK, Blyth A, Stotler RL et al. 2013. Deep fluids in the continents. In Treatise on Geochemistry: Second Edition. Vol 7. Elsevier. Pp. 517–62.

Fredrickson JK, Balkwill DL. 2006. Geomicrobial processes and biodiversity in the deep terrestrial subsurface. Geomicrobiol J;23:345–56.

Fredrickson JK, McKinley JP, Bjornstad BN et al. 1997. Pore-size constraints on the activity and survival of subsurface bacteria in a late cretaceous shale-sandstone sequence, northwestern New Mexico. Geomicrobiol J;14:183–202.

Fry NK, Fredrickson JK, Fishbain S et al. 1997. Population structure of microbial communities associated with two deep, anerobic, alkaline aquifers. Appl Environ Microbiol;63:1498–504.

Fry JC, Horsfield B, Sykes R et al. 2009. Prokaryotic Populations and Activities in an Interbedded Coal Deposit, Including a Previously Deeply Buried Section (1.6–2.3 km) Above ~ 150 Ma Basement Rock. Geomicrobiol J; 26:163-78.

Fukuda A, Hagiwara H, Ishimura T et al. 2010. Geomicrobiological properties of ultra-deep granitic groundwater from the Mizunami Underground Research Laboratory (MIU), central Japan. Microb Ecol;60:214–25.

Gagen EJ, Denman SE, Padmanabha J et al. 2010. Functional gene analysis suggests different acetogen populations in the bovine rumen and tammar wallaby forestomach. Appl Environ Microbiol;**76**:7785–95.

Geets J, Borremans B, Diels L et al. 2006. *DsrB* gene-based DGGE for community and diversity surveys of sulfate-reducing bacteria. J Microbiol Methods; **66**:194–205.

Giardine B, Riemer C, Hardison RC et al. 2005. Galaxy: a platform for interactive large-scale genome analysis. Genome Res;15:1451-5

Gihring TM, Moser DP, Lin L-HH et al. 2006. The distribution of microbial taxa in the subsurface water of the Kalahari Shield, South Africa. Geomicrobiol J;23:415–30.

Gniese C, Bombach P, Rakoczy J et al. 2014. Relevance of deep-subsurface microbiology for underground gas storage and geothermal energy production. In: Schippers A, Glombitza F, Sand W (Eds.). Geobiotechnology II. Springer; 95–121.

Goecks J, Nekrutenko A, Taylor J et al. 2010. Galaxy: a comprehensive approach for supporting accessible, reproducible, and transparent computational research in the life sciences. *Genome Biol*;**11**:R86.

Gold T. 1992. The deep, hot biosphere. Proc Natl Acad Sci U S A;89:6045–9.

Griffiths RI, Thomson BC, James P et al. 2011. The bacterial biogeography of British soils. Environ Microbiol;13:1642–54.

Grosskopf R, Janssen PH, Liesack W. 1998. Diversity and structure of the methanogenic community in anoxic rice paddy soil microcosms as examined by cultivation and direct 16S rRNA gene sequence retrieval. Appl Environ Microbiol;**64**:960–9.

Hales BA, Edwards C, Ritchie DA et al. 1996. Isolation and identification of methanogen-specific DNA from blanket bog peat by PCR amplification and sequence analysis. Appl Environ Microbiol;**62**:668–75.

Hallam SJ, Putnam N, Preston CM et al. 2004. Reverse methanogenesis: testing the hypothesis with environmental genomics. Science;305:1457–62.

Hallbeck L, Pedersen K. 2008a. Characterization of microbial processes in deep aquifers of the Fennoscandian Shield. Appl Geochem;23:1796-819

Hallbeck L, Pedersen K. 2008b.Explorative Analyses of Microbes, Colloids, and Gases Together with Microbial Modeling: Site Description Model SDM-Site Laxemar. SKB Report R-08-109, Swedish Nuclear Fuel and Waste Management Co., Stockholm.

Hallbeck L, Pedersen K. 2012. Culture-dependent comparison of microbial diversity in deep granitic groundwater from two sites considered for a Swedish final repository of spent nuclear fuel. FEMS Microbiol Ecol;81:66–77.

Hammer Ø, Harper D, Ryan P. 2001. Past: Paleontological Statistics Software Package for education and data analysis. Paleontología Electroníca;4: 1–9.

Haveman SA, Pedersen K. 2002a. Distribution of culturable microorganisms in Fennoscandian Shield groundwater. FEMS Microbiol Ecol;39:129–37.

Haveman SA, Pedersen K. 2002b. Microbially mediated redox processes in natural analogues for radioactive waste. J Contam Hydrol;55:161–74.

Haveman SA, Pedersen K, Ruotsalainen P. 1999. Distribution and metabolic diversity of microorganisms in deep igneous rock aquifers of Finland. Geomicrobiol J;16:277–94.

Head IM, Jones DM, Larter SR. 2003. Biological activity in the deep subsurface and the origin of heavy oil. Nature;426:344–52.

Heinonen S, Kukkonen IT, Heikkinen PJ et al. 2011. High resolution reflection seismics integrated with deep drill hole data in Outokumpu, Finland. In: Kukkonen IT (ed.) Geol Surv Finland, Spec Pap;51:105-18.

Higuchi R, Fockler C, Dollinger G. 1993. Kinetic PCR analysis: real-time monitoring of DNA amplification reactions. Nat Biotechnol;**11**:1026–30.

Hinrichs K-U, Inagaki F. 2012. Downsizing the deep biosphere. Science;338:204–5.

Hoehler TM, Jørgensen BB. 2013. Microbial life under extreme energy limitation. Nat Rev Microbiol;11:83–94.

Holland G, Lollar BS, Li L et al. 2013. Deep fracture fluids isolated in the crust since the Precambrian era. Nature;497:357–60.

Itävaara M, Nyyssönen M, Bomberg M et al. 2011a. Microbiological sampling and analysis of the Outokumpu Deep Drill Hole biosphere in 2007–2009. Geol Surv Finland, Spec Pap;51:199–206.

Itävaara M, Nyyssönen M, Kapanen A et al. 2011b. Characterization of bacterial diversity to a depth of 1500 m in the Outokumpu deep borehole, Fennoscandian Shield. FEMS Microbiol Ecol;77:295–309.

Javaherdashti R. 2011. Impact of sulphate-reducing bacteria on the performance of engineering materials. Appl Microbiol Biotechnol;91:1507–17.

Jones DM, Head IM, Gray ND et al. 2008. Crude-oil biodegradation via methanogenesis in subsurface petroleum reservoirs. Nature;451:176–80.

Jørgensen BB, D'Hondt S. 2006. Ecology. A starving majority deep beneath the seafloor. Science;314:932–4.

Kaija J, Blomqvist R, Ahonen, L et al. 1998. The Hydrogeochemical Database of Palmottu. The Palmottu Natural Analogue Project, Technical Report 98-08.

Kallmeyer J, Pockalny R, Adhikari RR et al. 2012. Global distribution of microbial abundance and biomass in subseafloor sediment. Proc Natl Acad Sci U S A;109:16213–6.

Kalyuzhnaya MG, Khmelenina VN, Kotelnikova S et al. 1999. Methylomonas scandinavica sp. nov., a new methanotrophic psychrotrophic bacterium isolated from deep igneous rock ground water of Sweden. Syst Appl Microbiol;**22**:565–72.

Katz A, Starinsky A, Marion GM. 2011. Saline waters in basement rocks of the Kaapvaal Craton, South Africa. Chem Geol;289:163–70.

Kepner RL, Pratt JR. 1994. Use of fluorochromes for direct enumeration of total bacteria in environmental samples: past and present. Microbiol Rev;**58**:603–15.

Kerrick M. 2001. Present and past nonanthorponenic CO_2 degassing from the solid earth. Rev Geophys;39:565–85.

Kieft TL, McCuddy SM, Onstott TC et al. 2005. Geochemically generated, energy-rich substrates and indigenous microorganisms in deep, ancient groundwater. Geomicrobiol J;**22**:325–35.

Kietäväinen R, Ahonen L, Kukkonen IT et al. 2013. Characterisation and isotopic evolution of saline waters of the Outokumpu Deep Drill Hole, Finland – Implications for water origin and deep terrestrial biosphere. Appl Geochemistry;32:37–51.

Kietäväinen R, Ahonen L, Kukkonen IT et al. 2014. Noble gas residence times of saline waters within crystalline bedrock, Outokumpu Deep Drill Hole, Finland. Geochim Cosmochim Acta;145:159–74.

Kietäväinen R, Purkamo L. 2015. The origin, source and cycling of methane in deep crystalline rock biosphere. Front Microbiol;6.

Kim BH, Gadd MG. 2008. Bacterial physiology and metabolism. Cambridge University Press.

Kirk JL, Beaudette LA, Hart M et al. 2004. Methods of studying soil microbial diversity. J Microbiol Methods;58:169–88.

Knittel K, Boetius A. 2009. Anaerobic oxidation of methane: progress with an unknown process. Annu Rev Microbiol;63:311–34.

Kotelnikova S. 2002. Microbial production and oxidation of methane in deep subsurface. Earth-Sci Rev;58:367-95

Kotelnikova S, Pedersen K. 1997. Evidence for methanogenic Archaea and homoacetogenic Bacteria in deep granitic rock aquifers. FEMS Microbiol Rev;**20**:339–49.

Kotelnikova S, Pedersen K. 1998. Distribution and activity of methanogens and homoacetogens in deep granitic aquifers at Äspö Hard Rock Laboratory, Sweden. FEMS Microbiol Ecol;26:121–34.

Krumholz LR, McKinley JP, Ulrich GA et al. 1997. Confined subsurface microbial communities in Cretaceous rock. Nature;386:64-6.

Kukkonen IT. 2011. Outokumpu Deep Drilling Project 2003–2010. In: Kukkonen IT (Ed.). Geol Surv Finland, Spec Pap;51.

Kukkonen IT, Rath V, Kivekäs L et al. 2011a. Geothermal studies of the Outokumpu Deep Drill Hole. Kukkonen IT (Ed.). Geol Surv Finland, Spec Pap;51:181-98

Kukkonen IT, Rath V, Kivekäs L et al. 2011b. Geothermal studies of the Outokumpu Deep Drill Hole: Vertical variation in heat flow and palaeoclimatic implications. Phys Earth Planet Inter;188:9-25

Laaksoharju M, Tullborg EL, Wikberg P et al. 1999. Hydrogeochemical conditions and evolution at the Aspo HRL, Sweden. Appl Geochemistry;14:835–59.

Lalucat J, Bennasar A, Bosch R et al. 2006. Biology of Pseudomonas stutzeri. Microbiol Mol Biol Rev;70:510–47.

Lang SQ, Butterfield DA, Schulte M et al. 2010. Elevated concentrations of formate, acetate and dissolved organic carbon found at the Lost City hydrothermal field. Geochim Cosmochim Acta;74:941–52.

Lang SQ, Früh-Green GL, Bernasconi SM et al. 2012. Microbial utilization of abiogenic carbon and hydrogen in a serpentinite-hosted system. Geochim Cosmochim Acta;92:82-99

Langille MGI, Zaneveld J, Caporaso JG et al. 2013. Predictive functional profiling of microbial communities using 16S rRNA marker gene sequences. Nature Biotech;8:1-10.

LaRowe DE, Amend JP. 2015. Power limits for microbial life. Front Microbiol;6.

Lau MCY, Cameron C, Magnabosco C et al. 2014. Phylogeny and phylogeography of functional genes shared among seven terrestrial subsurface metagenomes reveal N-cycling and microbial evolutionary relationships. Front Microbiol;5.

Lerm S, Westphal A, Miethling-Graff R et al. 2013. Thermal effects on microbial composition and microbiologically induced corrosion and mineral precipitation affecting operation of a geothermal plant in a deep saline aquifer. Extremophiles;17:311–27.

Lever MA. 2013. Functional gene surveys from ocean drilling expeditions–a review and perspective. FEMS Microbiol Ecol;84:1–23.

Lever MA, Heuer VB, Morono Y et al. 2010. Acetogenesis in Deep Subseafloor Sediments of The Juan de Fuca Ridge Flank: A Synthesis of Geochemical, Thermodynamic, and Gene-based Evidence. Geomicrobiol J; 27:183–211.

Lever MA, Rouxel O, Alt JC et al. 2013. Evidence for microbial carbon and sulfur cycling in deeply buried ridge flank basalt. Science;339:1305–8.

Lin L-H, Hall J, Lippmann-Pipke J et al. 2005a. Radiolytic H_2 in continental crust: nuclear power for deep subsurface microbial communities. Geochemistry, Geophys Geosystems;6.

Lin L-H, Slater GF, Sherwood Lollar B et al. 2005b. The yield and isotopic composition of radiolytic H2, a potential energy source for the deep subsurface biosphere. Geochim Cosmochim Acta;69:893–903.

Lin L-H, Hall J, Onstott TC et al. 2006a. Planktonic microbial communities associated with fracture-derived groundwater in a deep gold mine of South Africa. Geomicrobiol J;23:475–97.

Lin L-H, Wang P-L, Rumble D et al. 2006b. Long-term sustainability of a high-energy, low diversity crustal biome. Science;324:479-82

Lloyd D, Hayes AJ. 1995. Vigour, vitality and viability of microorganisms. FEMS Microbiol Lett;**133**:1–7.

Lloyd JR, Lovley DR. 2001. Microbial detoxification of metals and radionuclides. Curr Opin Biotech;12:248-53

Lollar BS, Lacrampe-Couloume G, Slater GF et al. 2006. Unravelling abiogenic and biogenic sources of methane in the Earth's deep subsurface. Chem Geol;226:328–39.

Lollar BS, Onstott TC, Lacrampe-Couloume G et al. 2014. The contribution of the Precambrian continental lithosphere to global H_2 production. Nature; 516:379–82.

López-Gutiérrez JC, Henry S, Hallet S et al. 2004. Quantification of a novel group of nitrate-reducing bacteria in the environment by real-time PCR. J Microbiol Methods;**57**:399–407.

Loukola-Ruskeeniemi K. 2011a. Graphite- and sulphide-bearing schists in the Outokumpu R2500 drill core: comparison with the Ni-Cu-Co-Zn-Mn-rich black schists at Talvivaara, Finland. In: Kukkonen IT (Ed.). Geol Surv Finland, Spec Pap;51:229–52

Lovley DR, Chapelle FH. 1995. Deep subsurface microbial processes. Rev Geophys;33:365.

Loy A, Duller S, Baranyi C et al. 2009. Reverse dissimilatory sulfite reductase as phylogenetic marker for a subgroup of sulfur-oxidizing prokaryotes. Environ Microbiol;11:289–99.

Luukkonen A, Pitkänen P, Ruotsalainen P et al. 1999. Hydrogeochemical Conditions at the Hästholmen site. Posiva report 99-26, Posiva Oy, Helsinki.

MacLean LCW, Pray TJ, Onstott TC et al. 2007. Mineralogical, chemical and biological characterization of an anaerobic biofilm collected from a borehole in a deep gold mine in South Africa. Geomicrobiol J;24:491–504.

Madigan MT, Martinko JM, Dunlap P V et al. 2008. Brock Biology of Microorganisms (12th Edition).

Margulies M, Egholm M, Altman WE et al. 2005. Genome sequencing in microfabricated high-density picolitre reactors. Nature;**437**:376–80.

Martiny JBH, Bohannan BJM, Brown JH et al. 2006. Microbial biogeography: putting microorganisms on the map. Nat Rev Microbiol;4:102–12.

McCollom TM. 2013. Laboratory simulations of abiotic hydrocarbon formation in Earth's deep subsurface. Rev Mineral Geochemistry;75:467–94.

McCollom TM, Amend JP. 2005. A thermodynamic assessment of energy requirements for biomass synthesis by chemolithoautotrophic microorganisms in oxic and anoxic environments. Geobiology;3:135–44.

McCollom TM, Bach W. 2009. Thermodynamic constraints on hydrogen generation during serpentinization of ultramafic rocks. Geochim Cosmochim Acta;73:856–75.

McCollom TM, Lollar BS, Lacrampe-Couloume G et al. 2010. The influence of carbon source on abiotic organic synthesis and carbon isotope fractionation under hydrothermal conditions. Geochim Cosmochim Acta;74:2717–40.

McCollom TM, Seewald JS. 2001. A reassessment of the potential for reduction of dissolved CO2 to hydrocarbons during serpentinization of olivine. Geochim Cosmochim Acta;65:3769–78.

McMahon S, Parnell J. 2014. Weighing the deep continental biosphere. FEMS Microbiol Ecol;87:113–20.

Meckenstock RU, Elsner M, Griebler C et al. 2015. Biodegradation: Updating the concepts of control for microbial clean-up in contaminated aquifers. Environ Sci Technol;49:7073-81

Meyer B, Kuever J. 2007. Phylogeny of the alpha and beta subunits of the dissimilatory adenosine-5′-phosphosulfate (APS) reductase from sulfate-reducing prokaryotes–origin and evolution of the dissimilatory sulfate-reduction pathway. Microbiology;153:2026–44.

Mills CT, Amano Y, Slater GF et al. 2010. Microbial carbon cycling in oligotrophic regional aquifers near the Tono Uranium Mine, Japan as inferred from δ 13 C and Δ 14 C values of in situ phospholipid fatty acids and carbon sources. Geochim Cosmochim Acta;74:3785–805.

Mitchell AC, Phillips AJ, Hamilton MA et al. 2008. Resilience of planktonic and biofilm cultures to supercritical CO_2. J Supercrit Fluids;47:318–25.

Morelli RM, Bell CC, Creaser R a. et al. 2010. Constraints on the genesis of gold mineralization at the Homestake Gold Deposit, Black Hills, South Dakota from rhenium-osmium sulfide geochronology. Miner Depos;**45**:461–80.

Moser DP, Onstott TC, Fredrickson JK et al. 2003. Temporal shifts in microbial community structure and geochemistry of an ultradeep South African gold mine borehole. Geomicrobiol J;20:517–48.

Moser DP, Gihring TM, Brockman FJ et al. 2005. Desulfotomaculum and Methanobacterium spp. dominate a 4- to 5-kilometer-deep fault. Appl Environ Microbiol;71:8773–83.

Mummey D, Holben W, Six J et al. 2006. Spatial stratification of soil bacterial populations in aggregates of diverse soils. Microb Ecol;51:404–11.

Muyzer G, de Waal EC, Uitterlinden AG. 1993. Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. Appl Environ Microbiol;59:695–700.

Nakagawa T, Hanada S, Maruyama A et al. 2002. Distribution and diversity of thermophilic sulfate-reducing bacteria within a Cu-Pb-Zn mine (Toyoha, Japan). FEMS Microbiol Ecol;41:199–209.

Nanba K, King GM, Dunfield K. 2004. Analysis of facultative lithotroph distribution and diversity on volcanic deposits by use of the large subunit of ribulose 1,5-bisphosphate carboxylase/oxygenase. Appl Environ Microbiol; **70**:2245–53.

Nealson KH, Inagaki F, Takai K. 2005. Hydrogen-driven subsurface lithoautotrophic microbial ecosystems (SLiMEs): do they exist and why should we care? Trends Microbiol;13:405–10.

Nercessian O, Bienvenu N, Moreira D et al. 2005. Diversity of functional genes of methanogens, methanotrophs and sulfate reducers in deep-sea hydrothermal environments. Environ Microbiol;7:118–32.

Newberry CJ, Webster G, Cragg B a. et al. 2004. Diversity of prokaryotes and methanogenesis in deep subsurface sediments from the Nankai Trough, Ocean Drilling Program Leg 190. Environ Microbiol;6:274–87.

Newby DT, Reed DW, Petzke LM et al. 2004. Diversity of methanotroph communities in a basalt aquifer. FEMS Microbiol Ecol;**48**:333–44.

Nicolaisen MH, Ramsing NB. 2002. Denaturing gradient gel electrophoresis (DGGE) approaches to study the diversity of ammonia-oxidizing bacteria. J Microbiol Methods;**50**:189–203.

Niibori Y. 2015. Radioactive Waste Disposal. In Radioactive Waste Engineering and Management. Springer. Pp. 153–73.

Nurmi PA, Kukkonen IT. 1986. A new technique for sampling water and gas from deep drill holes. Can J Earth Sci;23:1450-4

Nyyssönen M, Bomberg M, Kapanen A et al. 2012. Methanogenic and sulphate-reducing microbial communities in deep groundwater of crystalline rock fractures in Olkiluoto, Finland. Geomicrobiol J;29:863–78.

Nyyssönen M, Hultman J, Ahonen L et al. 2014. Taxonomically and functionally diverse microbial communities in deep crystalline rocks of the Fennoscandian shield. ISME J; 8:126-138.

O'Connell SP, Lehman RM, Snoeyenbos-West O et al. 2003. Detection of Euryarchaeota and Crenarchaeota in an oxic basalt aquifer. FEMS Microbiol Ecol;**44**:165–73.

Onstott TC, Lin LH, Davidson M et al. 2006. The origin and age of biogeochemical trends in deep fracture water of the Witwatersrand Basin, South Africa. Geomicrobiol J;23:369–414.

Onstott TC, McGown DJ, Bakermans C et al. 2009. Microbial communities in subpermafrost saline fracture water at the Lupin Au mine, Nunavut, Canada. Microb Ecol;58:786–807.

Orsi WD, Edgcomb VP, Christman GD et al. 2013. Gene expression in the deep biosphere. Nature;499:205–8.

Osburn MR, LaRowe DE, Momper LM et al. 2014. Chemolithotrophy in the continental deep subsurface: Sanford Underground Research Facility (SURF), USA. Front Microbiol;5.

Pace NR, Stahl DA, Lane DJ et al. 1986. The Analysis of Natural Microbial Populations by Ribosomal RNA Sequences. Adv Microb Ecol;**9**:1–55.

Parkes RJ, Linnane CD, Webster G et al. 2011. Prokaryotes stimulate mineral H_2 formation for the deep biosphere and subsequent thermogenic activity. Geology;39:219–22.

Paul K, Nonoh JO, Mikulski L et al. 2012. "Methanoplasmatales," Thermoplasmatales-related archaea in termite guts and other environments, are the seventh order of methanogens. Appl Environ Microbiol;78:8245–53.

Pedersen K. 1996. Investigations of subterranean bacteria in deep crystalline bedrock and their importance for the disposal of nuclear waste. Can J Microbiol;42:382–91.

Pedersen K. 1997. Microbial life in deep granitic rock. FEMS Microbiol Rev; 20:399–414.

Pedersen K. 2000. Exploration of deep intraterrestrial microbial life: current perspectives. FEMS Microbiol Lett;185:9–16.

Pedersen K. 2012a. Subterranean microbial populations metabolize hydrogen and acetate under in situ conditions in granitic groundwater at 450 m depth in the Äspö Hard Rock Laboratory, Sweden. FEMS Microbiol Ecol;81:217–29.

Pedersen K. 2012b. Influence of H_2 and O_2 on sulphate-reducing activity of a subterranean community and the coupled response in redox potential. FEMS Microbiol Ecol;82:653–65.

Pedersen K. 2013. Metabolic activity of subterranean microbial communities in deep granitic groundwater supplemented with methane and H_2. ISME J;7:839–49.

Pedersen K, Ekendahl S. 1990. Distribution and activity of bacteria in deep granitic groundwaters of southeastern Sweden. Microb Ecol;20:37–52.

Pedersen K, Ekendahl S. 1992. Assimilation of CO_2 and introduced organic compounds by bacterial communities in groundwater from southeastern Sweden deep crystalline bedrock. Microb Ecol;23:1–14.

Pedersen K, Haveman S. 1999. Analysis of Diversity and Distribution of Microorganisms in Palmottu Groundwater and Evaluation of their Influence on Redox Potential and Uranium (VI) Reduction. The Palmottu Natural Analogue Project, Technical Report 99-14.

Pedersen K, Arlinger J, Ekendahl S et al. 1996. 16S rRNA gene diversity of attached and unattached bacteria in boreholes along the access tunnel to the Äspö hard rock laboratory, Sweden. FEMS Microbiol Ecol;19:249–62.

Pedersen K, Arlinger J, Eriksson S et al. 2008. Numbers, biomass and cultivable diversity of microbial populations relate to depth and borehole-specific conditions in groundwater from depths of 4–450 m in Olkiluoto, Finland. ISME J;2:760–75.

Petitjean C, Deschamps P, López-García P et al. 2015. Rooting the domain archaea by phylogenomic analysis supports the foundation of the new kingdom Proteoarchaeota. Genome Biol Evol;7:191–204.

Petsch ST, Eglington TI, Edwards KJ. 2001. 14C-dead living biomass: evidence for microbial assimilation of ancient organic carbon during shale weathering. Science;292:1127–31.

Petsch ST, Edwards KJ, Eglinton TI. 2005. Microbial transformations of organic matter in black shales and implications for global biogeochemical cycles. Palaeogeogr Palaeoclimatol Palaeoecol;219:157–70.

Pikuta EV, Hoover RB, Tang J. 2007. Microbial extremophiles at the limits of life. Crit Rev Microbiol;33:183–209.

Pitkänen P, Partamies S. 2007. Origin and Implications of Dissolved Gases in Groundwater at Olkiluoto. Posiva report 2007-04, Posiva Oy, Olkiluoto.

Pitkänen P, Snellman M, Vuorinen U et al. 1996.Geochemical Modelling Study on the Age and Evolution of the Groundwater at the Romuvaara Site. Posiva report 96-06, Posiva Oy, Helsinki.

Pitkänen P, Luukkonen A, Ruotsalainen P et al. 1998. Geochemical Modelling of Groundwater Evolution and Residence Time at the Kivetty Site. Posiva report 98-07, Posiva Oy, Helsinki.

Posiva Oy. 2012. Olkiluoto site description 2011. Report POSIVA 2011-02, Posiva Oy.

Proskurowski G, Lilley MD, Seewald JS et al. 2008. Abiogenic hydrocarbon production at lost city hydrothermal field. Science;319:604–7.

Rajala P, Bomberg M, Kietäväinen R et al. 2015. Rapid Reactivation of Deep Subsurface Microbes in the Presence of C-1 Compounds. Microorganisms;3:17–33.

Rastogi G, Stetler LD, Peyton BM et al. 2009. Molecular analysis of prokaryotic diversity in the deep subsurface of the former Homestake gold mine, South Dakota, USA. J Microbiol;47:371–84.

Rastogi G, Osman S, Kukkadapu R et al. 2010. Microbial and Mineralogical Characterizations of Soils Collected from the Deep Biosphere of the Former Homestake Gold Mine, South Dakota. Microb Ecol;60:539–50.

Rinke C, Schwientek P, Sczyrba A et al. 2013. Insights into the phylogeny and coding potential of microbial dark matter. Nature;499:431–7.

Rosenberg E, DeLong EF, Lory S et al. 2013. The Prokaryotes. 4th Edition, Springer.

Rosewarne CP, Greenfield P, Li D et al. 2013. Draft Genome Sequence of Clostridium sp. Maddingley, Isolated from Coal-Seam Gas Formation Water. Genome Announc 2013;1:10.1128/genomeA.00081–12. Epub, Jan 24.

Russell MJ, Hall AJ, Martin W. 2010. Serpentinization as a source of energy at the origin of life. Geobiology;8:355–71.

Sahl JW, Schmidt R, Swanner ED et al. 2008. Subsurface microbial diversity in deep-granitic-fracture water in Colorado. Appl Environ Microbiol;74:143–52.

Sahl JW, Fairfield N, Harris JK et al. 2010. Novel microbial diversity retrieved by autonomous robotic exploration of the world's deepest vertical phreatic sinkhole. Astrobiology;10:201–13.

Schloss PD, Westcott SL, Ryabin T et al. 2009. Introducing mothur: Open-source, platform-independent, community-supported software for describing and comparing microbial communities. Appl Env Microb;75:7537-41

Schrenk MO, Brazelton WJ, Lang SQ. 2013. Serpentinization, carbon, and deep life. Rev Miner Geochem;75:575–606.

Scow KM, Hicks K. 2005. Natural attenuation and enhanced bioremediation of organic contaminants in groundwater. Curr Opin Biotechnol;16:246–53.

Sherwood Lollar B, Frape SK, Weise SM et al. 1993. Abiogenic methanogenesis in crystalline rocks. Geochim Cosmochim Acta;57:5087–97.

Sherwood Lollar B, Lacrampe-Couloume G, Slater GF et al. 2006. Unravelling abio-genic and biogenic sources of methane in the Earth's deep subsurface. Chem Geol;226:328-39

Sherwood Lollar B, Onstott TC, Lacrampe-Couloume G et al. 2014. The contribution of the Precambrian continental lithosphere to global H_2 production. Nature;516:379-82

Shimizu S, Akiyama M, Ishijima Y et al. 2006. Molecular characterization of microbial communities in fault-bordered aquifers in the Miocene formation of northernmost Japan. Geobiology;4:203–13.

Silver BJ, Onstott TC, Rose G et al. 2010. In situ cultivation of subsurface microorganisms in a deep mafic sill: implications for SLiMEs. Geomicrobiol J; 27:329–48.

Sogin ML, Morrison HG, Huber JA et al. 2006. Microbial diversity in the deep sea and the underexplored “rare biosphere.” Proc Natl Acad Sci U S A;103:12115–20.

Sohlberg E, Bomberg M, Miettinen H et al. 2015. Revealing the unexplored fungal communities in deep groundwater of crystalline bedrock fracture zones in Olkiluoto, Finland. Front Microbiol;6:573

Sorokin DY, Tourova TP, Mußmann M et al. 2008. Dethiobacter alkaliphilus gen. nov. sp. nov., and Desulfurivibrio alkaliphilus gen. nov. sp. nov.: Two novel representatives of reductive sulfur cycle from soda lakes. Extremophiles;12:431–9.

Spiridonova EM, Berg IA, Kolganova T V et al. 2004. An oligonucleotide primer system for amplification of the ribulose-1, 5-bisphosphate carboxylase/oxygenase genes of bacteria of various taxonomic groups. Microbiology;**73**:316–25.

Stahl D. A., Amann R. 1991. Development and application of nucleic acid probes in bacterial systematics. In: Stackebrandt E, Goodfellow M (eds.). Nucleic Acid Techniques in Bacterial Systematics. John Wiley & Sons Ltd, Chichester, England. 205–48.

Stevens T. 1997. Lithoautotrophy in the subsurface. FEMS Microbiol Rev. Vol 20, 327–37.

Stevens TO, McKinley JP, Fredrickson JK. 1993. Bacteria Associated with Deep, Alkaline, Anaerobic Groundwaters in Southeast Washington. Microb Ecol;25:35–50.

Stevens TO, McKinley JP. 1995. Lithoautotrophic Microbial Ecosystems in Deep Basalt Aquifers. Science;270:450–5.

Stotler RL, Frape SK, Ruskeeniemi T et al. 2009. Hydrogeochemistry of groundwaters in and below the base of thick permafrost at Lupin, Nunavut, Canada. J Hydrol;373:80–95.

Stotler RL, Frape SK, Freifeld BM et al. 2011. Hydrogeology, Chemical and Microbial Activity Measurement Through Deep Permafrost. Ground Water;49:348–64.

Stotler RL, Frape SK, Ruskeeniemi T et al. 2012. The interglacial–glacial cycle and geochemical evolution of Canadian and Fennoscandian Shield groundwaters. Geochim Cosmochim Acta;76:45–67.

Stroes-Gascoyne S, West JM. 1997. Microbial studies in the Canadian nuclear fuel waste management program. FEMS Microbiol Rev;20:573–90.

Swanner E, Templeton A. 2011. Potential for nitrogen fixation and nitrification in the granite-hosted subsurface at Henderson Mine, CO. Front Microbiol;2:254.

Szewzyk U, Szewzyk R, Stenström TA. 1994. Thermophilic, anaerobic bacteria isolated from a deep borehole in granite in Sweden. Proc Natl Acad Sci U S A;91:1810–3.

Szponar N, Brazelton WJ, Schrenk MO et al. 2013. Geochemistry of a continental site of serpentinization, the Tablelands Ophiolite, Gros Morne National Park: a Mars analogue. Icarus;224:286–96.

Takai K, Moser DP, DeFlaun M et al. 2001. Archaeal diversity in waters from deep South African gold mines. Appl Environ Microbiol;67:5750–60.

Takai K, Campbell BJ, Cary SC et al. 2005. Enzymatic and genetic characterization of carbon and energy metabolisms by deep-sea hydrothermal chemolithoautotrophic isolates of Epsilonproteobacteria. Appl Environ Microbiol;71:7310–20.

Takai K, Nakamura K, Toki T et al. 2008. Cell proliferation at 122 degrees C and isotopically heavy CH4 production by a hyperthermophilic methanogen under high-pressure cultivation. Proc Natl Acad Sci U S A;105:10949–54.

Takii S, Hanada S, Tamaki H et al. 2007. Dethiosulfatibacter aminovorans gen. nov., sp. nov., a novel thiosulfate-reducing bacterium isolated from coastal marine sediment via sulfate-reducing enrichment with Casamino acids. Int J Syst Evol Microbiol;**57**:2320–6.

Taran YA, Kliger GA, Sevastianov VS. 2007. Carbon isotope effects in the open-system Fischer-Tropsch synthesis. Geochim Cosmochim Acta;71:4474–87.

Taran LN, Onoshko MP, Mikhailov ND. 2011. Structure and composition of organic matter and isotope geochemistry of the Palaeoproterozoic graphite and sulphide-rich metasedimentary rocks from the Outokumpu Deep Drill Hole, Eastern Finland. Kukkonen IT (ed.). Geol Surv Finland, Spec Pap;51:219–28.

Teske A, Biddle JF. 2008. Analysis of deep subsurface microbial communities by functional genes and genomics. In Links Between Geological Processes, Microbial Activities and Evolution of Life. Springer. Pp. 159–76.

Tiago I, Veríssimo A. 2013. Microbial and functional diversity of a subterrestrial high pH groundwater associated to serpentinization. Environ Microbiol; 15:1687–706.

Trimarco E, Balkwill D, Davidson M et al. 2006. In situ enrichment of a diverse community of bacteria from a 4–5 km deep fault zone in South Africa. Geomicrobiol J;23:463–73.

Tyson GW, Chapman J, Hugenholtz P et al. 2004. Community structure and metabolism through reconstruction of microbial genomes from the environment. Nature;428:37–43.

Valdez B, Schorr M, Quintero M et al. 2009. Corrosion and scaling at Cerro Prieto geothermal field. Anti-Corrosion Methods Mater;56:28–34.

Västi K. 2011. Petrology of the drill hole R2500 at Outokumpu, eastern Finland–the deepest drill hole ever drilled in Finland. In: Kukkonen IT (ed.) Geol Surv Finland, Spec Pap;51:17–46.

Wagner M, Roger AJ, Flax JL et al. 1998. Phylogeny of dissimilatory sulfite reductases supports an early origin of sulfate respiration. J Bacteriol;180:2975–82.

Wang Q, Garrity GM, Tiedje JM, Cole JR. 2007. Naive Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. Appl Environ Microb;73: 5261-7

Wanger G, Southam G, Onstott TC. 2006. Structural and Chemical Characterization of a Natural Fracture Surface from 2.8 Kilometers Below Land Surface: Biofilms in the Deep Subsurface. Geomicrobiol J;23:443–52.

Ward JA, Slater GF, Moser DP et al. 2004. Microbial hydrocarbon gases in the Witwatersrand Basin, South Africa: Implications for the deep biosphere. Geochim Cosmochim *Acta*;68:3239–50.

Whitman WB, Coleman DC, Wiebe WJ. 1998. Prokaryotes: the unseen majority. Proc Natl Acad Sci U S A;95:6578–83.

Willems A, De Ley J, Gillis M et al. 1991. *Comamonadace*ae, a new family encompassing the Acidovorans rRNA Complex, including *Variovorax paradoxus* gen. nov., comb. nov., for *Alcaligenes paradoxus* (Davis 1969). Int J Syst Bacteriol;41:445–50.

Wilkins MJ, Daly R a., Mouser PJ et al. 2014. Trends and future challenges in sampling the deep terrestrial biosphere. Front Microbiol;5:1–8.

Woese CR. 1987. Bacterial Evolution Background. Microbiology;51:221–71.

Woese CR, Kandler O, Wheelis ML. 1990. Towards a natural system of organisms: proposal for the domains Archaea, Bacteria, and Eucarya. Proc Natl Acad Sci U S A;87:4576–9.

Wouters K, Moors H, Boven P et al. 2013. Evidence and characteristics of a diverse and metabolically active microbial community in deep subsurface clay borehole water. FEMS Microbiol Ecol;86:458–73.

Zhang G, Dong H, Xu Z et al. 2005. Microbial diversity in ultra-high-pressure rocks and fluids from the Chinese Continental Scientific Drilling Project in China. Applied Env Microbiol;71:3213–27.

Zhang G, Dong H, Jiang H et al. 2006. Unique Microbial Community in Drilling Fluids from Chinese Continental Scientific Drilling. Geomicrobiol J;23:499–514.

Zhang S, Mi J, He K. 2013. Synthesis of hydrocarbon gases from four different carbon sources and hydrogen gas using a gold-tube system by Fischer–Tropsch method. Chem Geol;349-350:27–35.

Øvreås L, Forney L, Daae FL et al. 1997. Distribution of bacterioplankton in meromictic lake Saelenvannet, as determined by denaturing gradient gel electrophoresis of PCR-amplified gene fragments coding for 16S rRNA. Appl Environ Microbiol;**63**:3367–73.

ARTICLE I

Dissecting the deep biosphere: retrieving authentic microbial communities from packer-isolated deep crystalline bedrock fracture zones

FEMS Microbiol Ecol. 2013, 85(2):324–37.

RESEARCH ARTICLE

Dissecting the deep biosphere: retrieving authentic microbial communities from packer-isolated deep crystalline bedrock fracture zones

Lotta Purkamo[1], Malin Bomberg[1], Mari Nyyssönen[1], Ilmo Kukkonen[2], Lasse Ahonen[2], Riikka Kietäväinen[2] & Merja Itävaara[1]

[1]VTT Technical Research Centre of Finland, Espoo, Finland; and [2]Geological Survey of Finland (GTK), Espoo, Finland

Correspondence: Lotta Purkamo, VTT Technical Research Centre of Finland, PO BOX 1000, Tietotie 2, 02044 VTT, Espoo, Finland. Tel.: +358 207 226 460; fax: +358 207 227 071; e-mail: lotta.purkamo@vtt.fi

Received 21 December 2012; revised 8 March 2013; accepted 21 March 2013. Final version published online 2 May 2013.

DOI: 10.1111/1574-6941.12126

Editor: Tillmann Lueders

Keywords
microbial communities; deep terrestrial fracture zones; packer method; *Archaea*; sulfate-reducing bacteria.

Abstract

Deep fracture zones in Finnish crystalline bedrock have been isolated for long, the oldest fluids being tens of millions of years old. To accurately measure the native microbial diversity in fracture-zone fluids, water samples were obtained by isolating the borehole fraction spanning a deep subsurface aquifer fracture zone with inflatable packers (500 and 967 m) or by pumping fluids directly from the fracture zone. Sampling frequency was examined to establish the time required for the space between packers to be flushed and replaced by indigenous fracture fluids. Chemical parameters of the fluid were monitored continuously, and samples were taken at three points during the flushing process. Microbial communities were characterized by comparison of 16S ribosomal genes and transcripts and quantification of *dsr*B (dissimilatory sulfate reduction) gene. Results suggest that fracture-zones host microbial communities with fewer cells and lower diversity than those in the drill hole prior to flushing. In addition, each fracture zone showed a community composition distinct from that inhabiting the drill hole at corresponding depth. The highest diversity was detected from the 967-m fracture zone. We conclude that the applied packer method can successfully isolate and sample authentic microbial fracture-zone communities of deep bedrock environments.

Introduction

A significant fraction of global biomass of Earth is located in the deep terrestrial bedrock environments (Whitman *et al.*, 1998). Microbial communities inhabiting these environments can vary greatly depending on the geography, geology, and the origin and residence time of fracture water. Rock–water interactions in deep subterranean crystalline bedrock fracture environments provide the fuel for the subsurface ecosystems (Stevens and McKinley, 1995), and host microbial communities with cell numbers ranging from 10^3 to 10^7 cells mL^{-1} (Pedersen *et al.*, 1996; Pedersen, 1997; Haveman & Pedersen, 2002; Lin *et al.*, 2006; Sahl *et al.*, 2008; Fukuda *et al.*, 2010; Itävaara *et al.*, 2011a, b; Nyyssönen *et al.*, 2012). Most of the studies characterizing the microbial communities of these environments have been conducted on borehole or free-flowing fluids from mines or caves (e.g. Northup & Lavoie, 2001; Moser *et al.*, 2003; Lin *et al.*, 2006; Barton & Northup, 2007; Sahl *et al.*, 2008; Davidson *et al.*, 2011) or from drill holes drilled from the surface (Onstott *et al.*, 1998; Shimizu *et al.*, 2006; Fukuda *et al.*, 2010). These studies have relied on several different methods for acquiring the samples. The samples from South African gold mines have been collected from free-flowing or packer-plugged boreholes as well as from a natural roof fracture (Takai *et al.*, 2001; Moser *et al.*, 2003; Onstott *et al.*, 2006). Sahl *et al.* (2008) studied free-flowing borehole water in the Henderson molybdenum mine. Studies of drill holes have shown some deep subsurface environments to consist of simple, single-species systems [e.g. *Firmicutes* bacterium: (Chivian *et al.*, 2008)] as well as diverse communities of *Bacteria, Archaea* (e.g. Fredrickson & Balkwill, 2006; Biddle *et al.*, 2008) and nematodes (Borgonie *et al.*, 2011). However, these findings are difficult to interpret because boreholes can be filled with

FEMS MICROBIOLOGY ECOLOGY

fluids from one or more fractures. In response to this perceived problem, a technique has been developed for obtaining groundwater samples from packer-sealed fractures with encouraging results (Haveman *et al.*, 1999; Nyyssönen *et al.*, 2012). To date, little is known about the indigenous microorganisms inhabiting fracture aquifers of crystalline bedrock. Haveman & Pedersen (2002) studied the aquifers of the Fennoscandian Shield bedrock, but the research relied on traditional cultivation techniques.

The Outokumpu scientific Deep Drill Hole (Kukkonen, 2011) in Eastern Finland has been well characterized with respect to its geology. Having known fracture systems (Ahonen *et al.*, 2011), it represents an excellent opportunity to study microbial communities living in the bedrock fracture zones. The drill hole water column can be divided into five different sections corresponding to separate fracture zones (Kietäväinen *et al.*, 2013). Microorganisms inhabiting these sections have been studied earlier by retrieving the entire water profile to 1500 m with polyamid (PA) tube segments (Itävaara *et al.*, 2011a, b). With this technique, it remains possible for microbial communities from different fracture zones to mix and interact in ways they would otherwise not. As the fractures have remained undisturbed for a long period of time (Kietäväinen *et al.*, 2013), it is assumed that the isolated fractures contain unique ecosystems. Properties of fracture zones in the form of characteristic gas profiles (i.e. methane, nitrogen, hydrogen, and helium), saline fluids and the slow chemical alteration of the fracture wall itself are factors that are expected to influence microbial diversity. Physiological isolation can play a major role in microbial evolution (Papke & Ward, 2004), and genetic variation in a population or community defines its capacity to future changes. Sulfate reduction coupled with H_2 oxidation is a common strategy for gaining energy in anaerobic deep subsurface environments with low organic carbon content (Lovley & Chappelle, 1995). Even though most sulfate-reducing bacteria (SRB) require organic carbon compounds as energy source, some can rely solely on H_2 as an electron donor (Muyzer & Stams, 2008). By quantifying SRB activity and abundance, the importance of this metabolic strategy in Outokumpu deep subsurface fracture zones can be estimated.

The aim of this study was to evaluate the packer sampling method as a technique for sampling the indigenous microbial community from bedrock fractures. It was hypothesized that the microbial communities inhabiting fracture zones at Outokumpu were distinct from those inhabiting the drill hole fluid. Samples were obtained from 500, 967, and 2260 m depths of the Outokumpu Deep Drill Hole, each representing a fracture zone with unique microbial communities, hydrogeochemistry and geology. We present the results of microbial community studies and discuss the utility of the packer sampling technique.

Materials and methods

Site description

The Outokumpu Deep Drill Hole (Kukkonen, 2011) (62.72°N, 29.07°E) located is in a Palaeoproterozoic, (*c.* 2 billion years old) rock formation in Eastern Finland. Metamorphosed layers of marine sediments and slices of oceanic lithosphere cut by slightly younger granitic rocks dominate the Outokumpu area. The drilling site is located in a topographically flat area, with about 33 m of glacial sand and silt on top of the crystalline bedrock. The drill hole is 2516 m deep and spans Precambrian bedrock, which comprises metasediments (mica schist, gneiss), ophiolitic (serpentinite, diopside, and tremolite skarn), and granitic rocks. The Outokumpu drill hole spans five groundwater types, each discharging into the drill hole from different fracture zones with minimal mixing and interflow and are characterized by unique stable isotope and geochemical profiles. Based on water stable isotope data, fracture-zone fluids are estimated to have residence times of up to tens of millions of years (Kietäväinen *et al.*, 2013). Thus, microbial communities are assumed to be well adapted to these nutrient-poor environments.

The drill hole has a diameter of 22 cm and is encased only to a depth of 39 m. Fracture-zone fluids are saline, anoxic, and increase in salinity and temperature with depth (Ahonen *et al.*, 2011; Kukkonen *et al.*, 2011). Rock types around the fracture zones are as follows: highly fractured chlorite-sericite schist at 500 m, mica schist at 967 m, and biotite-gneiss at 2260 m. Temperature in the drill hole increases approximately linearly with depth (Fig. 1), being approximately 12, 18, and 36 °C at 500, 967, and 2260 m, respectively. Electrical conductivity (EC; i.e. salinity) also increases with depth but with abrupt changes at *c.* 1400 and 2300 m. The pH is typically between 8 and 9 throughout the drill hole water column, except for the uppermost 400 m, where the pH exceeds 10 and is most likely influenced by the concrete reinforcement of the drill hole near the surface.

Isolation of the sampling sections

Fluid samples were collected from fracture zones located at three different depths. The fracture zone at 500 m was sampled during September and October 2010, at 967-m zone August and September 2009, and the one at 2260 m in June and July 2010. Two isolation techniques were applied. The 500- and 967-m fracture zones were isolated with expandable rubber and stainless steel packers placed above and below each zone, as described by Ahonen *et al.* (2011). The 2260-m fracture zone could not be packer-isolated in the same way because packers could not be

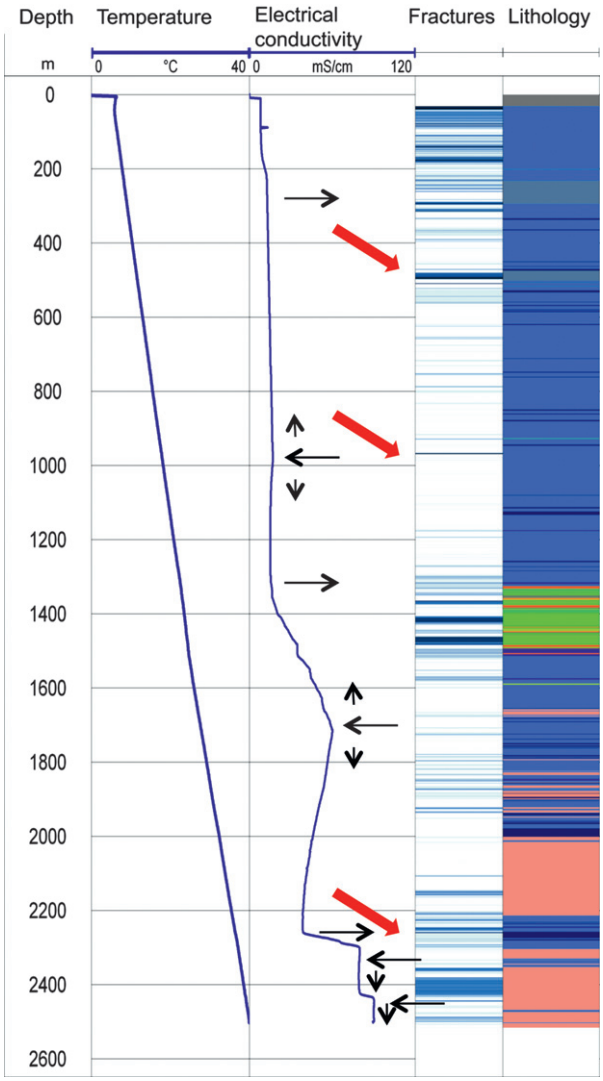

Fig. 1. Schematic of *in situ* temperature, EC, fractures and lithology of the Outokumpu drill hole. Black arrows indicate flow direction of fracture fluids: left = flow from fracture to drill hole; right = flow from drill hole to fracture. Red arrows denote locations of studied fracture zones. Gray and blue indicate metasediments, green and orange indicate ophiolite-derived rock types and pink pegmatitic granite (modified from Ahonen *et al.*, 2011).

passed through a constriction of *c.* 17 cm in the drill hole at 1480 m. Instead, one packer was placed at 1190 m to close the upper end of the column, while the end of the sampling tube was lowered to 2260 m. The packers were expanded with tap water to seal and isolate each fracture zone. The PA tube was initially filled with autoclaved ultrapure (MQ) water at 967 m or filter-sterilized reverse osmosis (RO) water at 2260 m. The 500- and 2260-m samples were collected with the same tube. Fluid from between the packers or from the 2260-m zone was slowly (about 2–5 L h^{-1}) pumped to the surface. This allowed the sample fluid to discharge from within the isolated fracture zone.

As the 2260-m fracture zone could not be isolated by packers, care was taken to adjust the pumping rate to equal the flow of fluid into the borehole. The characteristics of the fluid at this depth are unique and have previously been observed to be different from above lying water (Kietäväinen *et al.*, 2013). The sampled water had the same characteristics as observed for this depth before and was considered representative of the fracture-zone fluid and not the borehole water.

Sample collection

In order to confirm that the indigenous fracture fluids were sampled, EC, oxygen levels, and pH of the pumped water were continuously monitored. Depending on depth, water was pumped for 21 (500-m fracture), 28 (967 m), or 63 (2260 m) days from each fracture zone. Stable parameters of the pumped fluid were considered an indication that indigenous water was obtained. The 2260-m zone was deliberately pumped longer than the other two zones because of the lack of packers.

An anaerobic chamber (MBRAUN, Garching, Germany) was used to handle and manipulate samples taken from anoxic environments. The chamber was flushed with constant flow of N_2 gas o/n, and residual oxygen was removed with Anaerocult (Merck, NJ) anaerobic atmosphere developers. The chamber was considered anaerobic when Anaerocult indicator strips placed in different parts of the chamber turned white. Sample fluids were fed directly into the anaerobic chamber via a sterile PA tube to prevent the exposure to oxygen. In the anaerobic chamber, water samples were transferred to an acid-washed and sterile glass bottle (Schott) for subsequent concentration of microbial biomass. Microbial biomass was collected on sterile cellulose acetate filters of 0.2 μm pore size (Corning Inc., NY). Filters were removed from filter funnels, placed into sterile 50-mL tubes (Corning Inc.) and immediately frozen in dry ice prior to storage at −80 °C until further processing. Microscopy samples of 100 mL each were placed into acid-washed, sterile headspace glass flasks in the anaerobic chamber and closed with butyl rubber stoppers and aluminum crimp caps. Samples were collected at three time points to demonstrate the gradual change from drill hole water to authentic fracture fluid: (1) one hour after the start of pumping; (2) 1–3 weeks later; and (3) 3–6 weeks later. In general, three replicates were collected at each time point for each analysis, that is, 3 × 1 L each for analysis of community composition, and 3 × 0.5-L each for qPCR analysis (Supporting Information, Table S1). A control sample of 1 L was collected from the MQ- or RO-filled sampling tube.

Analyses of groundwater chemistry

EC, pH, reduction potential, temperature, and oxygen levels were continuously monitored and recorded during pumping. In addition, the chemical composition of the water was regularly analyzed. Samples for cation analysis (100 mL) were filtered (< 0.45 μm) in the field and acidified with 0.5 mL ultrapure HNO_3 and analyzed by ICP-MS (Perkin Elmer and Agilent Technologies) or ICP-OES (Thermo Jarrell Ash Corp.). Anions were analyzed by ion chromatography. All analyses were conducted at Labtium Oy (Espoo, Finland).

Total cell counts by microscopy

The number of microorganisms in the 500- and 2260-m samples was determined with fluorescent staining with 4′-6 –diamidino-2-phenylindole (DAPI). A 5-mL water sample was stained with 50 μL of 2.5 mg mL^{-1} of DAPI (Sigma) suspended in 200 μL of 2.5% glutaraldehyde (Merck, NJ). Staining was conducted in dark at room temperature for 15 min. Microbial cells in the 967-m fluid samples were stained with the BacLight Bacterial Viability Kit (Molecular Probes, Life Technologies, Ltd, UK), as described in Nyyssönen *et al.* (2012). The stained samples were collected on 0.2-μm GTPB membrane filter (Millipore, Billercia, MA) using a Millipore filtering unit (Millipore) and rinsed twice with 1 mL of filter-sterilized 0.9% NaCl and placed on a microscopy slide. The microscopy slides were examined with an epifluorescence microscope (Olympus BX60, Olympus Optical Ltd, Tokyo, Japan) with the Cell^P software (Olympus Optical Ltd), and the number of cells was counted from 30 random microscopy fields, as described in Nyyssönen *et al.* (2012). Microbial cell number was determined from an average of two replicate filters of each sample, and from two replicate samples from each depth.

DNA and RNA extraction

DNA was extracted with the PowerSoil DNA isolation kit (MoBio Laboratories, Inc., CA). Prior to extraction, the cellulose acetate membrane was cut with a sterile scalpel into 2 × 40 mm slices and placed into the PowerBead Tube. Extraction was performed according to the manufacturer's protocol, and DNA was eluted with 50 or 100 μL of molecular-grade water (0.5 and 1 L samples, respectively). The extracted DNA was stored at −80 °C.

RNA was extracted with the PowerWater RNA isolation kit (MoBio Laboratories, Inc.). The membranes were briefly thawed on ice and then immediately placed into the PowerWater Bead Tube. RNA was extracted according to manufacturer's protocol and eluted as for DNA extractions.

Negative reagent controls for DNA and RNA extractions were included in each extraction batch. The RNA extracts were checked for possible DNA contamination by performing a PCR with bacterial 16S rRNA gene primers. An additional DNase treatment was applied to samples containing residual DNA. The DNase treatment was performed in 20 μL reaction volumes containing 16 μL of RNA, 1 μL of 10× buffer, and 1 U of RNase-free DNase (Promega, Madison, WI). The mixture was kept at 25 °C for 30 min. After the incubation, 2 μL of DNase Stop solution RQ1 (Promega) was added and the mixture kept at 65 °C for 10 min.

Reverse transcription of RNA

RNA samples were translated to cDNA with the SuperScript III (Invitrogen, Life Technologies, Ltd, UK) and Random Hexamers (Promega). The cDNA was synthesized in triplicate, and a negative reagent control was included in every batch.

The reverse transcription reaction was performed in 20 μL (0.5 L samples) or 40 μL (1 L samples) reaction volumes. The reverse transcription reactions contained per 20 μL reaction mix 250 ng of random primers (Promega), 10 ng dNTP (Promega), 11 μL of RNA sample on nuclease-free water to a total volume of 13 μL. The reaction mix was kept at 65 °C for 5 min, chilled on ice, and centrifuged briefly. The remaining reagents were subsequently added, that is, 4 μL of 5× reaction buffer, 40 units DTT, 40 units RNasin inhibitor (Promega) and 200 units of Superscript III reverse transcriptase (Invitrogen, Life Technologies, Ltd). The reaction mix was incubated at 25 °C for 5 min and inactivated at 70 °C for 15 min. The triplicate sample reactions were pooled and stored at −80 °C.

Quantitative PCR analysis

Quantitative PCR (qPCR) was applied to estimate the number and activity of sulfate reducers by quantifying the number of *dsr*B gene copies and transcripts mL^{-1} in fracture-zone fluid. For the qPCR analyses, the KAPA SYBR FAST Roche LightCycler 480 2× qPCR Master Mix (Kapa Biosystems, Woburn, MA) was applied. The PCR reactions were performed in 10 μL reactions, containing 2.5 pmol of each primer, 1× KAPA SYBR FAST Roche LightCycler 480 qPCR Master Mix, and 1-μL template DNA or cDNA. The 370-bp fragment of *dsr*B gene and transcript was amplified with the primer pair DSRp2060f and DSR4r (Wagner *et al.*, 1998; Geets *et al.*, 2006). The qPCR reactions were conducted in triplicate using the following protocol in the LightCycler 480 (Roche, Basel, Switzerland): an initial denaturation step at 95 °C for 15 min, 50 cycles of

amplification step including 10 s at 95 °C, 35 s at 55 °C, 30 s at 72 °C, a final extension step for 3 min at 72 °C. Melting curve analysis was performed with a denaturation step for 10 s at 95 °C followed by a 1-min annealing step at 65 °C before a continuous heating from 65 to 95 °C with a ramp rate 0.11 °C s^{-1} and 5 acquisitions per 1 °C. A standard curve for each run was conducted in triplicate reaching from 1.5×10^6 to 1.5×10^0 copies of *dsr*B gene from *Desulfobulbus propionicus*. Inhibition of qPCR reactions was evaluated by mixing 1 μL of each sample DNA or cDNA and 1.5×10^5 copies of the *dsr*B-standard DNA dilution and comparing the amplification efficiency of the added control *dsr*B to the standard curve. No-template controls were included in every run.

Bacterial 16S ribosomal RNA gene and transcript PCR-DGGE

Bacterial 16S rRNA genes and transcripts were amplified from the DNA and cDNA of the 1 L samples using the P2 and P3 primer pair (Muyzer *et al.*, 1993) targeting the variable V3 region of the gene. PCR amplification was performed in 50 μL reactions containing 1× Dynazyme II buffer (10 mM Tris-HCl, pH 8.8, 1.5 mM $MgCl_2$, 50 mM KCl, and 1% Triton-X 100), 1% formamide, 0.2 mM final concentration of each deoxynucleoside triphosphate dNTP, 20 pmol of each primer, 2 units of Dynazyme II polymerase enzyme (ThermoFisher Scientific, Waltham, MA), and 1 μL of template. The PCR program consisted of an initial denaturation step at 94 °C for 5 min, 35 cycles of 1 min at 94 °C, 1 min at 54 °C, and 1 min at 72 °C. A final elongation step of 10 min was performed at 72 °C. The 193-bp PCR products were verified with agarose gel electrophoresis, and amplicons were separated with DGGE in 8% acrylamide and 20–65% denaturing gradient at 60 V and 60 °C for 18 h. The DGGE gel was stained with SYBR Green I and visualized with a Bio-Rad 168 GelDoc imager (Bio-Rad, Hercules, CA).

Archaeal 16S ribosomal RNA gene and transcript PCR-DGGE

Archaeal 16S rRNA gene fragments were amplified with a nested PCR approach, as described in Nyyssönen *et al.* (2012). First, an approximately 800-bp product of the archaeal 16s rRNA gene or transcript was amplified with primers A109f and Arch915r (Grosskopf *et al.*, 1998; Stahl & Amann, 1991). This PCR product was used as template for a second PCR with primer pair A344f and 519rP (Bano *et al.*, 2004; Lane, 1991) flanking the V3 region of the archaeal 16S rRNA gene, producing a 227-bp fragment. PCR amplification was performed in 50 μL reactions containing 1× Dynazyme II buffer (10 mM Tris-HCl, pH 8.8, 1.5 mM $MgCl_2$, 50 mM KCl, and 1% Triton-X 100), 0.2 mM each dNTP, 50 pmol of each primer, 3 U of Dynazyme II polymerase (ThermoFisher Scientific), and 4 or 2 μL of template (first-round PCR and second-round PCR, respectively). The PCR program for the larger product was conducted as described above for the bacteria, with the exception that the amplification was performed with 30 cycles. The nested PCR program consisted of an initial denaturation step at 95 °C for 5 min, 40 cycles of 1 min at 95 °C, 1 min at 50 °C, and 1 min at 72 °C. A final elongation step for 10 min was performed at 72 °C. The PCR products were run on a SYBR safe-stained 1% agarose gel after the nested PCR. The DGGE analysis was performed in 8% acrylamide and 40–65% denaturing gradient at 85 V and 60 °C for 20 h. The gel staining and imaging were conducted as described above.

*dsr*B gene PCR-DGGE

A 470-bp fragment of the *dsrB* gene or transcript was amplified as described in Nyyssönen *et al.* (2012) in 50-μL mixtures containing 1× Dynazyme II buffer (10 mM Tris-HCl, pH 8.8, 1.5 mM $MgCl_2$, 50 mM KCl, and 1% Triton-X 100), 0.2 mM of each dNTP, 50 pmol of each primer (Dsr4R and 2060F+GC, Wagner *et al.*, 1998; Geets *et al.*, 2006), 2 U of Dynazyme II polymerase enzyme (ThermoFisher Scientific) and 4 μL of template. The PCR amplification was performed as described for 16S rRNA gene, with the exception that the annealing temperature was 55 °C. PCR products were confirmed in 1× SYBR safe-stained 1% agarose gel electrophoresis. DGGE was performed in 8% acrylamide and 40–70% denaturing gradient at 85V and 60 °C for 20 h, and gel stained and imaged as described above.

Sequencing and phylogenetic analysis

The most prominent DGGE bands obtained by the different primer sets were excised from the gels and immediately frozen at −20 °C. Prior to reamplification, the excised bands were incubated overnight in 20 μL molecular-biology-grade water at 4 °C to elute the DNA. Gene fragments were reamplified using the same primers and conditions as in the original PCR. The PCR products were then purified and sequenced by staff at Macrogen, Inc. (South Korea).

Sequences were manually checked and edited in Geneious Pro (v. 5.5.7, Biomatters Ltd, Auckland, New Zealand) prior to comparison with NCBI's nucleotide database with BLAST tool (Altschul *et al.*, 1997) and to the Ribosomal Database Project II with the Hierarchy Classifier tool (Wang *et al.*, 2007). Sequences obtained from the DGGE together with the relevant reference sequences from the databases were aligned with ClustalW and Muscle with default settings in Geneious Pro. The Jukes-Cantor 69

substitution model (Jukes & Cantor, 1969) was applied for 16S rRNA gene sequences and the WAG substitution model (Whelan & Goldman, 2001) for the translated *dsr*B amino acid sequences. The phylogenetic tree was constructed with the PhyML program (Guindon & Gascuel, 2003), and bootstrap support was calculated with 1000 random repeats.

Sequences were submitted to European Nucleotide Archive with accession numbers HF565417-HF565444 (bacterial 16S rRNA gene), HF565395-HF565416 (archaeal 16S rRNA gene) and HF565370-HF565394 (dsrB). For 8 archaeal sequences, which were too short (< 100 bps) for submission to the archive we can provide upon request.

Statistical analyses

DGGE gel images were normalized with the Bionumerics software package (v. 5.10, Applied Maths, Sint-Martens-Latem, Belgium), calculating the similarity of band profiles using Dice's coefficient of similarity. Dendrograms were constructed via UPGMA clustering.

Results

Changes in water chemistry during the pumping

Water chemistry was monitored during flushing of the space between the packers. Chemical measurements showed that the predominant cations in the fluids were Ca^{2+} and Na^{+}, and the most abundant anion was Cl^{-} (Table 1). Stabilization of pH and EC of the pumped fluid indicated that constant conditions at the fracture zones were reached rapidly after the start of pumping. At 500 m, oxygen concentration fell below 0.1 mg L^{-1} (Fig. S1) after 1 h of pumping, with a slight increase toward the second sampling at 11 days. After the second sampling, the oxygen concentration again fell to 0.1 mg L^{-1}, pH was constant with an average of 9.4, and EC decreased slightly from 19.4 to 19.2 mS cm^{-1}. At 967 m, oxygen concentration fell to the detection limit after 2 weeks, pH level was around 9.4, and EC was highest after 1 h of pumping but then declined to an average of 17.9 mS cm^{-1}. At 2260 m, oxygen level decreased to 0.6 mg L^{-1} within an hour of pumping. pH slightly decreased during the pumping from 9.2 to 8.8, and EC stabilized after a lag phase to 41 mS cm^{-1} before slowly rising toward the end of the pumping to 45 mS cm^{-1}. The deepest fracture zone contained significantly more ions than the other two fracture fluids, resulting in an EC that was twice that of the two shallower fractures. Compared with other fracture zones, the deepest fracture fluid also contained a high level of lithium (> 2 mg L^{-1}).

Table 1. Chemical composition of indigenous fracture fluids from three depths of the Outokumpu Deep Drill hole at the final sampling point

Depth (m)	Ca (mg L^{-1})	Na (mg L^{-1})	Cl (mg L^{-1})	Sr (mg L^{-1})	K (mg L^{-1})	Mg (mg L^{-1})	S (mg L^{-1})	Si (mg L^{-1})	B (µg L^{-1})	Li (µg L^{-1})	Mn (µg L^{-1})	Rb (µg L^{-1})	Mo (µg L^{-1})	Ni (µg L^{-1})	Pb (µg L^{-1})	pH	Alkalinity (mM)	EC (mS cm^{-1} 25 °C)
500	2250	1820	7970	22	18.6	12.8	3.24	1.54	120	94	27.4	39.1	17.7	3.14	4.1	9.4	0.19	19
967	2010	1800	5870	20.7	11	0.87	4.58	5.15	157	15.9	22.2	27.4	3.61	14	8.77	9.38	0.21	17.2
2260	7660	2460	17000	72	56	21.4	4.81	1.63	49.7	2070	192	176	68.5	3.16	0.08	9.1	0.25	47.5

The effect of the pumping on the total number of microbial cells

Cell numbers in the final sample were highest at 500 m and lowest at 2260 m, that is, decreased with increasing depth (Fig. 2). The fracture zone at 500 m had the highest cell count of all fractures (7.5×10^4 cells mL^{-1}) after 3 weeks of pumping. The total number of microorganisms in the 967-m fracture-zone fluid ranged from 1.3×10^4 to 4.4×10^5 cells mL^{-1}. At 2260 m, the cell count increased at the second sampling point to approximately five times greater than in the beginning, but then fell to 1.5×10^3 cells mL^{-1} after 63 days.

Activity staining of the 967-m sample revealed that 59% of the cells stained green (presumed to stain active cells) at the start of the pumping. The number of putative active cells was clearly higher after 11 days of pumping than in the first sample (average 78%). The number of cells presumed to be active decreased as pumping continued and after 21 days only 44.5% of cells stained green.

Measurement of sulfate-reducing bacteria with *dsr*B qPCR

The number of *dsr*B gene copies was used to measure the quantity of sulfate-reducing (SRB) microorganisms present in fracture water, while the number of *dsr*B transcripts was used to infer SRB activity. Microorganisms carrying the *dsr*B gene were most abundant in the indigenous fracture water derived from the 500-m fracture zone after 3 weeks of continuous pumping (Fig. 3). The number of *dsr*B gene copies was 7.4×10^3 copies mL^{-1}, with the ratio of dsrB : total number of cells 1 : 7.7. Transcription of *dsr*B in Outokumpu fracture fluids was low; the ratio of *dsr*B gene : transcript was 89 : 1 in the 500 m fracture fluid. At 967 m, the total number of *dsr*B copies decreased from 3.8×10^2 to 1.5×10^1 mL^{-1} during the pumping period, and the ratio of *dsr*B gene : transcript was 5.5 : 1. Similar to the total number of cells, the *dsr*B gene and transcript copy numbers increased at 21 days at the 2260-m fracture. Copy numbers were up to 20 times higher in the second sample at 21 days than after 1 h or at the end of the pumping. The detection limit of the qPCR assay was 80 copies mL^{-1}, and results below that were considered uncertain.

Changes in bacterial community structure over time

All three fracture zones had different bacterial communities. Community structure changed more during pumping in the 967- and 2260-m zones than in the 500-m zone.

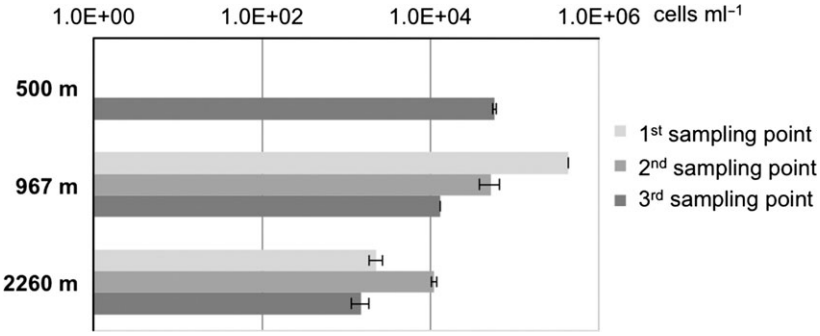

Fig. 2. Number of microbial cells in fracture fluid samples taken on three separate occasions. Bars represent the average number of cells over three sample times at each fracture zone. Cell numbers at 967 m decreased during pumping. At 2260 m, the total number of cells in the second sample rose but then returned to that at the start of pumping by the time, the third sample was taken. Cell numbers from 500 m at the first and second sample time are not available.

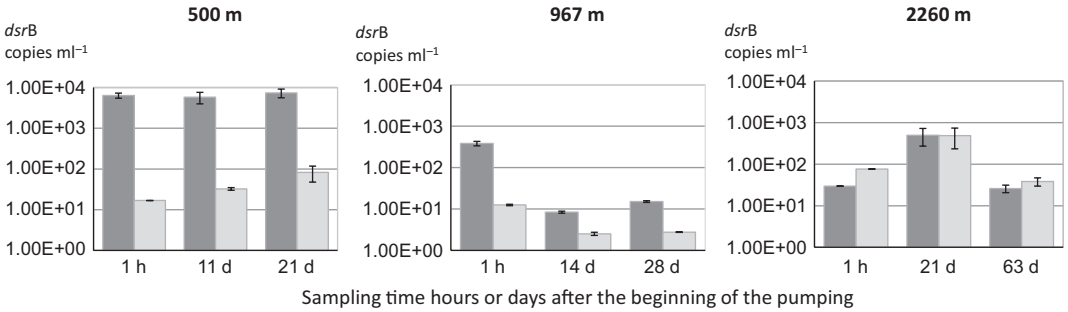

Fig. 3. The amount of *dsr*B gene and transcript copies in 1 mL of fracture fluid. The number of *dsr*B gene copies in the bedrock fluid is shown in dark gray and the number of *dsr*B transcript copies in light gray. The amount of *dsr*B copies at 500 m rose during pumping and dropped at 967 m. At 2260 m, the highest *dsr*B copy number was observed in the second sample.

At 500 m, cluster analysis of the DNA and RNA PCR-DGGE data suggested similar community profiles for samples taken after 1 h and 11 days of pumping (Fig. 4a). Another cluster comprised the DNA and RNA data after 21 days of pumping and RNA data after 11 days of pumping. The microbial community at 500 m was rich, and the banding pattern was up to 21% different among sample times. Of the bacterial phylotypes derived from the DNA-based profiles from the 500-m fracture zone, 64% represented *Alphaproteobacteria* (Fig. 4a) that clustered in the maximum-likelihood tree (Fig. S2a). The rest of the sequenced phylotypes affiliated with *Betaproteobacteria* and *Mollicutes*. A similar pattern was seen in the distribution of OTUs obtained from the RNA data. All but a few phylotypes were present throughout the sampling period.

The most diverse bacterial communities were found at the 967-m fracture zone. Community profiles from three different sample times were up to 38% dissimilar (Fig. 5a). After 1 h of pumping, communities were 21% dissimilar. The DNA and RNA community profiles were similar but formed separate branches in the cluster analysis. The community consisted of seven different phylogenetic groups, that is, *Beta-*, *Gamma-*, *Deltaproteobacteria*, *Mollicutes*, *Peptococcaceae*, undetermined *Firmicutes*, and *Flavobacteria*. *Betaproteobacteria* dominated the community slightly, but the relative amount of betaproteobacterial phylotypes decreased during pumping. After 14 days of pumping, two new *Firmicutes* phylotypes were detected (bands 37 and 44, Fig. 4a). Both of these were closely related (above 97% pairwise identity) to an uncultured *Peptococcaceae* found from deep sedimentary rock groundwater in Japan (AB237717, Shimizu *et al.*, 2006) clustering with *Desulfotomaculum thermosubterraneum* (Fig. S2a). In the indigenous fracture water after 28 days, phylotype 27 belonging to *Gammaproteobacteria* was absent from the active community, but one new phylotype (band 45) belonging to *Deltaproteobacteria* emerged.

The deepest fracture zone at 2260 m had the lowest bacterial diversity. The DNA and RNA community profiles differed from each other, with RNA suggesting a higher number of phylotypes than the DNA-targeted profile. In addition, the RNA community profile of the final sample was distinct. Four or five DNA phylotypes were detected throughout the pumping period and were up to 20% dissimilar. The sequences obtained belonged to *Actinobacteria*, *Firmicutes*, and *Alphaproteobacteria*. Three phylotypes fell with *Actinobacteria* in the phylogenetic tree, but two of these (54 and 64, Figs 4a and S2a) represented the one

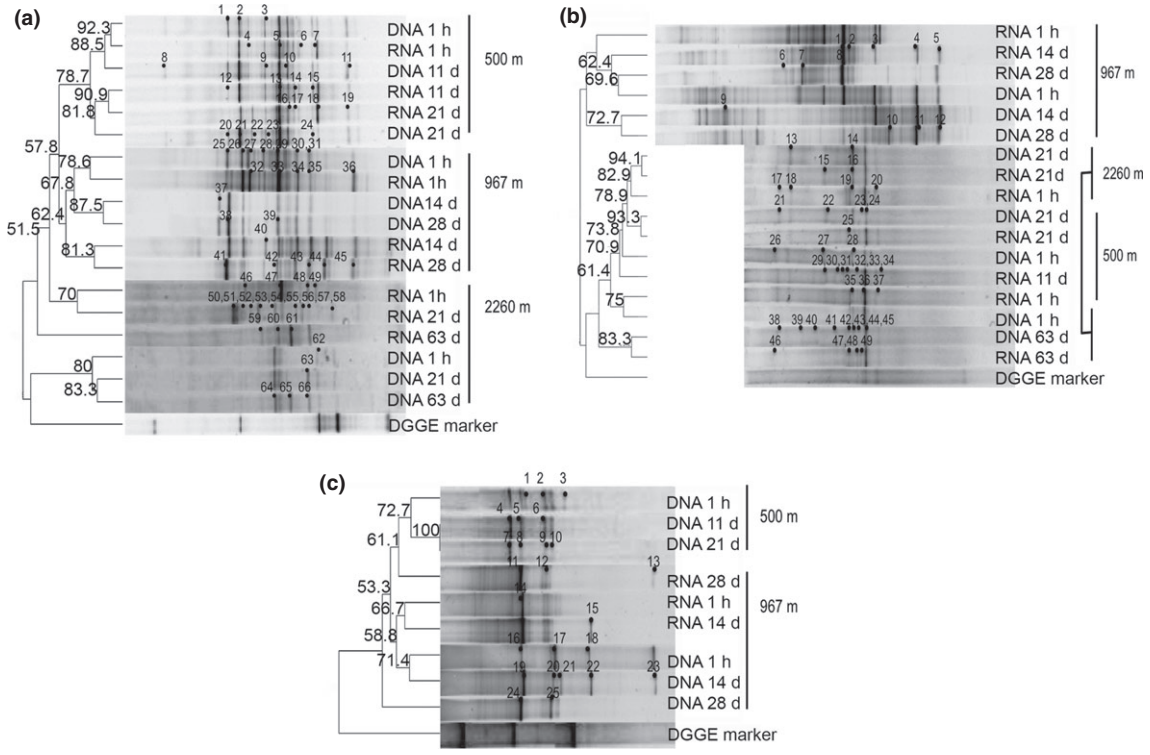

Fig. 4. Changes in the bacterial communities (a) and archaeal communities (b) as determined by DGGE analysis of 16s rRNA gene fragments and SRB communities (c) as determined by DGGE analysis of *dsr*B gene fragments. DNA and RNA extracted from fluid samples from the three depths and time points were amplified with PCR. The UPGMA dendrogram was generated with Dice's coefficient of similarity. Percentages at each node of dendrogram represent similarity between clusters. Numbered black points indicate sequenced phylotypes.

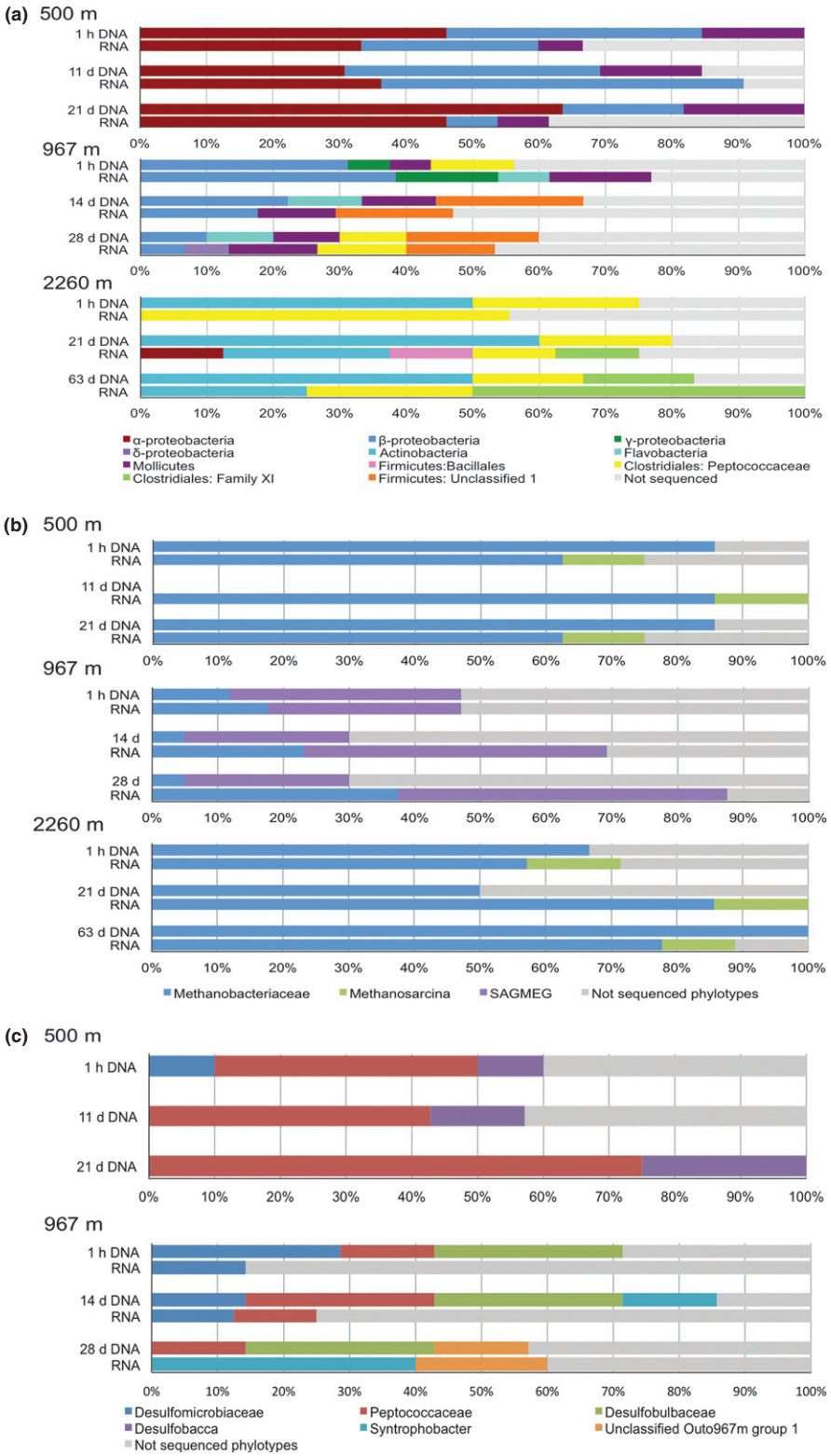

Fig. 5. Phylogenetic distribution of the recognized and unknown bacterial (a) and archaeal (b) 16S rRNA gene and transcript fragments and SRB (c) *dsr*B gene and transcript fragments at three time points.

fragment that was found from the lysis control as a faint band in DGGE (data not shown). After 21 days of pumping, several new phylotypes appeared in the RNA and DNA communities. Two of these (bands 57 and 66) representing the same phylotype were related to a cultured actinobacterium *Arthrobacter subterraneus* isolated from deep subsurface water from the south coast of Korea (Chang *et al.*, 2007). Interestingly, the RNA community profile contained three phylotypes (bands 50, 55, 58) that affiliated with the fungal clades *Cryptococcus* and *Taphrina* (phylogenetic tree not shown). Only two phylotypes in the 21-day sample were also detected at the end of the pumping (bands 57 and 60). Sequences were not obtained for two of the phylotypes found only from the RNA community at 21 days. However, two new phylotypes (59 and 65) were detected after 9 weeks of pumping and were similar to *Dethiosulfatibacter aminovorans*, a thiosulfate-reducing bacterium originally isolated from coastal marine sediment (Takii *et al.*, 2007).

In general, all replicates yielded similar profiles in the DGGE (data not shown).

Changes in archaeal community structure over time

According to community profiling, the most diverse archaeal community was found in the 967-m fracture (Fig. 4b). The other two fracture zones represented communities with only a few archaeal phylotypes. The archaeal DNA and RNA community profiles of the 500-m zone from 1 h, and 11-day samples were 6% dissimilar. *Archaea* inhabiting the 500 m-fracture belonged almost entirely to *Methanobacteraceae* (Fig. 4b). One phylotype (34) was affiliated with *Methanosarcina*. The RNA community profile changed more than that based on DNA. After 11 days of pumping, phylotypes 30, 32, 33, and 34 appeared but after 21 days, bands 30 and 32 could no longer be detected. The 500 m community was most similar to an uncultured archaeon detected in a continuous flow system for long-term pentachlorophenol mineralization (AB635384, Z. Li, Y. Inoue, D. Suzuki, L. Ye and A. Katayama 2011, unpublished data) (Fig. S2b).

The archaeal community profiles from 967 m clustered together but were up to 64% dissimilar (Fig 5b). While the RNA profiles grouped close together, DNA profiles were divided into two subclusters where the 1-h sample was more similar to RNA profiles, and the 14- and 28-day profiles formed an exclusive cluster of 27% dissimilarity. The 967-m fracture represented a different type of archaeal community compared with the other two zones. The majority of sequenced phylotypes belonged to the SAGMEG group (Fig. S2b). The DNA community profiles had three SAGMEG-related phylotypes (bands 10, 11, and 12) that were detected throughout the study period. Of these, bands 11 and 12 were also found in the RNA profiles after 2 and 4 weeks of pumping. *Methanobacteraceae* (phylotypes 1 and 2) were detected in the RNA profiles throughout the entire pumping period.

At the 2260 m, active and detected communities grouped together (Fig. 4b). Dissimilarities of the communities after 21 and 63 days of pumping were 6% and 17%, respectively. *Methanobacteraceae* again dominated the archaeal community at 2260 m. Only one phylotype (band 20) was affiliated to *Methanosarcina*. New phylotypes were detected after 3 weeks (bands 14, 15, 16) and 63 days of pumping (39, 44). All of these affiliated with *Methanobacteraceae*.

Community changes of sulfate-reducing microorganisms over time

SRB diversity inferred from the *dsr*B gene was identical in the last two samples from 500 m (Fig. 4c). Two phylotypes present at the beginning of the pumping (bands 1 and 3) were not detected after 11 days. Phylotypes related to *Peptococcaceae* dominated the SRB community at 500 m (Fig. 5c). Closely related sequences were found from other deep subsurface environments, including the Outokumpu drill hole (99.6% pairwise identity with band 3) (Fig. S2c). The other three identified phylotypes belonged to *Desulfobacca* (bands 2, 6, and 9) (Fig. 4c) and *Desulfomicrobiaceae* (band 1) (Fig. 4c).

At the 967 m fracture, SRB communities after 28 days of pumping differed from samples collected earlier. At the start of pumping, the majority of sequenced phylotypes affiliated with *Desulfomicrobiaceae* and *Desulfobulbaceae* (Fig. 5c). After 14 days of pumping the fracture, a phylotype affiliating with *Syntrophobacter dsr*B was detected (band 19). The *dsr*B community profiles based on DNA shared similar bands after 1 h and 14 days of pumping, as well as with the profiles of the *dsr*B gene transcripts from the same times (Fig. 4c). Only 26% of *dsr*B transcript fragments were sequenced from second time point, but profiles based on *dsr*B gene and transcripts were largely similar. After 28 days of pumping, several *dsr*B phylotypes belonged to *Syntrophobacter* and the unclassified Outo967 m group. DGGE band 13 was unique for the RNA profile after 28 days, when the majority of the *dsr*B genes belonged to *Desulfobulbaceae*, *Peptococcaceae* and two unidentified phylotypes. These two *dsr*B gene fragments (bands 12 and 24) were most similar to uncultured *Clostridiales* (EU258838 and EU258882, Liu *et al.*, 2009) but the nearest cultured species belonged to Nitrospirales (*Thermodesulfovibrio islandicus*). The rest of the *dsr*B gene transcripts (i.e. 40% of sequences) were similar to *Syntrophobacter*-type *dsr*B gene sequences.

Discussion

Research on microbial communities in bedrock has mainly focused on mines (Onstott *et al.*, 2006; Takai *et al.*, 2001; Sahl *et al.*, 2008; Trimarco *et al.*, 2006; Gihring *et al.*, 2006; Chivian *et al.*, 2008; Davidson *et al.*, 2011), where mining and related activities can disturb to the steady state. Three years after the end of drilling, salinity in the deep part of the Outokumpu drill hole gradually increased to indicate a slow inflow of formation fluids (Ahonen *et al.*, 2011; Kukkonen *et al.*, 2011). As such, the Outokumpu drill hole provides an opportunity to study microbial communities at pristine fracture zones. In order to evaluate the utility of the packer-isolation sampling method, we monitored changes in the number of microbial cells and community diversity. Chemical analyses revealed that the isolated space between the packers quickly stabilized after their installation. Microbial communities detected at 500 m varied only slightly, whereas those at 967 and 2260 m changed during the pumping period. Stable isotopes of water at the 500-m facture zone remained constant during the pumping period whereas those at 967 and 2260 m fluctuated over time (Kietäväinen *et al.*, 2013). Microbial communities of the drill hole water and the fracture fluid were 20–60% dissimilar (Fig. 5a), and diversity throughout the drill hole has been shown to be up to 10% dissimilar between 300 and 1300 m (Itävaara *et al.*, 2011b). Similar to microbial diversity estimates, cell numbers were lower in fracture fluids compared with the drill hole at each sample depth, with numbers gradually decreasing as indigenous fracture fluid replaced that in the drill hole. Cell numbers also decreased with increasing depth, similar to that reported in a previous study (Itävaara *et al.*, 2011b).

While the physicochemical characteristics of the deep bedrock environment at Outokumpu are only moderately extreme, the most probable limiting factor for microbial diversity is the availability of suitable nutrients, carbon sources, and terminal electron acceptors. New interactions between microorganisms in different water types can occur when the stable state of the drill hole and its fracture zones is disturbed. Such interactions can increase the microbial cell number and activity (Pedersen *et al.*, 2008), and we observed activation in terms of the number of viable and nonviable cells in the second fluid sample from 967 m. Pumping increased the number of viable cells to almost 80% of the total amount. In addition, an activation effect was detected as an increased number of *dsr*B gene copies and transcripts and also in the PCR-DGGE data of the active bacterial population at the 2260-m fracture. At the second time point, *dsr*B copy number was highest and active bacterial population the richest. Chemical factors stabilized more rapidly than the microbial community. Thus, the increased mixing caused by pumping probably affected the microbial community by first introducing nutrients, carbon and energy sources or by detaching microorganisms from surfaces and suspending or redistributing them, but after flushing the isolated zone reached a steady state and the microbial community responded accordingly.

Sequencing the major phylotypes inferred from PCR-DGGE revealed that although community profiles of the sample times were different, composition at the class level did not change dramatically. Most of the community in the 500-m fracture was composed of *Alpha-* and *Betaproteobacteria*. The sequences affiliated to *Alphaproteobacteria* were closely related to the type strain *Loktanella maricola*, as well as other *Rhodobacteraceae* found in seawater (Yoon *et al.*, 2007; Lai *et al.*, 2011). Itävaara *et al.* (2011a, b) considered *Alphaproteobacteria* to be a minor component of the microbial community in the Outokumpu drill hole. Lin *et al.* (2006) described a similar community with *Alpha-* and *Beta* at 700 m in a South African gold mine and Shimizu *et al.* (2006) at 458 m in a hard shale aquifer. *Betaproteobacteria* in this study were most similar to *Hydrogenophaga* sp., which from other habitats have been shown to be aerobic chemolithotrophs that oxidize hydrogen as an energy source (Yoon *et al.*, 2008). Hydrogen concentration in the Outokumpu drill hole water increases with depth, from 0.008 mM at 500 m to 3.1 mM detected at 2480 m (Kietäväinen *et al.*, 2013). The origin of the hydrogen in Outokumpu has yet to be confirmed but could be related to radiolysis of water or serpentinization (Sherwood Lollar *et al.*, 2007). Although the oxygen and hydrogen concentrations measured at the 500-m fracture were low, this type of oxic-anoxic transition zone environment may represent a niche for bacteria such as *Hydrogenophaga*. *Hydrogenophaga* were also observed in drill hole communities of a previous study (Itävaara *et al.*, 2011b). The majority of the archaeal community at the 500-m fracture belonged to *Methanobacteriaceae*, which in general have been shown to be hydrogenotrophic. This indicates that hydrogen may play an important role for deep subsurface microbial communities in Outokumpu.

The most diverse microbial community was observed at the 967-m fracture zone. *Firmicutes* were detected after 2 weeks of pumping from the 967-m fracture zone and covered approximately 20% of the population profile. The number of betaproteobacterial phylotypes decreased during the pumping period, suggesting that while *Betaproteobacteria* thrived in the upper part of the drill hole, they did not represent a majority of the community at 967 m. Several archaeal phylotypes from 967 m that were most similar to the SAGMEG group (Takai *et al.*, 2001) were not detected from other two fracture zones. SAGMEG *Archaea* are globally distributed in subsurface environ-

ments, but their energy metabolism and nutrient demands remain unknown (Inagaki & Nakagawa, 2008). The 967-m fracture zone is suggested to be methane rich (Ahonen *et al.*, 2011), but 16S archaeal sequences did not correspond to any known methanogens. Thus, the methane could be due to abiogenic processes (Sherwood Lollar *et al.*, 1993) or produced by a small number of methanogens that were not observed with our PCR-DGGE method or primers. The primer pair used in this study was found out to have an overall coverage over 70%, but it still misses five archaeal phyla (Klindworth *et al.*, 2013). In addition, it is possible that methanogens were attached to surfaces of the fractures and therefore were not be detected by sampling only fracture fluids. Increased microbial diversity and therefore complexity of interactions at the 967-m fracture could be a result of the fluid mixing between the studied and unknown fractures. Detaching biofilms during the pumping could explain the increased number of detected phylotypes, but no evident aggregates or broken biofilm structures were detected with microscopy. Kietäväinen *et al.* (2013) speculated that this depth might be subject to a natural or artificial (i.e. drill hole) supply of fresh water. However, sequences were most similar to taxa previously detected in other deep subsurface environments.

Firmicutes (*Bacillales*, *Peptococcaceae*, and *Clostridiales* Family XI) were a significant part of the community at 2260 m, similar to the findings of an earlier study that characterized microbial communities at Outokumpu drill hole fluid to a depth of 1500 m (Itävaara *et al.*, 2011b). Most of the other important phylotypes at 2260 m were *Actinobacteria*, among which the best match was *A. subterraneus*, which is a halophile that can tolerate fluids of up to 13% of NaCl (Chang *et al.*, 2007). With this ability, it is well suited for life in the most saline fluids of the Outokumpu deep bedrock. The archaeal community at 2260 m was dominated by hydrogenotrophic *Methanobacteriaceae*.

SRB were detected with PCR-DGGE in the upper two fractures. The largest SRB community was found at 967 m, including SRB phylotypes that could not be matched to any known taxa. qPCR results suggest that there were some SRB in the 2260-m fracture zone. However, these could not be detected with our PCR-DGGE method and primers. Interestingly, sequences derived from PCR-DGGE of 16S rRNA from the 2260 m were related to *Desulfovibrio* and *Dethiosulfatibacter*. *Dethiosulfatibacter aminovorans* is not a sulfate reducer, but its growth with casamino acids or glutamate is enhanced in co-culture with *Desulfovibrio* sp., both of which have been isolated from the same marine sediment (Takii *et al.*, 2007). More studies are, however, required to verify whether a similar type of metabolic integration among microorganisms occurs in the 2260-m fracture at Outokumpu.

Conclusions

When studying fluids from deep fracture zones, sampling accuracy (i.e. specific fracture fluid) and efficiency must be given special consideration. The drill hole opens hydraulic connections between fracture zones that enable the mixing of their fluids and suspended microorganisms. Therefore, samples should preferably be taken from sections isolated with hydraulic packers. When this is not technically possible, careful pumping with a low rate combined with continuous monitoring of fluid characteristics over weeks and months can be successful and generate representative samples of the indigenous fluids and their microbial flora. In the Outokumpu deep drill hole, microbial diversity was higher in the samples taken from the open drill hole. When samples were pumped from sections isolated with packers, the chemical and physical properties of the fluids changed rapidly but microbial communities developed more slowly and stabilized after only several weeks of pumping. By sampling the indigenous fracture fluids from three different depths of the Outokumpu drill hole, we observed how the microbial community became less diverse during the pumping and all fractures contained different indigenous communities. The number of microbial cells was lower in each fracture zone compared with the drill hole at the same depth and cell numbers decreased with increasing depth. Microbial abundance and phylogenetic affiliations are in agreement with findings from several other studies of deep subsurface environments.

Acknowledgements

The research project was funded by the Academy of Finland, the Foundation for Research of Finnish Natural Resources and Finnish research programme on nuclear waste management (KYT). Arto Pullinen, Maija Nuppunen-Puputti, and Pauliina Rajala are acknowledged for skillful sampling at the Outokumpu drill hole. Michael Hardman (PhD) is thanked for critical language correction.

References

Ahonen L, Kietäväinen R, Kortelainen N *et al.* (2011) Hydrogeological characteristics of the Outokumpu Deep Drill Hole. *Geol Surv Finland Spec Pap* **51**: 151–168.

Altschul SF, Madden TL, Schäffer AA, Zhang J, Zhang Z, Miller W & Lipman DJ (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res* **25**: 3389–3402.

Bano N, Ruffin S, Ransom B & Hollibaugh JT (2004) Phylogenetic composition of Arctic Ocean archaeal assemblages and comparison with antarctic assemblages. *Appl Environ Microb* **70**: 781–789.

Barton HA & Northup DE (2007) Geomicrobiology in cave environments: past, current and future perspectives. *J Cave Karst Stud* **69**: 163–178.

Biddle JF, Fitz-Gibbon S, Schuster SC, Brenchley JE & House CH (2008) Metagenomic signatures of the Peru Margin subseafloor biosphere show a genetically distinct environment. *P Natl Acad Sci USA* **105**: 10583–10588.

Borgonie G, García-Moyano A, Litthauer D, Bert W, Bester A, van Heerden E, Möller C, Erasmus M & Onstott TC (2011) Nematoda from the terrestrial deep subsurface of South Africa. *Nature* **474**: 79–82.

Chang HW, Bae JW, Nam YD *et al.* (2007) *Arthrobacter subterraneus* sp. nov., isolated from deep subsurface water of the South Coast of Korea. *J Microbiol Biotechnol* **11**: 1875–1879.

Chivian D, Brodie EL, Alm EJ *et al.* (2008) Environmental genomics reveals a single-species ecosystem deep within Earth. *Science* **322**: 275–278.

Davidson MM, Silver BJ, Onstott TC *et al.* (2011) Capture of planktonic microbial diversity in fractures by long-term monitoring of flowing drill holes, Evander Basin, South Africa. *Geomicrobiol J* **28**: 275–300.

Fredrickson JK & Balkwill DL (2006) Geomicrobiological processes and biodiversity in the deep terrestrial subsurface. *Geomicrobiol J* **23**: 345–356.

Fukuda A, Hagiwara H, Ishimura T, Koudoka M, Ioka S, Amano Y, Tsunogai U, Suzuki Y & Mizuno T (2010) Geomicrobiological properties of ultra-deep granitic groundwater from Mizunami Underground Research Laboratory (MIU), Central Japan. *Microb Ecol* **60**: 214–225.

Geets J, Borremans B, Diels L, Springael D, Vangronsveld J, Van Der Lelie D & Vanbroekhoven K (2006) *DsrB* gene-based DGGE for community and diversity surveys of sulfate-reducing bacteria. *J Microbiol Methods* **66**: 194–205.

Gihring TM, Moser DP, Lin L-H *et al.* (2006) The distribution of microbial taxa in the subsurface water of the Kalahari Shield, South Africa. *Geomicrobiol J* **23**: 415–430.

Grosskopf R, Janssen PH & Liesack W (1998) Diversity and structure of the methanogenic community in anoxic rice paddy soil microcosms as examined by cultivation and direct 16s rRNA gene sequence retrieval. *Appl Environ Microbiol* **64**: 960–969.

Guindon S & Gascuel O (2003) A simple, fast, and accurate algorithm to estimate large phylogenies by maximum likelihood. *Syst Biol* **52**: 696–704.

Haveman SA & Pedersen K (2002) Distribution of culturable microorganisms in Fennoscandian Shield groundwater. *FEMS Microbiol Ecol* **39**: 129–137.

Haveman SA, Pedersen K & Ruotsalainen P (1999) Distribution and metabolic diversity of microorganisms in deep igneous rock aquifers of Finland. *Geomicrobiol J* **16**: 277–294.

Inagaki F & Nakagawa S (2008) Spatial distribution of the subseafloor life: diversity and biogeography. *Links Between Geological Processes, Microbial Activities & Evolution of Life* (Dilek Y, Furnes H & Muehlenbachs K, eds), pp. 135–158. Springer, Netherlands.

Itävaara M, Nyyssönen M, Bomberg M, Nousiainen A, Kapanen A, Ahonen L, Hultman J, Paulin L, Auvinen P & Kukkonen I (2011a) Microbiological sampling and analysis of Outokumpu Deep Drill hole biosphere in 2007–2008. *Outokumpu Deep Drilling Project 2003–2010 Geological Survey of Finland, Special Paper, Vol. 51* (Kukkonen I, Ed), pp. 199–206. Geological Survey of Finland, Finland.

Itävaara M, Nyyssönen M, Kapanen A, Nousiainen A, Ahonen L & Kukkonen I (2011b) Characterization of bacterial diversity down to a depth of 1500 m in the Outokumpu deep drill hole, Fennoscandian Shield. *FEMS Microbiol Ecol* **77**: 295–309.

Jukes TH & Cantor CR (1969) *Evolution of Protein Molecules*, pp. 21–132. Academic Press, New York.

Kietäväinen R, Ahonen L, Kukkonen I, Hendriksson N, Nyyssönen M & Itävaara M (2013) Characterisation and isotopic evolution of saline waters of the Outokumpu Deep Drill Hole, Finland – implications for water origin and deep terrestrial biosphere. *Appl Geochem* **32**: 37–51.

Klindworth A, Pruesse E, Schweer T, Peplies J, Quast C, Horn M & Glöckner FO (2013) Evaluation of general 16S ribosomal RNA gene PCR primers for classical and next-generation sequencing-based diversity studies. *Nucleic Acids Res* **41**: e1.

Kukkonen IT (Ed) (2011) Outokumpu Deep Drilling Project 2003–2010. Geological Survey of Finland, Special Paper, 51, 252 pp.

Kukkonen IT, Rath V, Kivekäs L, Safanda J & Cermak V (2011) Geothermal studies of the Outokumpu Deep Drill Hole, Finland: vertical variation in heat flow and palaeoclimatic implications. *Phys Earth Planet Inter* **188**: 9–25.

Lai Q, Zhong H, Wang J, Yuan J, Sun F, Wang L, Zheng T & Shao Z (2011) *Roseovarius indicus* sp. nov., isolated from deep-sea water of the Indian Ocean. *Int J Syst Evol Microbiol* **61**: 2040–2044.

Lane DJ (1991) 16S/23S rRNA sequencing. *Nucleic Acid Techniques in Bacterial Systematics*, 1st edn (Stackebrandt E & Goodfellow M, eds), pp. 115–175. John Wiley and Sons, New York, NY.

Lin LH, Wang PL, Rumble D *et al.* (2006) Long-term sustainability of a high-energy low-diversity crustal biome. *Science* **314**: 479–482.

Liu XZ, Zhang LM, Prosser JI & He JZ (2009) Abundance and community structure of sulfate reducing prokaryotes in a paddy soil of southern China under different fertilization regimes. *Soil Biol Biochem* **41**: 687–694.

Lovley DR & Chappelle FH (1995) Deep subsurface microbial processes. *Rev Geophys* **33**: 365–381.

Moser DP, Onstott TC, Fredrickson JK *et al.* (2003) Temporal shifts in the geochemistry and microbial community structure of an ultradeep mine drill hole following isolation. *Geomicrobiol J* **20**: 517–548.

Muyzer G & Stams AJM (2008) The ecology and biotechnology of sulphate-reducing bacteria. *Nat Rev Microbiol* **6**: 441–454.

Muyzer G, De Waal EC & Uitterlinden AG (1993) Profiling of complex microbial populations by denaturing gradient gel

electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16s rRNA. *Appl Environ Microbiol* **59**: 695–700.

Northup DE & Lavoie KH (2001) Geomicrobiology of caves: a review. *Geomicrobiol J* **18**: 199–222.

Nyyssönen M, Bomberg M, Kapanen A, Nousiainen A, Pitkänen P & Itävaara M (2012) Methanogenic and sulphate-reducing microbial communities in deep groundwater of crystalline rock fractures in Olkiluoto, Finland. *Geomicrobiol J* **29**: 863–878.

Onstott TC, Phelps TJ, Colwell FS *et al.* (1998) Observations pertaining to the origin and ecology of microorganisms recovered from the deep subsurface of Taylorsville Basin, Virginia. *Geomicrobiol J* **15**: 353–385.

Onstott TC, Lin LH, Davidson M, Mislowack B, Borcsik M, Hall J, Slater G, Ward J, Sherwood Lollar B & Lippmann-Pipke J (2006) The origin and age of biogeochemical trends in deep fracture water of the Witwatersrand Basin, South Africa. *Geomicrobiol J* **23**: 369–414.

Papke RT & Ward DM (2004) The importance of physical isolation to microbial diversification. *FEMS Microbiol Ecol* **48**: 293–303.

Pedersen K (1997) Microbial life in deep granitic rock. *FEMS Microbiol Ecol* **20**: 399–414.

Pedersen K, Arlinger J, Ekendahl S & Hallbeck L (1996) 16S rRNA gene diversity of attached and unattached bacteria in drill holes along the access tunnel to the Äspö hard rock laboratory. *Sweden FEMS Microbiol Ecol* **19**: 249–262.

Pedersen K, Arlinger J, Eriksson S, Hallbeck A, Hallbeck L & Johansson J (2008) Numbers, biomass and cultivable diversity of microbial populations relate to depth and drill hole-specific conditions in groundwater from depths of 4–450 m in Olkiluoto, Finland. *ISME J* **2**: 760–775.

Sahl JW, Schmidt R, Swanner ED, Mandernack KW, Templeton AS, Kieft TL, Smith RL, Sanford WE, Callaghan RL & Mitton JB (2008) Subsurface microbial diversity in deep-granitic-fracture water in Colorado. *Appl Environ Microbiol* **74**: 143–152.

Sherwood Lollar B, Frape SK, Weise SM, Fritz P, Macke SA & Welhan JA (1993) Abiogenetic methanogenesis in crystalline rocks. *Geochim Cosmochim Acta* **57**: 5087–5097.

Sherwood Lollar B, Voglesonger K, Lin LH, Lacrampe-Couloume G, Telling J, Abrajano TA, Onstott TC & Pratt LM (2007) Hydrogeologic controls on episodic H_2 release from Precambrian fractured rocks – energy for deep subsurface life on Earth and Mars. *Astrobiology* **7**: 971–986.

Shimizu S, Akiyama M, Ishijima Y, Hama K, Kunimaru T & Naganuma T (2006) Molecular characterization of microbial communities in fault-bordered aquifers in the Miocene formation of northernmost Japan. *Geobiology* **4**: 203–213.

Stahl DA & Amann R (1991) Development and application of nucleic acid probes. *Nucleic Acid Techniques in Bacterial Systematics* (Stackebrandt E & Goodfellow M, eds), pp. 205–248. John Wiley & Sons Ltd, Chichester, England.

Stevens TO & McKinley JP (1995) Lithoautotrophic microbial ecosystems in deep basalt aquifers. *Science* **270**: 450–455.

Takai K, Moser DP, DeFlaun M, Onstott TC & Fredrickson JK (2001) Archaeal diversity in waters from deep South African gold mines. *Appl Environ Microbiol* **67**: 5750–5760.

Takii S, Hanada S, Tamaki H, Ueno Y, Sekiguchi Y, Ibe A & Matsuura K (2007) *Dethiosulfatibacter aminovorans* gen. nov., sp. nov., a novel thiosulfate-reducing bacterium isolated from coastal marine sediment via sulfate-reducing enrichment with Casamino acids. *Int J Syst Evol Microbiol* **57**: 2320–2326.

Trimarco E, Balkwill D, Davidson M & Onstott T (2006) *In situ* enrichment of a diverse community of bacteria from a 4–5 km deep fault zone in South Africa. *Geomicrobiol J* **23**: 463–473.

Wagner M, Roger AJ, Flax JL, Brusseau GA & Stahl DA (1998) Phylogeny of dissimilatory sulfite reductases supports an early origin of sulfate respiration. *J Bacteriol* **180**: 2975–2982.

Wang Q, Garrity GM, Tiedje JM & Cole JR (2007) Naive Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. *Appl Environ Microbiol* **73**: 5261–5267.

Whelan S & Goldman N (2001) A general empirical model of protein evolution derived from multiple protein families using a maximum-likelihood approach. *Mol Biol Evol* **18**: 691–699.

Whitman WB, Coleman DC & Wiebe WJ (1998) Prokaryotes: the unseen majority. *P Natl Acad Sci USA* **95**: 6578–6583.

Yoon JH, Kang SJ, Le ST & Oh TK (2007) *Loktanella maricola* sp. nov., isolated from seawater of the East Sea in Korea. *Int J Syst Evol Microbiol* **57**: 1799–1802.

Yoon JH, Kang SJ, Ryu SH, Jeon CO & Oh TK (2008) *Hydrogenophaga bisanensis* sp. nov., isolated from wastewater of a textile dye works. *Int J Syst Evol Microbiol* **58**: 393–397.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Monitoring of electrical conductivity (green, mS cm^{-1}), dissolved oxygen (red, mg L^{-1}) and pH (blue) at three different fracture zone fluids pumped continuously from the Outokumpu deep drill hole.

Fig. S2. (a) Maximum likelihood tree of bacterial 16s rRNA gene fragments from the indigenous fracture fluids. All sequences from the final sampling point are included. (b) Maximum likelihood tree of archaeal 16s rRNA gene fragments from the final sampling point. (c) Maximum likelihood tree of *dsr*B gene from the DGGE-analysis.

Table S1. Samples taken from each depth and time point and the analyses performed.

ARTICLE II

Heterotrophic communities supplied by ancient organic carbon pre-dominate in deep Fennoscandian bedrock fluids

Microb Ecol. 2015, 69(2):319–32.

ARTICLE III

The keystone species of Precambrian deep bedrock biosphere belong to *Burkholderiales* and *Clostridiales*

Accepted to Biogeosciences Discussions.

Biogeosciences Discuss., 12, 18103–18150, 2015
www.biogeosciences-discuss.net/12/18103/2015/
doi:10.5194/bgd-12-18103-2015

Biogeosciences
Discussions
Open Access
EGU

The keystone species of Precambrian deep bedrock biosphere belong to *Burkholderiales* and *Clostridiales*

L. Purkamo[1], M. Bomberg[1], R. Kietäväinen[2], H. Salavirta[1], M. Nyyssönen[1], M. Nuppunen-Puputti[1], L. Ahonen[2], I. Kukkonen[2,a], and M. Itävaara[1]

[1]VTT Technical Research Centre of Finland Ltd., Espoo, Finland
[2]Geological Survey of Finland (GTK), Espoo, Finland
[a]now at: University of Helsinki, Helsinki, Finland

Received: 13 September 2015 – Accepted: 23 October 2015 – Published: 11 November 2015

Correspondence to: L. Purkamo (lotta.purkamo@vtt.fi)

Published by Copernicus Publications on behalf of the European Geosciences Union.

Abstract

The bacterial and archaeal community composition and the possible carbon assimilation processes and energy sources of microbial communities in oligotrophic, deep, crystalline bedrock fractures is yet to be resolved. In this study, intrinsic microbial communities from six fracture zones from 180–2300 m depths in Outokumpu bedrock were characterized using high-throughput amplicon sequencing and metagenomic prediction. *Comamonadaceae*-, *Anaerobrancaceae*- and *Pseudomonadaceae*-related OTUs form the core community in deep crystalline bedrock fractures in Outokumpu. Archaeal communities were mainly composed of *Methanobacteraceae*-affiliating OTUs. The predicted bacterial metagenomes showed that pathways involved in fatty acid and amino sugar metabolism were common. In addition, relative abundance of genes coding the enzymes of autotrophic carbon fixation pathways in predicted metagenomes was low. This indicates that heterotrophic carbon assimilation is more important for microbial communities of the fracture zones. Network analysis based on co-occurrence of OTUs revealed the keystone genera of the microbial communities belonging to *Burkholderiales* and *Clostridiales*. Bacterial communities in fractures resemble those found from oligotrophic, hydrogen-enriched environments. Serpentinization reactions of ophiolitic rocks in Outokumpu assemblage may provide a source of energy and organic carbon compounds for the microbial communities in the fractures. Sulfate reducers and methanogens form a minority of the total microbial communities, but OTUs forming these minor groups are similar to those found from other deep Precambrian terrestrial bedrock environments.

1 Introduction

The microbial communities in deep terrestrial subsurface biosphere contribute significantly to the overall biomass on Earth (Whitman et al., 1998; McMahon and Parnell, 2014). It is essential to understand the metabolic capacity and energy sources of the

microbial communities in deep biosphere in order to evaluate their role in global biogeochemical cycles, assess the risks these communities might cause to for example geological long-term storage of nuclear waste, and even to estimate the possibility of microbial life in deep subsurface of other planetary bodies. In general, chemolithoautotrophic organisms are thought to be the primary producers in deep crystalline rock environments, into which sunlight, or the organic carbon or oxygen produced in photosynthesis, do not penetrate (Gold, 1992; Pedersen, 1997, 2000). Therefore, energy and carbon sources for deep biosphere have to be geochemical. The most important source of reducing power in deep subsurface is H_2. It is produced in abiotic reactions such as through radiolysis of H_2O, water-rock interactions such as serpentinization, but also by microbial activity (Pedersen, 2000; Lin et al., 2005; McCollom, 2013; Szponar et al., 2013). Carbon sources for microbes in deep subsurface are usually in the form of CO_2, CH_4 or other small hydrocarbons. Abiotic synthesis of organic carbon may take place through Fischer–Tropsch type reactions and provide a photosynthesis-independent carbon source for heterotrophic organisms in deep terrestrial biosphere (Proskurowski et al., 2008; McCollom et al., 2010; Etiope and Sherwood Lollar, 2013; Kietäväinen and Purkamo, 2015). This process may be triggered and enhanced by continuous H_2 flux provided by for example serpentinization. Numerous studies have characterized microbial communities of deep Precambrian rock formations (e.g. Pedersen et al., 1996, 2008; Hallbeck and Pedersen, 2008, 2012; Lin et al., 2006; Gihring et al., 2006; Silver et al., 2010; Itävaara et al., 2011; Nyyssönen et al., 2012; Purkamo et al., 2013, 2015; Osburn et al., 2014; Bomberg et al., 2015a, b). Although some of these studies have explored the energy and carbon sources or electron accepting processes in these environments, attention has been focused mainly on chemoautotrophic organisms utilizing H_2 and CO_2. After all, abiotic synthesis of organic carbon could also provide a photosynthesis-independent source of carbon and thus support heterotrophic organisms in deep biosphere (Amend and Teske, 2005; Schrenk et al., 2013). However, heterotrophic involvement to the carbon cycling and energy production in the deep continental bedrock biosphere has been rather neglected (Amend and Teske, 2005),

although it was recently suggested that heterotrophy might play a significant role in deep fluids of Fennoscandian crystalline rock (Purkamo et al., 2015).

While the microbial communities in deep marine subsurface environments have been intensively characterized within the last decade with next-generation sequencing methods (Sogin et al., 2006; Biddle et al., 2008, 2011; Brazelton et al., 2012), high-throughput (HTP) sequencing techniques have only recently emerged in characterization of the terrestrial deep subsurface microbial communities (Nyyssönen et al., 2014; Bomberg et al., 2014, 2015a, b; Lau et al., 2014; Mu et al., 2014). Vast amount of data obtained from HTP sequencing studies can be used to estimate ecological measures such as species richness, abundance and β-diversity, but it also allows the exploration of significant relationships between microbial taxa and their coexistence in a specific environment (Zhou et al., 2011; Barberan et al., 2012; Lupatini et al., 2014). These co-occurrence patterns, i.e. interactions between different microbial taxa and the complexity of the microbial communities can significantly contribute to the processes that will take place in the ecosystem (Zhou et al., 2011). In addition, keystone organisms can be identified from co-occurrence patterns of the community. Keystone organisms often have a greater role in the ecosystem functionality than their abundance refers (Power et al., 1996). For example, in hydrogen-driven lithoautotrophic ecosystems, autotrophic methanogens can be responsible of primary production of the whole ecosystem (Pedersen, 2000; Nealson et al., 2005). Moreover, these diverse minority groups with low abundance, i.e. the so-called rare biosphere, can be an almost infinite source of genetic potential to be distributed through the microbial populations via gene transfer (Sogin et al., 2006).

In this study, high-throughput amplicon sequencing, metagenome prediction and co-occurrence analysis were used (1) to describe the microbial community structure, (2) to detect key microbial genera of deep fracture fluids in Outokumpu, (3) to evaluate the possible carbon assimilation processes taking place in deep bedrock and ultimately, (4) to understand the origin of carbon and energy sources in Outokumpu Palaeoproterozoic deep bedrock and to establish links between microbial communities and

the geology and geochemistry in Outokumpu crystalline rock biosphere. Groundwater samples were collected from six different fracture zones located at depths ranging from 180 to 2300 m, and bacterial and archaeal communities in these fractures were characterized by their 16S rRNA genes and transcripts. In addition, two functional groups carrying out important electron accepting processes in deep subsurface, namely sulphate reduction and methanogenesis were characterized by dissimilatory sulfite reductase and methyl-coenzyme M reductase genes, respectively.

2 Materials and methods

2.1 Sample collection and geochemistry

Deep subsurface fracture fluids were collected during years 2009–2011 from the Outokumpu Deep Drill Hole, Eastern Finland. The sampling was conducted from overall six depths, 180, 500, 967, 1820, 2260 and 2300 m, as described previously (Purkamo et al., 2013). Shortly, 967 m and shallower depths were packer-isolated and purged for 21–42 days, and deeper fractures were sampled with slow continuous pumping of the fluid from the fracture depth for 9–63 days in order to flush the drill hole. Care was taken to ensure that the pumping rate did not exceed the rate of inflow from the fracture zone. The hydrogeological characteristics of these fluids differ with depth (Table 1). Kietäväinen et al. (2013) described five different water types in Outokumpu, and the fracture zones in this study represent the types I (180 m), II (500 and 967 m), IV (1820 and 2260 m) and V (2300 m). The type I water is characterized with high pH (around 10) and higher alkalinity than other water types in Outokumpu. High pH in the drill hole water column probably originates from cementation within the uppermost 200 m of the drill hole, while during long-term pumping of the 180 m fracture zone, pH dropped to the level of 8.5. Water type II contains the highest amount of dissolved gases in the whole water column, of which approximately 75 % (22–32 mmol L^{-1}) is methane. Distinctive greenish colour and unpleasant "rotten egg" odour are typical for water type IV, indi-

cating presence of reduced sulfur compounds. Water type V also has special features, such as high K and Li concentration due to the interaction with surrounding granitic rocks. In addition, the dominant dissolved gases in the two deepest water types IV and V are He and H_2, in contrast to the CH_4-dominated water types above 2 km depth (Kietäväinen et al., 2013).

The fluid from each fracture zone was collected in the field into sterile, acid-washed glass bottles (Schott) in an anaerobic chamber (MBraun, Germany). The anaerobic conditions in the chamber were achieved as previously described (Purkamo et al., 2013). The biomass for RNA and DNA extraction was collected on nitrocellulose acetate filters (Corning Inc., NY, USA) from 3 × 1 and 3 × 0.5 L of fracture fluid by vacuum suction. The filter was cut from the filter funnel with sterile scalpel and placed immediately to dry ice in a sterile 50 mL plastic tube (Corning Inc., NY, USA). In the laboratory, the samples were preserved at −80 °C before processing. In addition, duplicate 100 mL fluid samples for microbial cell enumeration were obtained from each fracture zone. Sterile, acid-washed 120 mL serum bottles were flushed with a small amount of fracture fluid in the anaerobic cabinet and subsequently filled with 100 mL of the sample fluid, capped with butyl rubber stoppers, sealed with aluminium crimp caps and kept refridgerated until further processing in the laboratory within five days after the sampling.

2.2 Enumeration of the total amount of microbes

In order to calculate the total amount of microbes in fracture fluids, microbes were stained with 4′-6-diamidino-2-phenylindole (DAPI). Preparation of the duplicate samples for examination by microscopy was conducted as in Purkamo et al. (2013). Stained microbes were collected from 5 mL fracture fluid samples by filtering, rinsed and filter was placed on microscopy slide. The total cell number in the samples was based on the sum of counted cells and the effective area of the filter divided by volume of filtrated sample, number of randomly selected microscopy fields and the surface area of the field at 100× magnification.

2.3 Nucleic acids preparation

DNA and RNA were extracted from the biomass with PowerSoil DNA or PowerWater RNA extraction kit (MO BIO Laboratories, Inc., CA, USA) as previously described (Purkamo et al., 2013). An additional DNase treatment was applied to RNA extracts that had DNA contamination detected by PCR performed with with P1 and P2 primers for bacterial 16S rRNA gene (Muyzer et al., 1993) as previously described in Purkamo et al. (2013). RNA was reverse-transcribed in triplicate reactions with random hexamers using the Superscript III Reverse Transcriptase kit (Invitrogen, ThermoFisherScientific, MA, USA) as described in Purkamo et al. (2013). The triplicate reactions were pooled and subsequent cDNA as well as DNA were stored at −80 °C. Negative controls for reagents were included in every extraction and translation step.

2.4 Quantitative estimation of bacterial and archaeal communities

The bacterial and archaeal numbers in each fracture were estimated with quantitative PCR from DNA extracts. 16S rRNA gene copy number was used as a proxy of the quantity of bacteria and archaea. In addition, qPCR was conducted to calculate the abundance of genes representing the key metabolic processes in anaerobic subsurface environments, namely sulfate reduction and methanogenesis with dissimilatory sulphite reductase (*dsr*B) and methyl-coenzyme M reductase (*mcr*A) genes, respectively. Bacterial 16S rRNA gene copy numbers were determined with V3 region-targeted primers P1 and P2 (Muyzer et al., 1993) resulting in a 190 bp product. A 370 bp fragment of *dsrB* gene and transcript was amplified with the primer pair DSRp2060f and DSR4R (Wagner et al., 1998; Geets et al., 2006). Archaeal 16S rRNA genes were amplified with ARC344f (Bano et al., 2004) and Ar774r (modified from Barns et al., 1994) primers producing a 430 bp product. A 330 bp fragment of *mcr*A gene was amplified with the primer pair ME1 and ME3rc (Hales et al., 1996; Nyyssönen et al., 2012).

Bacterial 16S rRNA gene-targeted qPCR was performed in triplicate reactions of each sample with KAPA™ SYBR® Fast 2× Master mix for Roche LightCycler 480 (Kapa

Biosystems, Inc., MA, USA) and 0.3 µM each of forward and reverse primer. The qPCR was performed on a Roche LightCycler 480 (Roche Applied Science, Germany) on white 96-well plates (4titude, UK) and sealed with transparent adhesive seals (4titude, UK). The qPCR conditions consisted of an initial denaturation at 95 °C for 10 min followed by 45 amplification cycles of 15 s at 95 °C, 30 s at 55 °C and 30 s at 72 °C and a final extension step of 3 min at 72 °C. After the quantification analysis, the melting curves for each reaction were determined. The melting curve analysis consisted of a denaturation step for 10 s at 95 °C followed by an annealing step at 65 °C for 1 min prior to a gradual temperature rise to 95 °C at a rate of 0.11 °C s^{-1} during which the fluorescence was continuously measured. Amplification of *dsr*B, archaeal 16S rRNA and *mcr*A genes were performed in triplicate for each sample as described in Purkamo et al. (2013), Bomberg et al. (2015b) and in Nyyssönen et al. (2014), respectively. The gene copy numbers were calculated by comparing the amplification result to a standard dilution series. Bacterial 16S rRNA and *dsr*B gene copy numbers were determined in each sample by comparing the amplification result to a standard dilution series ranging from 0 to 10^7 of plasmid DNA containing *Esherichia coli* ATCC 31608 or from 1.5×10^1 to 1.5×10^7 copies of *Desulfobulbus propionicus* DSM 2554 *dsr*B gene, respectively. Archaeal 16S rRNA and *mcr*A gene copy numbers were determined by comparing the amplification result to a dilution series of genomic DNA of *Halobacterium salinarum* DSM 3754 or to 5 to 5×10^6 copies of *Methanothermobacter thermautotrophicus* DSM 1053 *mcr*A gene, respectively. No-template controls as well as nucleic acid extraction and translation reagent controls were analysed with the corresponding samples in the same run. The inhibition effect of the samples was evaluated by mixing a specified amount of standard dilution to each sample DNA or cDNA. Spiked reactions were then subsequently amplified using the same protocols as described above. The inhibition in each sample could be evaluated by comparing the amplification efficiency of the sample-spiked standard DNA to the corresponding standard dilution quantity in the standard curve. Inhibition was found to be low in all samples (data not shown).

2.5 High-throughput amplicon sequencing

PCR amplicon libraries from hypervariable region V1-V3 of bacterial 16S rRNA gene were generated with barcoded 8f and P2 primers (Edwards et al., 1989; Muyzer et al., 1993). Amplification of the *dsr*B gene fragment for dissimilatory sulphate reduction was done with 2060f and 4R primers with barcode sequences (Wagner et al., 1998; Geets et al., 2006). Archaeal libraries were produced with nested PCR method, first using A109f and A915r primers (Großkopf et al., 1998 and Stahl and Amann, 1991, respectively) to amplify ca. 800 bp long fragment of the archaeal 16S rRNA gene and using the resulting product as template in PCR reaction with barcoded A344f and A744 primers (Bano et al., 2004 and modified from Barns et al., 1994, respectively). *Mcr*A amplicons were also produced with nested PCR, first applying mcrA463f and mcrA1615r primers (Nyyssönen et al., 2012) and secondly barcoded primers Me1 and Me3 (modified from Hales et al., 1996). PCR reaction mix composed of one unit of proofreading Phusion DNA Polymerase (ThermoScientific), 1× high fidelity buffer and dNTP mix (2.5 mM each), filled to 50 µL with molecular biological grade H_2O. Dimethylsulfoxide was used in all PCR reactions to enhance the template availability to polymerase. The amplification cycle consisted of an initial denaturation at 98 °C for 30 s, 35 (bacteria and *dsr*B) or 40 (archaea and *mcr*A) times repetition of 10 s at 98 °C, 15 s at 55 °C and 30 s at 72 °C, and a final extension step of 5 min at 72 °C. Three samples were used for each fracture zone community (RNA or DNA) and two amplification reactions of each sample replicate, thus resulting to maximum of six positive reactions (verified with agarose gel electrophoresis). Succesful reactions were pooled prior to sequencing. PCR reactions were performed also for nucleic acid extraction and reagent control samples. The sequencing of the 180 m samples was performed at Research and Testing Laboratory (Texas, USA) and the rest of the samples, at the institute of Biotechnology (Helsinki, Finland) using the FLX 454 Titanium – platform (454 Life Sciences, Branford, CT, USA).

2.6 Quality control, classification and phylogenetic analysis of sequences

Sequences were analyzed using mothur (v. 1.32.1) (Schloss et al., 2009) and QIIME programs (MacQIIME v. 1.7.0, Caporaso et al., 2010). The QIIME pipeline was used with 16S rRNA gene sequences and mothur with the functional gene sequences. In QIIME, sequences were compared against Greengenes representative OTU set version gg_13_8 with 97 % similarity and the taxonomy was assigned with RDP. With 16S rRNA sequences, the quality score window was set to 50 and sequences shorter than 360 and longer than 450 base pairs were discarded. The proximal primer sequences were allowed to have two or six nucleotide mismatches for bacterial and archaeal sequences, respectively. The high mismatch rate allowed for archaeal sequences was due to an extra guanine nucleotide in the primer sequence of the 500 m sample. Sequences of the functional genes representing sulphate reducers (*dsr*B) and methanogens (*mcr*A) were analyzed with mothur. Raw flowgrams were denoised with the PyroNoise algorithm to reduce PCR and sequencing noise in the data (Quince et al., 2009). All *dsr*B sequences shorter than 200 bp were discarded and no mismatches in the forward primer sequence were allowed. The length limit for *mcr*A sequences was set to 100 bp and four mismatches in primer sequence were allowed due to ambiguous bases in the primer sequence. The resulting sequences were further aligned with model alignments of *dsr*B and *mcr*A sequences from the Fungene repository (Fish et al., 2013) and sequences were assigned to OTUs with nearest neighbour clustering method. Final phylogeny of the representative OTUs was done using the Geneious Pro software package, version 6.1.7 (Biomatters Inc., New Zealand) and blastn and blastx for comparison of the representative OTU sequences to NCBI's databases (Altschul et al., 1990). All sequence data were uploaded to ENA database with accession numbers ERS846377-ERS846388 (bacteria), ERS846389-ERS846397 (archaea), ERS846399-ERS846407 (dsrB) and ERS846408-ERS846414(mcrA).

2.7 Ecological indices and statistical analyses

Chao1 richness estimates were calculated for the bacterial and archaeal communities with 97 % sequence similarity using the alpha.diversity.py command in QIIME. The estimates of diversity, richness and rarefaction were calculated from random subsample of 3030 sequences per sample for bacteria and 270 sequences per sample for archaea. Same α-diversity estimates for *dsr*B and *mcr*A datasets were calculated in mothur from subsamples of 115 and 1712 sequences, respectively. Due to the low amount of *dsr*B sequences (47) retrieved from the 180 m fracture, these data were not subsampled. The bacterial and archaeal OTUs with resolved taxonomy were compared to the hydrogeochemical data as well as to the lithology of each fracture zone. Canonical correspondence analysis was performed with Past3 to the environmental metadata and taxonomical OTU matrix with all archaeal OTUs and bacterial OTUs with more than 0.1 % abundance in the fracture communities (Hammer and Harper, 2001).

2.8 Prediction of functionality and co-occurrence analysis

De novo OTUs were removed from the 16S rRNA OTU taxonomy file (.biom-table) prior to uploading to the Galaxy pipeline for PICRUSt analysis (Goecks et al., 2010; Blankenberg et al., 2010; Giardine et al., 2005; Langille et al., 2013). PICRUSt compares 16S rRNA marker gene data to reference genomes and provides a prediction of the metagenome of a sample. Data in the biom-file was normalized with 16S rRNA gene copy number. Prediction of the functionality of the metagenome of each sample was done by multiplying the normalized abundance of each OTU by each predicted functional feature abundance. A weighted nearest sequenced taxon index (NSTI) was calculated for all samples. The NSTI value describes the average branch length that separates each OTU in the sample from a reference genome, weighted by the abundance of that OTU in the sample. For example, the NSTI value of 0.03 means that the OTUs in the sample are on average 97 % similar to the genomes in the database.

In order to obtain complete metabolic pathway modules from the predicted metagenomes, the KEGG abundance data from the PICRUSt analysis was used as input for HUMAnN v. 0.99 (Abubucker et al., 2012), which was modified to include modules M00597 (Anoxygenic photosystem II), M00598 (Anoxygenic photosystem I), M00595 (Thiosulfate oxidation by SOX complex, thiosulfate => sulfate), K16952 (sulfur oxygenase/reductase), M00596 (Dissimilatory sulfate reduction, sulfate => H_2S), M00567 (Methanogenesis, CO_2 => CH_4), M00528 (Nitrification, ammonia => nitrite), and M00563 (Methanogenesis, methylamine/dimethylamine/trimethylamine => CH_4).

Co-occurrence of OTUs in total and active microbial communities of Outokumpu bedrock fractures was analyzed with the otu.association command in mothur. Based on pairwise Pearson correlations with significant p value (< 0.01), visualization of the co-occurrence network was constructed using Fruchtermann–Feingold layout in the Gephi program (Bastian et al., 2009). The keystone OTUs were revealed with the betweenness centrality calculation and the connectivity of the network with the closeness centrality estimate (Brandes, 2001). Modular structure of the community was evaluated with the modularity index calculation (Blondel et al., 2008; Lambiotte et al., 2009).

3 Results

3.1 Microbial density in the fracture zones

The total microbial cell numbers were highest in the 180 m fracture and declined according to the depth (Fig. 1) (Table 2). A similar trend was observed with the copy numbers of bacterial 16S rRNA gene. Archaeal 16S rRNA gene copy numbers varied more, but the highest number of archaeal 16S rRNA gene copies was detected from the fracture at 180 m depth (Table 2).

The number of *dsr*B and *mcr*A gene copies, used as an estimate for the amount of sulphate reducing bacteria and methane producing archaea, respectively, was assessed with quantitative PCR. The copy numbers were quantified also from RNA in

order to estimate the activity of sulphate reduction and methanogenesis. The *dsr*B copy numbers varied between 3–6 × 10^2 copies mL^{-1} in most fractures with the exceptions of the 500 and 967 m fractures where the *dsr*B copy number was 7.4 × 10^3 and 1.5 × 10^1 copies mL^{-1}, respectively (Table S1 in the Supplement). As a proxy of active transcription of *dsr*B genes, the number of mRNA transcripts was also quantified. The highest *dsr*B gene transcription was observed at 1820 m, where the number of *dsr*B transcripts was more than 6.0 × 10^2 transcripts mL^{-1}. All other fractures had below 1.0 × 10^2 transcripts mL^{-1}. Methanogenesis marker gene copies were detected only from the upper three fractures (180, 500 and 967 m). The *mcr*A gene copy numbers were just above the detection limit of the assay, i.e. less than 4.0 × 10^1 copies mL^{-1} in all. *Mcr*A gene transcripts were detected only from the 967 m fracture, where the copy number was just above 1.0 × 10^2 mL^{-1}.

3.2 The structure of the microbial communities and correlation to geochemistry

The sequencing data acquired from DNA were used as a representation of the total microbial community present in the fracture fluid samples whereas the microbial communities derived from RNA were used as a proxy of an active community. The microbial communities differed between the nucleic acid fractions as well as the sampling depth (Fig. 2). Pyrosequencing of the total and active bacterial communities based on the 16S rRNA gene resulted to 268 identified OTUs representing a total of 157 families in six fractures analysed (Table S2). Sulfate reducing communities (based on the *dsr*B gene) were successfully sequenced from all fracture fluid samples except the active community in the 2260 m fracture and total and active communities in 2300 m. The archaeal communities were overall less diverse than the bacterial communities. Archaeal sequences (16S rRNA gene) were retrieved from all fractures except the one at 1820 m. Only 17 different OTUs could be divided to 11 families (Table S3). Total methanogen communities were detected from 180, 500, 2260 and 2300 m fractures and active methanogen communities from 500 and 967 m fractures.

3.2.1 The 180 m fracture

The bacterial communities in the upmost fracture zone analysed in this study were dominated by OTUs resembling *Comamonadaceae* (Fig. 2). This β-proteobacterial family constituted over 70 % of the OTUs in total and active communities in the 180 m fracture (Table S2). The estimated richness of the community was 69 % of the observed OTUs of the 180 m bacterial communities (Table S4a). *Desulfatirhabdum* (54 % relative abundance) and *Desulfotomaculum* (98 % relative abundance) were most abundant in total and active sulfate reducing communities, respectively (Fig. 2). The archaeal community in the fracture zone at 180 m was dominated by OTUs affiliated with *Methanobacteriaceae* and *Methanoregula*, while *Methanosarcina* and methylotrophic *Methanolobus* OTUs represented minor groups (Table S3). This depth hosted the most diverse archaeal communities (the Shannon diversity index H′ 2.4 and 2.1 for total and active archaeal communities, respectively) (Table S4b). Similarly, the methanogen community was the most diverse at this fracture, and the dominating groups were similar to unclassified, uncultured methanogen sequences retrieved from wetland soil (LW-25) and acidic peat bog (MB04-15a).

3.2.2 The 500 m fracture

The total bacterial community in the 500 m fracture was dominated by *Comamonadaceae* (70 %) (Fig. 2, Table S2). The dominating OTUs in active bacterial community affiliated with α-proteobacterial order *Rhodobacterales* with 38 % relative abundance, otherwise this community comprised of OTUs affiliating to *Comamonadaceae* (23 %), *Dietzia* (23 %) and *Pseudomonas* (6 %). The amount of observed OTUs captured 77–86 % richness of the communities according to the Chao1 estimate at this depth (Table S4a). *Desulfotomaculum* and *Pelotomaculum* -affiliating OTUs were the most dominant sulfate reducers in this fracture (Fig. 2). The total and active archaeal communities comprised almost solely of methanogenic *Methanobacteriaceae*, while

Methanobrevibacter and *Methanosarcina* dominated the communities detected with methanogen-specific marker gene (*mcr*A) (Fig. 2).

3.2.3 The 967 m fracture

The total bacterial community in 967 m fracture zone comprised of *Natrananaerobiales, Clostridiales* and other *Firmicutes* in addition to mollicute *Acholeplasma*. In the active bacterial community, peptococcal *Syntrophobotulus* dominated and otherwise the community resembled the total community (Fig. 2). The observed richness was 84 or 88 % of the estimated richness of the total and active communities, respectively (Table S4a). Based on the Shannon diversity index H' (2.3) the total archaeal community in the 967 m fracture was among the most diverse of the archaeal communities. It was dominated by OTUs affiliating with SAGMEG-1 Euryarchaeota. In the active archaeal community in this fracture *Methanobacteraceae* dominated and SAGMEG OTUs represented only a minority of the OTUs. *Methanosarcina* dominated the active methanogen community in the 967 m fracture (Fig. 2).

3.2.4 The 1820 m fracture

The number of observed bacterial OTUs was among the highest in both total and active bacterial communities in the fracture zone at 1820 m. *Pseudomonadales* (29 % relative abundance), *Burkholderiales* (22 %) with *Comamonadaceae* and *Oxalobacteriaceae*, *Clostridiales* (13 %) comprised mainly of *Dethiosulfatibacter* and other Firmicutes with unresolved phylogeny dominated the total community in this fracture zone. In the active community OTU 86 belonging to Firmicutes was dominant with 39 % relative abundance (Fig. 2). The sequenced communities at this depth were estimated to have captured in average 80 % of the richness of the total communities (Table S4a). The total SRB community in this fracture was entirely composed of *Desulfovibrio*-affiliating OTU. The estimated diversity was low because only 115 sequences were retained. On the other hand, the active SRB community was diverse, with OTUs affiliating with

Desulfatirhabdum, *Desulfobulbus* and *Desulfoarculus*. Amplification of archaeal and methanogen communities was not succesfull from this fracture indicating low abundance of these groups.

3.2.5 The 2260 m fracture

The fracture at 2260 m hosted a bacterial community mainly comprising of actinobacterial OBP41 class (53 % relative abundance) and *Burkholderiales* (34 %) (Fig. 2). The active community in this fracture had the highest amount of observed OTUs of the whole dataset and best success in capturing the richness (91 %) of the community. OTUs belonging to *α*-proteobacterial *Bradyrhizobium* (20 %) and *Rickettsiales* (11 %) in addition to Firmicutes and Actinobacteria dominated this active community. *Desulfotomaculum* and *Desulforudis*-affiliating OTUs dominated the total SRB community at this fracture and *Methanobacterium* dominated both archaeal and methanogen communities (Fig. 2).

3.2.6 The 2300 m fracture

The most frequent OTUs in the bacterial communities in the fracture zone at 2300 m represented *Burkholderiales* (31 % of the OTUs) and *Pseudomonadales* (25 %) such as *Moraxellaceae* and *Pseudomonadaceae*. In addition, OTUs belonging to other Firmicutes, *Clostridiales*, *Actinomycetales* and *Natranaerobiales* were detected (Fig. 2). The sequenced DNA community covered 86 % of the estimated richness. The active community of this fracture mainly composed of unclassifiable OTUs: only 4 % of the community could be determined to more specifically than to phylum level, while half of the community could be determined only to phylum level (Firmicutes) leaving the rest of the community, 46 % unresolved. This reflected also to the richness and coverage indicator values: only 51 % of the richness was captured and the coverage was barely half (51 %) of the total abundance of the community.

Sulfate reducers were not detected at this depth, and *Methanobacterium* dominated the total archaeal and methanogen communities.

3.2.7 The core microbial community in Outokumpu bedrock fractures

Only a few OTUs that were present in all communities constituted the core community in the Outokumpu deep bedrock. *Pseudomonas* and *Dethiosulfatibacter* in addition to two OTUs with uncertain taxonomic classification (Firmicutes OTU 86 and bacterial OTU1) were detected in all total and active bacterial communities. When observing only the total bacterial communities, most abundant members of the core community were *Comamonadaceae*, *Dethiobacter* and *Pseudomonas*.

3.2.8 The relationship of the microbial community structure to geochemistry

Microbial OTUs formed three loose clusters in canonical correspondence analysis based on the depth where fracture fluid samples were retrieved (Fig. 3). A cluster of bacterial OTUs belonging to orders *Burkholderiales* and *Rhodobacterales* plotted near 180 and 500 m depths and correlated with biotite gneiss and concentration of its main elemental components, iron and magnesium. Microbial OTUs affiliating with the most abundant groups in the 967 m fracture (*Peptococcaceae*, *Anaerobrancaceae*, *Thermoanaerobacterales*, SAGMEG archaea) grouped loosely around the 967 m depth with sulfur concentration pointing to this ordination. The depths of 1820 and 2300 m correlated with sulfide concentration and defining rock types were black schist and pegmatititic granite. Clostridial *Dethiosulfatibacter* and other Firmicutes-affiliating OTUs in addition to several *Burkholderiales*-affiliating OTUs clustered close to these depths.

3.3 The functionality estimation of the microbial communities

The physiology of the members of the microbial communities was estimated from classified OTUs based on the prevalent physiology of the cultured representatives of each OTU at family level according to the Prokaryotes handbook (Rosenberg et al., 2014)

(Fig. 4). Bacterial physiotypes with capacity to use versatile metabolic pathways for carbon assimilation and energy production were characteristic in the fracture communities at shallower depths (180 and 500 m), while lithotrophic bacterial physiotypes are more frequently detected in 967 and 1820 m fractures. Overall, physiotypes with unknown metabolism became more frequent in the communities at fractures located deeper in the bedrock due to the lack of exact taxonomic classification of the OTUs detected in these fractures (Fig. 4a). In the archael communities the most dominant archaeal physiotype was hydrogenotrophic methanogenesis except in the 967 m fracture (Fig. 4b). The total archaeal community in this fracture was dominated by SAGMEG-affiliating OTUs with undetermined physiology.

3.3.1 Predicted bacterial metagenomes

The metagenomes of the microbial communities of different fracture zones representing six different biotopes were predicted from the 16S rRNA gene sequences, i.e. from the different OTUs with resolved taxonomy. In order to evaluate the accuracy of the prediction of metagenomes, nearest sequenced taxon index (NSTI) was calculated for each sample (Table S5). The NSTI's varied between the bacterial communities from 0.07 (the communities in 1820 and 2300 m fractures) to 0.30 for the community in 2260 m fracture. The archaeal communities represented NSTI's from 0.04 to 0.07 with the exception of the total community in 967 m fracture of which the NSTI was 0.29. Overall, the predicted metagenomes of the total communities did not vary greatly from the active community metagenomes. Top-level functionality estimates revealed differences between bacterial and archaeal communities. The average values for cellular processes and environmental information processing were more abundant in the predicted bacterial metagenomes than in the archaeal metagenomes. In contrast, genetic information processing and unknown features were more abundant in the predicted archaeal metagenomes (Fig. 5).

The most abundant group of bacterial predicted on the basis of PICRUSt analysis were those involved in amino acid metabolism (21–22 % of all metabolism genes),

carbohydrate metabolism (19–21 %) and energy metabolism (11–13 % of all genes involved in metabolism) (Table 3a). The predicted bacterial metagenomes differed mostly between 180 m and other fractures. In all fractures, the most abundant amino acid metabolism genes were amino acid related enzymes and arginine and proline metabolism genes (11–15 % and 11–13 % respectively) (Table S6a). In the predicted bacterial metagenome in the 180 m fracture, genes involved in phenylalanine, tyrosine, tryptophan and lysine biosynthesis were more abundant than in other fractures. On the other hand, branched-chain amino acid (valine, leucine and isoleucine) degradation represented 7–11 % of the predicted amino acid metabolism genes in all other bacterial communities than those at 180 m fracture, where it was approximately only half of this (4–5 %). Gene predictions on carbohydrate utilization revealed a highly similar pattern in all other fracture communities than those at 180 m (Table S6a). Amino sugar metabolism genes were more abundant in the 180 m, while in other fractures propanoate and butanoate metabolism genes were dominant. The most abundant energy metabolism genes were involved in oxidative phosphorylation in the predicted metagenomes of the bacterial communities (22–26 % in total bacterial communities, and 20–28 % in active communities) (Table S6a). Genes involved in carbon fixation pathways in prokaryotes were almost as common, in addition to methane metabolism genes.

To evaluate the operational capacity of different metabolic pathways detected with PICRUSt, HUMAnN analysis was performed on the predicted metagenomes. Genes needed for several amino acid biosynthesis pathways and transport systems to function were present. The relative abundance of the genes involved in carbon fixation pathways such as Arnon–Buchanan, Wood–Ljungdahl and Calvin cycle were low according to the HUMAnN analysis (Table S7). Calvin cycle genes were only present in the predicted metagenomes of the active bacterial communities in 1820 and 2260 m fractures. The coverage of pathways involved in carbohydrate metabolism (KEGG modules 1–4, 6–9, 11) was high.

The genes needed for coding of the enzymes in sulfur and sulphate reduction pathways were only covered in the communities in the 180 m fracture. Aerobic methane oxidation pathway on the other hand was considered operational in communities from 500 m fracture and other fractures below this (Table S7).

3.3.2 Predicted archaeal metagenomes

Similar to bacteria, half of the genes in the predicted archaeal metagenomes of each fracture zone were involved in metabolism (Fig. 5b). Energy metabolism genes were most common (18–25 %) in addition to amino acid metabolism and carbohydrate metabolism genes (21–25 and 17–20 %, respectively) (Table 3b). The predicted metagenome of the total archaeal community in the 967 m fracture zone had the highest abundance of amino acid metabolism genes (25 % of all metabolism genes) and the lowest abundance of energy metabolism genes (18 %) and thus differed from the community metagenome derived from RNA in the same fracture. Otherwise the predicted metabolism genes were similar in the different archaeal communities in the fracture zones. The predicted metagenome of the total archaeal community of the 967 m fracture differed also in predicted amino acid usage, carbohydrate metabolism and in the energy metabolism gene predictions. As expected, genes representing methane metabolism were the most dominant in all archaeal communities (Table S6b). However, oxidative phosphorylation genes were twice as frequent (16 % relative abundance) in the metagenome based on the total community in the 967 m fracture as in all other predicted metagenomes. Additionally, the community in the 180 m fracture differed from the other archaeal communities in predicted energy metabolism: less methane metabolism genes were detected in this community (61 % in the total community and 64 % in the active community), and genes involved in carbon fixation pathways were more abundant in the community in this fracture (16 % in DNA community and 14 % in RNA community).

The methanogenesis pathway from CO_2 to CH_4 was present and complete in all archaeal communities according to the HUMAnN analysis (Table S8). Methanogenesis

pathway from methylamines or methanol was detected and likely operational only in the communities in the 180 m fracture.

3.4 Co-occurrence of microbial OTUs in deep crystalline bedrock

From all detected microbial OTUs in deep crystalline fractures, only 15 % showed positive correlation ($r > 0.8$) with other members of the microbial communities. Only three significantly negative correlations ($p \leq 0.01$, $r < -0.8$) were detected among the total microbial communities and none in the active communities. The network analysis of the total microbial community divided significantly correlating OTUs into 8 modules with number of nodes ranging from 4 to 41 (Fig. 6). The closeness of centrality (CC) values varied only slightly between most of the OTUs indicating that the network had high connectance between different members (variance 0.5) (Table 4). The keystone OTUs were detected based on their above 300 betweenness of centrality (BC) value and these belonged to *Burkholderiaceae*-like OTU, *Desulfitobacter* and *Clostridiaceae*-affiliating OTU, all of which had relatively low abundance (0–3 %) in the communties, in addition to *Dethiobacter* with 0–5 % relative abundance in other total bacterial communities except in the 967 m fracture, in which the relative abundance of this OTU in the total bacterial community was higher, 25 % (Figs. 2a and 6). The most connected OTUs in the network belonged to *Rhodococcus* and *Herbaspirillum* (48 connections each) in addition to OTUs resembling *Renibacterium*, *Gemellaceae*, *Trabulsiella* and *Novosphingobium* (46–47 connections each). The positively correlating OTUs of the active microbial community divided into 8 clusters with number of nodes ranging from 2 to 64 (Fig. 7). The active community network had also small variation in the CC values (variance 0.3). The keystone genera of the active microbial community network were *Comamonas*, *Curvibacter* and *Sphingomonas*, with BC values above 470 each. *Comamonadaceae*-affiliating OTU was determined to be part of the core community in this Fennoscandian deep subsurface site, as it was frequently found in all depths with relatively high abundance ranging from 7–72 % of the total community. *Curvibacter* and

Sphingomonas were both present in the active communities, but with a very low relative abundance.

4 Discussion

The bacterial community structure in the Outokumpu fracture zones varies between the different depths. In addition, the structure of the total and active communities differs within the fracture zones. The core bacterial community of the deep crystalline bedrock in Outokumpu was composed of few OTUs found from all microbial communities in the fractures. Most abundant of these were *Comamonadaceae*-, *Firmicutes*-, *Anaerobrancaceae*- and *Pseudomonadaceae*-affiliating OTUs. However, the majority of the bacterial OTUs discovered in this study could be regarded as members of the so-called "rare biosphere" with their relatively low abundance and uneven distribution throughout the fracture zones (Sogin et al., 2006).

A keystone species has greater impact on its community or living environment than would be expected from its relative abundance or total biomass (Paine, 1995). Several keystone genera of both the active and the total microbial community were representatives of *Burkholderiales* (e.g. *Comamonas*, *Curvibacter*, *Oxalobacter*, *Herbaspirillum*, *Pelomonas*, *Cupriavidus*). In addition, some clostridial phylotypes were among the keystone genera. Several keystone genera detected with the co-occurrence networks were members of the rare biosphere, thus providing further evidence for the significance of the less abundant microorganisms for the whole community (Sogin et al., 2006; Brown et al., 2009). Hence, we propose that these have a major role in the functionality of the network. The metabolic flexibility of *Burkholderiales* species, for example the ability to use both autotrophic and heterotrophic carbon fixation mechanisms is beneficial in isolated deep biosphere environments where concentrations of different carbon substrates fluctuate over time (Moser et al., 2005; Magnabosco et al., 2015).

4.1 Serpentinization as a source for energy in Outokumpu

Hydrogen oxidizing, facultatively chemolithotrophic members of the *Comamonadaceae*-family were dominating the 180 and 500 m fracture zone communities. These microbes are commonly found from hydrogen-enriched subsurface environments. These include findings of *Ralstonia* and *Hydrogenophaga* in Lost City Hydrothermal Field and Tablelands Ophiolite serpentinite springs, respectively (Brazelton et al., 2012, 2013). Likewise, *Comamonadaceae*-affiliating and clostridial sequences formed the majority of the bacterial community in serpentinization-driven subsurface aquifer in Portugal (Tiago and Veríssimo 2013). *Comamonadaceae* were also dominant in the drill hole water communities in Outokumpu at shallow depths (0–100 m) (Itävaara et al., 2011), and at 200 and 2300 m depths (Nyyssönen et al., 2014). Additionally, *Comamonadaceae* were detected from other depths in the drill hole water column, such as at 1100–1500 m depth that was characterized by ophiolitic rock sequence (Nyyssönen et al., 2014). However, Kietäväinen et al. (2013) detected substantial amounts of H_2 only in fractures below 1500 m in Outokumpu, which might indicate that the source for H_2 for abundant hydrogen-oxidizers is in the bedrock aquifer that 180 and/or 500 m fractures intersect or lead into. The seismic reflectors of Outokumpu bedrock demonstrate ophiolite-derived rock types in the vicinity of the drill hole, some of which are located also at shallow depths (Kukkonen et al., 2011). Thus, we assume that these may affect the two shallowest fracture fluids and explain the similarity of the microbial results with other ophiolitic, serpentinizing environments.

The bacterial community of the 967 m fracture zone also resembles those of serpentinizing environments. One major member of the total bacterial community at 967 m fracture was hydrogen-oxidizing *Dethiobacter* that has previously been detected also from groundwaters associated with ophiolithic rock sequence with active serpentinization processes (Tiago and Veríssimo, 2013). Brazelton et al. (2013) detected acetyl-CoA synthase gene affiliating with *Dethiobacter* from bacterial shotgun-sequenced metagenomes from bacterial Winter House Canyon (WHCB) samples from Tablelands

Ophiolite. Moreover, similarities between serpentinizing environments and the deep biosphere of Outokumpu bedrock include the detection of abundant clostridial phylotypes such as *Anaerobrancaceae* from the deeper parts of the bedrock (Itävaara et al., 2011; Brazelton et al., 2013; Purkamo et al., 2013; Nyyssönen et al., 2014). The keystone species of the total and active microbial communities detected in this study reflect the similarity between the serpentinizing environments and Outokumpu deep biosphere with *Comamonadaceae*, *Burkholderiaceae*, *Clostridiaceae* and *Dethiobacter* as the recognized keystone families.

Overall, the spatial distribution of *Burkholderiales* at shallower depths and *Clostridiales* in the fractures located deeper in the bedrock can be explained to some level with the availability of electron acceptors. Both of these groups are able to use H_2 as electron donor, but *Comamonadaceae* are mainly aerobic chemoorganotrophs using a wide variety of different organic carbon compounds for energy and using oxygen as terminal electron acceptor (Willems et al., 1991), while *Dethiobacter* is a strict anaerobe that reduces sulphur compounds but not sulphate (Sorokin et al., 2008). However, as their co-occurrence in the 1820 m fracture suggests, these organisms can prevail in same environment. In Outokumpu, low concentrations of oxygen were measured during the pumping of the fracture fluids ($< 0.1\,mg\,L^{-1}$) (Purkamo et al., 2013). However, it is likely that this is due to the atmospheric contamination during the measurements in the field. In addition, small amounts of oxygen can be produced in radiolysis of water in bedrock (Pedersen, 1997; Lin et al., 2005). This might explain the detection of aerobic bacteria such as *Comamonadaceae* in the deep fluids of Outokumpu.

Higher hydrogen concentration in the two deepest fracture fluids (Kietäväinen et al., 2013) could indicate that something else than the electron donor is limiting the communities at these depths. Abundant bacterial groups of the communities in the 2260 m fracture belonged to Actinobacteria and α-proteobacteria. Little is known about metabolic capacities of the OPB41 candidate phylum, but as an actinobacterial phylotype, they may be chemoorganotrophs, while *Bradyrhizobiaceae* are mixotrophs with the capacity to oxidize H_2. The fractures located below 2 km depth, mostly dominated

by unclassifiable phylotypes, might suggest that these depths harbour life that differs considerably from the currently known microorganisms.

4.2 Comparison of the microbial community structure and functionality between different Precambrian deep subsurface sites

Bacterial and archaeal communities from the Outokumpu fracture zones resemble the drill hole water communities described in a previous study (Nyyssönen et al., 2014). This is not surprising, as drill hole fluids are a mixture of the different fracture fluids emanating to the drill hole from the fractures at different depths of the bedrock. *Comamonadaceae* form a major part of the bacterial community at most depths in the drill hole, as they are abundant in 180, 500, 2260 and 2300 m fracture communities. At 967 and 1820 m fracture communities, phylotypes affiliating with *Clostridiales* are dominating, while clostridial phylotypes represent a major fraction of the drill hole communities at 1000–1500 m. Many sulfate reducer phylotypes detected in this study were similar to those detected from the drill hole fluids (Itävaara et al., 2011; Purkamo et al., 2015) and from fracture zones (Purkamo et al., 2013) with DGGE. These included *Desulfotomaculum* and *Desulfovibrio*. Archaeal communities in the fractures and in the drill hole are mainly dominated by *Methanobacterium*, but SAGMEG archaea are also abundant in the drill hole communities at 1000 m and above (Nyyssönen et al., 2014), possibly originating from the 967 m fracture where SAGMEG archaea were dominating the total archaeal community.

Members of *Comamonadaceae* and *Pseudomonadaceae* belong to the core microbial community in Outokumpu. These were also detected from all studied microbial communities in another Fennoscandian crystalline bedrock environment in Olkiluoto fracture zones (Bomberg et al., 2015a). To emphasize the importance of these microbes to the total communities in deep crystalline bedrock environments, members of *Comamonadaceae* were recognized as the keystone genera of the active microbial community in the Outokumpu deep biosphere.

The microbial communities in Outokumpu deep crystalline bedrock share common features with those of the deep ecosystems in Witwatersrand Basin, South Africa. Clones affiliating with *Comamonadaceae* have been found from a deep drill hole outlet in Driefontein gold mine in South Africa. In addition, clostridial sulphate reducers, such as *Desulfotomaculum*, are dominating the SRB communities in Outokumpu as well as the deep borehole communities in Driefontein (Baker et al., 2003; Moser et al., 2003, 2005; Silver et al., 2010). Candidatus *Desulforudis audaxviator* was a minor component (with 1–35 % relative abundance) of the SRB communities in Outokumpu fractures at depths of 180–2260 m, in contrast to a microbial community where *D. audaxviator* formed a single-species ecosystem in deep bedrock fracture in Mponeng mine (Chivian et al., 2008). The archaeal community in the 967 m fracture was dominated by SAGMEG archaea that were first discovered from gold mines in South Africa (Takai et al., 2001). The predicted metagenome of the archaeal community at this depth showed notably higher amount of genes involved in oxidative phosphorylation than other fracture communities. Whether this is a bias induced by the absence of complete genomes of closely related species in the database or an indication of oxidative phosphorylation genes having a specific role in SAGMEGs remains to be resolved. Furthermore, this study supports the biogeographical trend that methanogens in different deep Precambrian sites are similarly distributed within depth (Kietäväinen and Purkamo, 2015). Methanogens with wider substrate range were found in the fractures located at shallower depths both in Outokumpu and several deep subsurface sites in South Africa. On the other hand, obligately hydrogenotrophic methanogens were detected in the fracture zones located deeper (Moser et al., 2005; Gihring et al., 2006; Lin et al., 2006). Archaeal communities represented much less diversity, and interestingly, while almost all fracture zones were dominated by methanogenic archaea, the archaeal fracture community in 180 m fracture was the most diverse, where for example archaea belonging to Miscellaneous Crenarchaeal Group (MCG) and Terrestrial Miscellaneous Euryarchaeal Group (TMEG) were detected. In Olkiluoto, the highest archaeal species richness was detected from a fracture at a depth of 296 m (Bomberg

et al., 2015a). In another study of archaeal communities in Olkiluoto, some indication of correlation between increasing depth and decreasing diversity could be detected (Bomberg et al., 2015b).

Many of the abundant bacterial groups in Outokumpu bedrock are organotrophic with capacity to use a wide range of substrates for biosynthesis and either fermentation or anaerobic respiration for energy conservation. Hence, depending on the available sources of energy and carbon, these organisms can switch to the best energy mechanism currently available. With the low relative abundance of the genes involved in autotrophic carbon fixation pathways in the predicted metagenomes, we propose that in Outokumpu, heterotrophic carbon metabolism is important also for the intrinsic fracture zone communities as it is for the drill hole water column communities (Purkamo et al., 2015). Archaeal communities in Outokumpu fracture zones are mainly methanogenic, using most likely the hydrogenotrophic methanogenesis pathway from CO_2 to CH_4.

5 Conclusions

The microbial communities of Outokumpu Precambrian crystalline bedrock fractures share features with serpenization-driven microbial communities in alkaline springs and subsurface aquifers. These include members belonging to *Burkholderiales* and Clostridia. Additionally, these phylotypes were regarded as the keystone species in Outokumpu deep biosphere. Additionally, *Comamonadaceae* are part of the core microbial community in Fennoscandian crystalline bedrock environments. Sulfate reducing microbes and methanogens are present, but they represent marginal groups of the microbial communities. The dominating taxa of the sulphate reducing communities observed in this study are similar to the ones detected from the deep subsurface of Driefontein mine in South Africa. Similarly, the total archaeal community at 967 m fracture is dominated by SAGMEG archaea, initially described at deep gold mines of South Africa. Otherwise hydrogenotrophic methanogens, mainly *Methanobacterium*,

dominate the archaeal communities. The fracture zone at 180 m in Outokumpu hosts the most diverse archaeal communities. Many keystone species of Outokumpu deep biosphere belong to rare biosphere, with low abundance but a wide range of carbon substrates and a capacity for H_2 oxidation. Metagenomic predictions of the bacterial communities revealed that heterotrophy is also important in the deep fracture zones in Outokumpu.

The Supplement related to this article is available online at doi:10.5194/bgd-12-18103-2015-supplement.

Author contributions. L. Purkamo planned and conducted the research, analysed the data and is the corresponding author. M. Bomberg aided in planning, sampling, analysis of the data and writing the manuscript. R. Kietäväinen took part in sampling, provided hydrogeochemistry data and participated in writing the manuscript. H. Salavirta assisted in analysing the data and writing the manuscript. M. Nyyssönen took part in sampling and writing the manuscript. M. Nuppunen-Puputti assisted in sampling and aided in laboratory research, L. Ahonen assisted in planning and executing the sampling and hydrogeochemistry analysis and writing the manuscript. I. Kukkonen provided access to Outokumpu Deep Drill Hole, supported sampling, writing the manuscript and provided funding and M. Itävaara took part in sampling and writing the manucript and provided funding.

Acknowledgements. Mirva Pyrhönen is acknowledged for the excellent laboratory skills and help in preparing the sampling trips. Sirpa Jylhä, Leea Ojala, Arto Pullinen and Pauliina Rajala and are acknowledged for the professional sampling assistance at the Outokumpu field site. This study was funded by Kone Foundation, The Academy of Finland (DEEP LIFE project, grant number 133348/2009), The Foundation for Research of Finnish Natural Resources (grant number 1718/09) and Finnish research program on nuclear waste management (KYT, grants GEOMOL, KABIO, SALAMI and RENGAS).

References

Abubucker, S., Segata, N., Goll, J., Schubert, A. M., Izard, J., Cantarel, B. L., Rodriguez-Mueller, B., Zucker, J., Thiagarajan, M., and Henrissat, B.: Metabolic reconstruction for metagenomic data and its application to the human microbiome, PLoS Comput. Biol., 8, e1002358, doi:10.1371/journal.pcbi.1002358, 2012.

Altschul, S. F., Gish, W., Miller, W., Myers, E. W., and Lipman, D. J.: Basic local alignment search tool, J. Mol. Biol., 215, 403–410, 1990.

Amend, J. P. and Teske, A.: Expanding frontiers in deep subsurface microbiology, Palaeogeogr. Palaeocl., 219, 131–155, 2005.

Baker, B. J., Moser, D. P., MacGregor, B. J., Fishbain, S., Wagner, M., Fry, N. K., Jackson, B., Speolstra, N., Loos, S., and Takai, K.: Related assemblages of sulphate-reducing bacteria associated with ultradeep gold mines of South Africa and deep basalt aquifers of Washington State, Environ. Microbiol., 5, 267–277, 2003.

Bano, N., Ruffin, S., Ransom, B., and Hollibaugh, J. T.: Phylogenetic composition of Arctic Ocean archaeal assemblages and comparison with Antarctic assemblages, Appl. Environ. Microbiol., 70, 781–789, 2004.

Barns, S. M., Fundyga, R. E., Jeffries, M. W., and Pace, N. R.: Remarkable archaeal diversity detected in a Yellowstone National Park hot spring environment, P. Natl. Acad. Sci. USA, 91, 1609–1613, 1994.

Bastian, M., Heymann, S., and Jacomy, M.: Gephi: an open source software for exploring and manipulating networks, ICWSM, 8, 361–362, 2009.

Biddle, J. F., Fitz-Gibbon, S., Schuster, S. C., Brenchley, J. E., and House, C. H.: Metagenomic signatures of the Peru margin subseafloor biosphere show a genetically distinct environment, P. Natl. Acad. Sci. USA, 105, 10583–10588, 2008.

Biddle, J. F., White, J. R., Teske, A. P., and House, C. H.: Metagenomics of the subsurface Brazos–Trinity Basin (IODP site 1320): comparison with other sediment and pyrosequenced metagenomes, ISME J., 5, 1038–1047, 2011.

Blankenberg, D., Von Kuster, G., Coraor, N., Ananda, G., Lazarus, R., Mangan, M., Nekrutenko, A., and Taylor, J.: Galaxy: a web-based genome analysis tool for experimentalists, Curr. Protoc. Mol. Biol., 19, 19.10.1–19.10.21, 2010.

Blondel, V. D., Guillaume, J.-L., Lambiotte, R., and Lefebvre, E.: Fast unfolding of communities in large networks, J. Stat. Mech.-Theory E., 10, P10008, doi:10.1088/1742-5468/2008/10/P10008, 2008.

Bomberg, M., Nyyssönen, M., Nousiainen, A., Hultman, J., Paulin, L., Auvinen, P., and Itävaara, M.: Evaluation of molecular techniques in characterization of deep terrestrial biosphere, Open J. Ecol., 4, 468–487, 2014.

Bomberg, M., Nyyssönen, M., Pitkänen, P., Lehtinen, A., and Itävaara, M.: Active microbial communities inhabit sulphate–methane interphase in deep bedrock fracture fluids in Olkiluoto, Finland, BioMed Research International, 979530, doi:10.1155/2015/979530, 2015a.

Bomberg, M., Lamminmäki, T., and Itävaara, M.: Estimation of microbial metabolism and co-occurrence patterns in fracture groundwaters of deep crystalline bedrock at Olkiluoto, Finland, Biogeosciences Discuss., 12, 13819–13857, doi:10.5194/bgd-12-13819-2015, 2015b.

Brandes, U.: A faster algorithm for betweenness centrality, J. Math. Sociol., 25, 163–177, 2001.

Brazelton, W. J., Nelson, B., and Schrenk, M. O.: Metagenomic evidence for H_2 oxidation and H_2 production by serpentinite-hosted subsurface microbial communities, Front. Microbiol., 2, 268, doi:10.3389/fmicb.2011.00268, 2012.

Brazelton, W. J., Morrill, P. L., Szponar, N., and Schrenk, M. O.: Bacterial communities associated with subsurface geochemical processes in continental serpentinite springs, Appl. Environ. Microbiol., 79, 3906–3916, doi:10.1128/AEM.00330-13, 2013.

Brown, M. V., Philip, G. K., Bunge, J. A., Smith, M. C., Bissett, A., Lauro, F. M., Fuhrman, J. A., and Donachie, S. P.: Microbial community structure in the North Pacific ocean, ISME J., 3, 1374–1386, doi:10.1038/ismej.2009.86, 2009.

Caporaso, J. G., Kuczynski, J., Stombaugh, J., Bittinger, K., Bushman, F. D., Costello, E. K., Fierer, N., Pena, A. G., Goodrich, J. K., and Gordon, J. I.: QIIME allows analysis of high-throughput community sequencing data, Nat. Methods, 7, 335–336, 2010.

Chivian, D., Brodie, E. L., Alm, E. J., Culley, D. E., Dehal, P. S., DeSantis, T. Z., Gihring, T. M., Lapidus, A., Lin, L. H., Lowry, S. R., Moser, D. P., Richardson, P. M., Southam, G., Wanger, G., Pratt, L. M., Andersen, G. L., Hazen, T. C., Brockman, F. J., Arkin, A. P., and Onstott, T. C.: Environmental genomics reveals a single-species ecosystem deep within Earth, Science, 322, 275–278, doi:10.1126/science.1155495, 2008.

Edwars, U., Rogall, T., Blocker, H., Emde, M., and Bottger, E. C.: Isolation and direct complete nucleotide determination of entire genes. Characterization of a gene coding for 16S ribosomal RNA, Nucleic Acids Res., 17, 7843–7853, 1989.

Etiope, G. and Sherwood Lollar, B.: Abiotic methane on Earth, Rev. Geophys., 51, 276–299, 2013.

Fish, J. A., Chai, B., Wang, Q., Sun, Y., Brown, C. T., Tiedje, J. M., and Cole, J. R.: FunGene: the functional gene pipeline and repository, Front. Microbiol., 4, 291, doi:10.3389/fmicb.2013.00291, 2013.

Geets, J., Borremans, B., Diels, L., Springael, D., Vangronsveld, J., van der Lelie, D., and Vanbroekhoven, K.: *Dsr*B gene-based DGGE for community and diversity surveys of sulfate-reducing bacteria, J. Microbiol. Meth., 66, 194–205, 2006.

Giardine, B., Riemer, C., Hardison, R. C., Burhans, R., Elnitski, L., Shah, P., Zhang, Y., Blankenberg, D., Albert, I., Taylor, J., Miller, W., Kent, W. J., and Nekrutenko, A.: Galaxy: A platform for interactive large-scale genome analysis, Genome Res., 15, 1451–1455, 2005.

Gihring, T., Moser, D., Lin, L. H., Davidson, M., Onstott, T., Morgan, L., Milleson, M., Kieft, T., Trimarco, E., and Balkwill, D.: The distribution of microbial taxa in the subsurface water of the Kalahari Shield, South Africa, Geomicrobiol. J., 23, 415–430, 2006.

Goecks, J., Nekrutenko, A., Taylor, J., and The Galaxy Team: Galaxy: a comprehensive approach for supporting accessible, reproducible, and transparent computational research in the life sciences, Genome Biol., 11, R86, doi:10.1186/gb-2010-11-8-r86, 2010.

Gold, T.: The deep, hot biosphere, P. Natl. Acad. Sci. USA, 89, 6045–6049, 1992.

Grosskopf, R., Janssen, P. H., and Liesack, W.: Diversity and structure of the methanogenic community in anoxic rice paddy soil microcosms as examined by cultivation and direct 16S rRNA gene sequence retrieval, Appl. Environ. Microb., 64, 960–969, 1998.

Hales, B. A., Edwards, C., Ritchie, D. A., Hall, G., Pickup, R. W., and Saunders, J. R.: Isolation and identification of methanogen-specific DNA from blanket bog peat by PCR amplification and sequence analysis, Appl. Environ. Microb., 62, 668–675, 1996.

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

Hallbeck, L. and Pedersen, K.: Culture-dependent comparison of microbial diversity in deep granitic groundwater from two sites considered for a Swedish final repository of spent nuclear fuel, FEMS Microbiol. Ecol., 81, 66–77, 2012.

Hammer, Ø., Harper, D., and Ryan, P.: Past: paleontological statistics software package for education and data analysis, Paleontología Electrónica, 4, 1–9, available at: http://palaeo-electronica.org/2001_1/past/past.pdf (last access: 6 November 2015), 2001.

Itävaara, M., Nyyssönen, M., Kapanen, A., Nousiainen, A., Ahonen, L., and Kukkonen, I.: Characterization of bacterial diversity to a depth of 1500 m in the Outokumpu Deep Borehole, Fennoscandian Shield, FEMS Microbiol. Ecol., 77, 295–309, 2011.

Kietäväinen, R. and Purkamo, L.: The origin, source and cycling of methane in deep crystalline rock biosphere, Frontiers in Microbiology, 6, 725, doi:10.3389/fmicb.2015.00725, 2015.

Kietäväinen, R., Ahonen, L., Kukkonen, I. T., Hendriksson, N., Nyyssönen, M., and Itävaara, M.: Characterisation and isotopic evolution of saline waters of the Outokumpu Deep Drill Hole, Finland – Implications for water origin and deep terrestrial biosphere, Appl. Geochem., 32, 37–51, 2013.

Kukkonen, I. T., Rath, V., Kivekäs, L., Šafanda, J., and Čermák, V.: Geothermal studies of the Outokumpu Deep Drill Hole, in: Outokumpu Deep Drilling Project 2003–2010, edited by: Kukkonen, I. T., Geological Survey of Finland, Finland, Special Paper 51, 181–198, 2011.

Lambiotte, R., Delvenne, J.-C., and Barahona, M.: Laplacian Dynamics and Multiscale Modular Structure, Networks, Laplacian dynamics and multiscale modular structure in networks, arXiv:0812.1770v3, 2009.

Langille, M. G., Zaneveld, J., Caporaso, J. G., McDonald, D., Knights, D., Reyes, J. A., Clemente, J. C., Burkepile, D. E., Thurber, R. L. V., and Knight, R.: Predictive functional profiling of microbial communities using 16S rRNA marker gene sequences, Nat . Biotechnol., 31, 814–821, 2013.

Lau, M. C., Cameron, C., Magnabosco, C., Brown, C. T., Schilkey, F., Grim, S., Hendrickson, S., Pullin, M., Lollar, B. S., and van Heerden, E.: Phylogeny and phylogeography of functional genes shared among seven terrestrial subsurface metagenomes reveal N-cycling and microbial evolutionary relationships, Frontiers in Microbiology, 5, 531, doi:10.3389/fmicb.2014.00531, 2014.

Lin, L., Hall, J., Lippmann-Pipke, J., Ward, J. A., Sherwood Lollar, B., DeFlaun, M., Rothmel, R., Moser, D., Gihring, T. M., and Mislowack, B.: Radiolytic H_2 in continental crust: nuclear power for deep subsurface microbial communities, Geochem. Geophy. Geosy., 6, Q07003, doi:10.1029/2004GC000907, 2005.

Lin, L., Hall, J., Onstott, T., Gihring, T., Lollar, B. S., Boice, E., Pratt, L., Lippmann-Pipke, J., and Bellamy, R. E.: Planktonic microbial communities associated with fracture-derived groundwater in a deep gold mine of South Africa, Geomicrobiol. J., 23, 475–497, 2006.

Lupatini, M., Suleiman, A. K., Jacques, R. J., Antoniolli, Z. I., de Siqueira Ferreira, A., Kuramae, E. E., and Roesch, L. F.: Network topology reveals high connectance levels

and few key microbial genera within soils, Frontiers in Environmental Science, 2, 10, doi:10.3389/fenvs.2014.00010, 2014.

Magnabosco, C., Ryan, K., Lau, M. C., Kuloyo, O., Sherwood Lollar, B., Kieft, T. L., van Heerden, E., and Onstott, T.: A metagenomic window into carbon metabolism at 3 km depth in Precambrian continental crust, ISME J., in press, doi:10.1038/ismej.2015.150, 2015.

McCollom, T. M.: Laboratory simulations of abiotic hydrocarbon formation in Earth's deep subsurface, Rev. Mineral. Geochem., 75, 467–494, doi:10.2138/rmg.2013.75.15, 2013.

McCollom, T. M., Lollar, B. S., Lacrampe-Couloume, G., and Seewald, J. S.: The influence of carbon source on abiotic organic synthesis and carbon isotope fractionation under hydrothermal conditions, Geochim. Cosmochim. Ac., 74, 2717–2740, 2010.

Moser, D., Onstott, T., Fredrickson, J., Brockman, F., Balkwill, D., Drake, G., Pfiffner, S., White, D., Takai, K., and Pratt, L.: Temporal shifts in microbial community structure and geochemistry of an ultradeep South African gold mine borehole, Geomicrobiol. J., 20, 517–548, 2003.

Moser, D. P., Gihring, T. M., Brockman, F. J., Fredrickson, J. K., Balkwill, D. L., Dollhopf, M. E., Lollar, B. S., Pratt, L. M., Boice, E., Southam, G., Wanger, G., Baker, B. J., Pfiffner, S. M., Lin, L. H., and Onstott, T. C.: *Desulfotomaculum* and *Methanobacterium* spp. dominate a 4- to 5-kilometer-deep fault, Appl. Environ. Microb., 71, 8773–8783, 2005.

Mu, A., Boreham, C., Leong, H. X., Haese, R. R., and Moreau, J. W.: Changes in the deep subsurface microbial biosphere resulting from a field-scale CO_2 geosequestration experiment, Frontiers in Microbiology, 5, 209, doi:10.3389/fmicb.2014.00209, 2014.

Muyzer, G., de Waal, E. C., and Uitterlinden, A. G.: Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA, Appl. Environ. Microb., 59, 695–700, 1993.

Nyyssönen, M., Bomberg, M., Kapanen, A., Nousiainen, A., Pitkänen, P., and Itävaara, M.: Methanogenic and sulphate-reducing microbial communities in deep groundwater of crystalline rock fractures in Olkiluoto, Finland, Geomicrobiol. J., 29, 863–878, 2012.

Nyyssönen, M., Hultman, J., Ahonen, L., Kukkonen, I., Paulin, L., Laine, P., Itävaara, M., and Auvinen, P.: Taxonomically and functionally diverse microbial communities in deep crystalline rocks of the Fennoscandian shield, ISME J., 8, 126–138, 2013.

Osburn, M. R., LaRowe, D. E., Momper, L. M., and Amend, J. P.: Chemolithotrophy in the continental deep subsurface: Sanford Underground Research Facility (SURF), USA, Frontiers in Microbiology, 5, 610, doi:10.3389/fmicb.2014.00610, 2014.

Paine, R. T.: A conversation on refining the concept of keystone species, Conserv. Biol., 9, 962–964, 1995.

Pedersen, K.: Investigations of subterranean bacteria in deep crystalline bedrock and their importance for the disposal of nuclear waste, Can. J. Microbiol., 42, 382–391, 1996.

Pedersen, K.: Microbial life in deep granitic rock, FEMS Microbiol. Rev., 20, 399–414, 1997.

Pedersen, K.: Exploration of deep intraterrestrial microbial life: current perspectives, FEMS Microbiol. Lett., 185, 9–16, 2000.

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

Power, M. E., Tilman D., Estes, J. A., Menge, B. A., Bond, W. J., Mills, L. S., Gretchen, D. , Castilla, J. C., Lubchenco, J., and Paine, R. T.: Challenges in the quest for keystones, BioScience, 4, 609–620, 1996.

Proskurowski, G., Lilley, M. D., Seewald, J. S., Fruh-Green, G. L., Olson, E. J., Lupton, J. E., Sylva, S. P., and Kelley, D. S.: Abiogenic hydrocarbon production at lost city hydrothermal field, Science, 319, 604–607, doi:10.1126/science.1151194, 2008.

Purkamo, L., Bomberg, M., Nyyssönen, M., Kukkonen, I., Ahonen, L., Kietäväinen, R., and Itävaara, M.: Dissecting the deep biosphere: retrieving authentic microbial communities from packer-isolated deep crystalline bedrock fracture zones, FEMS Microbiol. Ecol., 85, 324–337, 2013.

Purkamo, L., Bomberg, M., Nyyssönen, M., Kukkonen, I., Ahonen, L., and Itävaara, M.: Heterotrophic communities supplied by ancient organic carbon predominate in deep Fennoscandian bedrock fluids, Microb. Ecol., 69, 319–332, 2015.

Quince, C., Lanzén, A., Curtis, T. P., Davenport, R. J., Hall, N., Head, I. M., Read, L. F., and Sloan, W. T.: Accurate determination of microbial diversity from 454 pyrosequencing data, Nature Meth., 6, 639–641, 2009.

Rosenberg, E., DeLong, E. F., Lory, S., Stackebrandt, E., and Thompson, F. (Eds): The Prokaryotes, 4th Edn., Springer-Verlag Berlin Heidelberg, 2014.

Schloss, P. D., Westcott, S. L., Ryabin, T., Hall, J. R., Hartmann, M., Hollister, E. B., Lesniewski, R. A., Oakley, B. B., Parks, D. H., and Robinson, C. J.: Introducing mothur: open-source, platform-independent, community-supported software for describing and comparing microbial communities, Appl. Environ. Microbiol., 75, 7537–7541, 2009.

Silver, B., Onstott, T., Rose, G., Lin, L. H., Ralston, C., Sherwood-Lollar, B., Pfiffner, S., Kieft, T., and McCuddy, S.: In situ cultivation of subsurface microorganisms in a deep mafic sill: implications for SLiMEs, Geomicrobiol. J., 27, 329–348, 2010.

Sogin, M. L., Morrison, H. G., Huber, J. A., Mark Welch, D., Huse, S. M., Neal, P. R., Arrieta, J. M., and Herndl, G. J.: Microbial diversity in the deep sea and the underexplored "rare biosphere", P. Natl. Acad. Sci. USA, 103, 12115–12120, 2006.

Stahl, A. and Amann, R.: Development and application of nucleic acid probes, in: Nucleic acid techniques in bacterial systematics, edited by: Stackebrandt, E. and Goodfellow, M., John Wiley & Sons, Chichester, 205–248, 1991.

Szponar, N., Brazelton, W. J., Schrenk, M. O., Bower, D. M., Steele, A., and Morrill, P. L.: Geochemistry of a continental site of serpentinization, the Tablelands Ophiolite, Gros Morne National Park: a Mars analogue, Icarus, 224, 286–296, 2013.

Tiago, I. and Veríssimo, A.: Microbial and functional diversity of a subterrestrial high pH groundwater associated to serpentinization, Environ. Microbiol., 15, 1687–1706, 2013.

Wagner, M., Roger, A. J., Flax, J. L., Brusseau, G. A., and Stahl, D. A.: Phylogeny of dissimilatory sulfite reductases supports an early origin of sulfate respiration, J. Bacteriol., 180, 2975–2982, 1998.

Willems, A., De Ley, J., Gillis, M., and Kersters, K.: Comamonadaceae, a new family encompassing the acidovorans rRNA complex, including *Variovorax paradoxus* gen. nov., comb. nov. for *Alcaligenes paradoxus* (Davis 1969), Int. J. Syst. Bacteriol., 41, 445–450, 1991.

Zhou, J., Deng, Y., Luo, F., He, Z., and Yang, Y.: Phylogenetic molecular ecological network of soil microbial communities in response to elevated CO_2, MBio, 2, e00122-11, doi:10.1128/mBio.00122-11, 2011.

Table 1. Hydrogeochemical characteristics of six fracture zones of Outokumpu. Concentrations for cations and anions are given in mg mL^{-1}, EC in mS m^{-1} and alkalinity in mmol mL^{-1}.

Depth m	Prevalent rock type	Ca	Fe	Mg	Na	S	Br	Cl	SO_4	NO_3	Sulfide	TOC	DOC	TIC	DIC	pH	EC[1]	Alkalinity
180	Mica schist, biotite gneiss	1060	0.34	16.7	1070	1.27	23	3280	1.5	< 20	0.057	12.8	9	0.7	0.6	7.4	1060	0.31
500	Chlorite-sericite schist	2250	< 0.03	12.9	1810	3.49	< 50	8180	1.0	< 100	bd	bd	bd	bd	bd	8.3	1900	0.19
967	Mica schist, chlorite-sericite schist	2000	< 0.03	0.8	1770	17.1	62.2	5790	0.6	< 40	bd	6.93	6.4	< 0.2	< 0.2	8.9	1740	0.29
1820	Mica and black schist, granite	11800	0.03	15.1	3820	44.4	159	30300	2.6	< 200	0.87	30.33	29.7	0.4	0.51	9.0	6930	0.37
2260	Biotite gneiss	8130	0.03	21	2630	4.8	< 1000	16400	< 2	< 2000	bd	bd	bd	bd	bd	8.2	4890	0.25
2300	Mica schist, granite	9480	< 0.03	18.7	3120	7.42	123	24500	< 2	< 200	0.086	34.33	34	0.4	< 0.2	8.6	4370	0.29

[1] Kietäväinen et al. (2013).
[2] Electrical conductivity at 25 °C.
bd = below detection limit.

Table 2. The total number of cells and the 16S rRNA gene copy numbers of microbial communities in six fractures of Outokumpu. Values are given in mL^{-1}.

Fracture depth	Total cell amount		Bacterial 16S rRNA gene		Archaeal 16S rRNA gene	
m	cell number	SEM*	copy number	SEM*	copy number	SEM*
180	2.97×10^5	6.25×10^4	5.13×10^6	1.49×10^5	6.24×10^3	1.25×10^0
500	5.72×10^4	3.04×10^3	1.88×10^6	2.99×10^5	8.62×10^1	1.23×10^0
967	1.00×10^4	8.91×10^2	1.26×10^5	2.15×10^4	4.90×10^2	1.24×10^0
1820	4.74×10^3	1.17×10^3	9.05×10^2	2.29×10^1	bd	NA
2260	1.51×10^3	3.52×10^2	9.01×10^2	3.72×10^1	2.32×10^1	1.07×10^0
2300	6.30×10^3	1.89×10^3	9.00×10^2	2.07×10^1	bd	NA

* SEM = standard error of mean.
bd = below detection limit.
NA = not available.

Table 3. The most abundant metabolism-related **(a)** bacterial and **(b)** archaeal genes in the predicted metagenomes.

(a)	180 m		500 m		967 m		1820 m		2260 m		2300 m	
	DNA	RNA	DNA	RNA	DNA	RNA	DNA	RNA	DNA	RNA	DNA	RNA
Carbohydrate Metabolism	19%	21%	20%	19%	20%	19%	19%	20%	21%	19%	19%	19%
Amino Acid Metabolism	21%	21%	22%	23%	22%	22%	21%	22%	21%	21%	22%	21%
Energy Metabolism	13%	13%	12%	11%	11%	13%	11%	12%	13%	13%	11%	11%
Metabolism of Cofactors and Vitamins	9%	0%	9%	9%	8%	10%	8%	8%	9%	8%	8%	9%
Lipid Metabolism	6%	0%	7%	8%	8%	7%	8%	8%	7%	7%	8%	7%
Nucleotide Metabolism	8%	0%	7%	6%	7%	8%	6%	7%	7%	7%	6%	8%
Xenobiotics Biodegradation and Metabolism	4%	0%	7%	8%	7%	6%	9%	6%	7%	9%	9%	7%
Metabolism of Terpenoids and Polyketides	4%	0%	4%	5%	5%	4%	4%	4%	5%	5%	4%	4%
Metabolism of Other Amino Acids	4%	0%	4%	4%	4%	3%	4%	4%	4%	4%	4%	4%
Enzyme Families	4%	0%	3%	3%	4%	4%	4%	4%	3%	3%	4%	4%
Glycan Biosynthesis and Metabolism	7%	0%	3%	3%	4%	4%	4%	3%	3%	3%	4%	4%
Biosynthesis of Other Secondary Metabolites	2%	0%	2%	2%	2%	2%	1%	1%	1%	2%	2%	1%

(b)	180 m		500 m		967 m		1820 m		2260 m		2300 m	
	DNA	RNA	DNA	RNA	DNA	RNA	DNA	RNA	DNA	RNA	DNA	RNA
Energy Metabolism	23%	23%	25%	25%	18%	24%			24%	24%	24%	
Amino Acid Metabolism	21%	21%	21%	21%	25%	22%			22%	22%	22%	
Carbohydrate Metabolism	18%	20%	17%	17%	19%	17%			17%	17%	17%	
Nucleotide Metabolism	11%	10%	11%	11%	13%	11%			11%	11%	11%	
Metabolism of Cofactors and Vitamins	10%	10%	11%	11%	10%	10%			10%	10%	10%	
Xenobiotics Biodegradation and Metabolism	3%	4%	3%	3%	2%	3%			3%	3%	3%	
Enzyme Families	3%	3%	3%	3%	3%	3%	nd	nd	3%	3%	3%	nd
Metabolism of Terpenoids and Polyketides	3%	3%	3%	3%	3%	3%			3%	3%	3%	
Glycan Biosynthesis and Metabolism	2%	2%	3%	3%	2%	2%			2%	2%	2%	
Biosynthesis of Other Secondary Metabolites	2%	2%	2%	2%	2%	2%			2%	2%	2%	
Lipid Metabolism	2%	1%	1%	1%	2%	1%			1%	1%	1%	
Metabolism of Other Amino Acids	1%	1%	1%	1%	1%	1%			1%	1%	1%	

nd = not detected.

Table 4. The keystone genera of the total microbial communities in Outokumpu fractures.

Keystone OTUs	Closeness Centrality	Betweenness Centrality	Degree	Family	Relative abundance*
Other *Burkholderiaceae*	2.0	394	41	*Burkholderiacaea*	1 %
Desulfitobacter	2.4	302	4	*Peptococcaceae*	6 %
Other *Clostridiaceae*	1.7	302	20	*Clostridiaceae*	2 %
Dethiobacter	2.4	302	26	*Anaerobrancaceae*	25 %
Herbaspirillum	1.8	248	48	*Oxalobacteracaea*	6 %
Pelomonas	1.8	218	41	*Comamonadaceae*	72 %
Novosphingobium	1.1	162	46	*Sphingomonadaceae*	2 %
Comamonas	1.8	151	29	*Comamonadaceae*	72 %
Average	1.4	21	25		

* Highest relative abundance in the family level in the community.

Table 5. The keystone genera of the active microbial communities in Outokumpu fractures.

Keystone OTUs	Closeness Centrality	Betweenness Centrality	Degree	Family	Relative abundance[a]
Curvibacter	1.8	797	41	*Comamonadaceae*	71 %
Comamonas	1.8	476	4	*Comamonadaceae*	71 %
Sphingomonas	1.7	474	20	*Sphingomonadaceae*	0.1 %
Bacilli OTU87	1.1	294	26	*Bacilli*[b]	1 %
Flavobacterium	1.8	276	48	*Flavobacteraceae*	< 0,1 %
Williamsia	1.1	256	41	*Williamsiacaea*	< 0,1 %
Staphylococcus	2.5	189	46	*Staphylococcaceae*	1 %
Oxalobacteraceae	1.8	172	29	*Oxalobacteraceae*	0.3 %
Herbaspirillum	1.9	155	57	*Oxalobacteraceae*	0.3 %
Average	1.3	21	37		

[a] Highest relative abundance in the family level in the community.
[b] Only identified to class level.

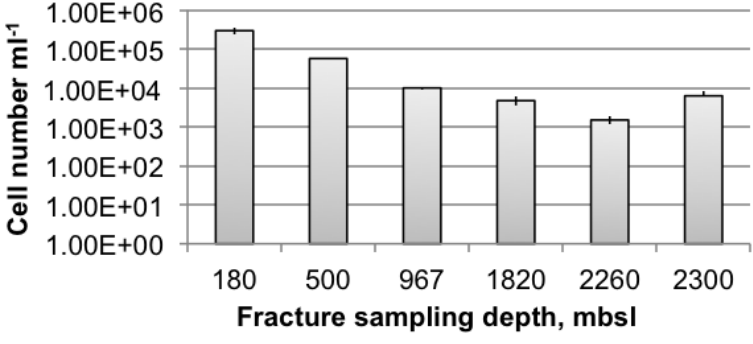

Figure 1. Total number of microbial cells in Outokumpu fracture fluids.

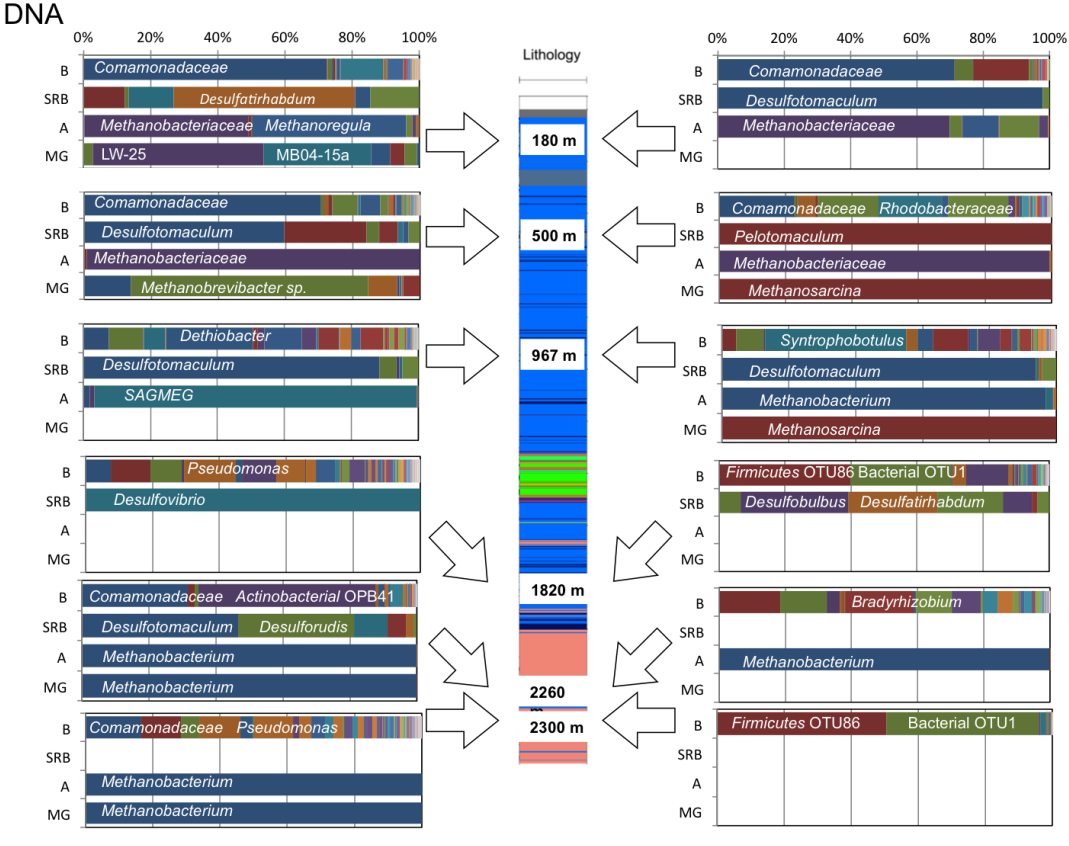
DNA
RNA
0%
20%
40%
60%
80%
100%
Lithology
B
SRB
A
MG
Comamonadaceae
Desulfatirhabdum
Methanobacteriaceae
Methanoregula
LW-25
MB04-15a
180 m
Comamonadaceae
Desulfotomaculum
Methanobacteriaceae
Rhodobacteraceae
Pelotomaculum
Methanosarcina
500 m
Methanobrevibacter sp.
Dethiobacter
SAGMEG
967 m
Syntrophobotulus
Methanobacterium
Pseudomonas
Desulfovibrio
Firmicutes OTU86 Bacterial OTU1
Desulfobulbus
1820 m
Actinobacterial OPB41
Desulforudis
Bradyrhizobium
2260
2300 m
Firmicutes OTU86
Bacterial OTU1

Figure 2. Microbial community structure at six different fractures in Outokumpu Precambrian crystalline bedrock. On the center a schematic presentation of the Outokumpu Deep Drill hole lithology (blue mica schist, green ophiolitic rocks, pink pegmatitic granite) with arrows pointing to the depths of the fractures studied. The composition of the total communities on the left and the active communities on the right side. The taxonomic classification of only the most abundant OTUs is shown. B: bacteria, SRB: sulphate reducing bacteria, A: archaea, MG: methanogens.

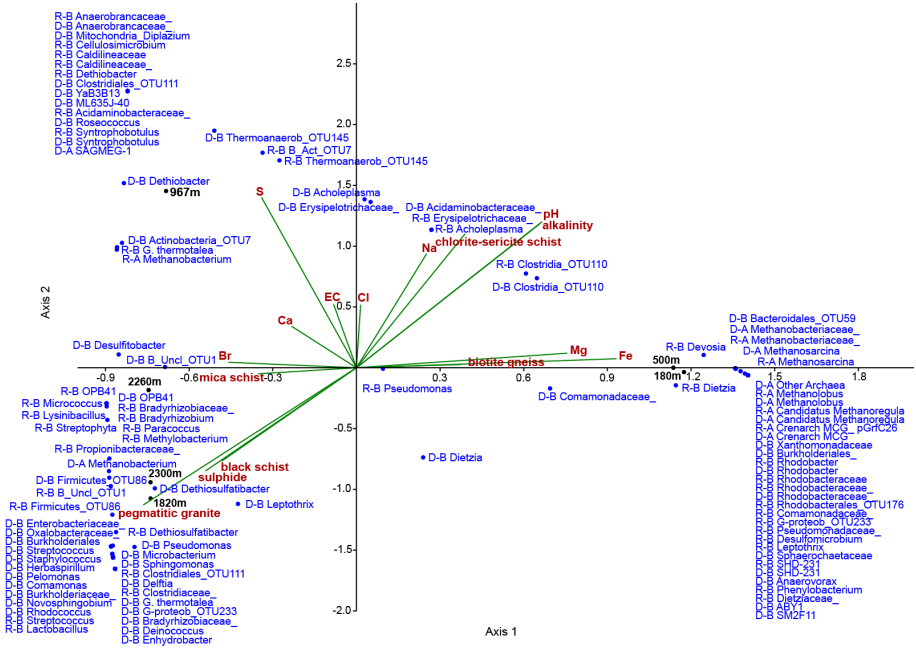

Figure 3. Canonical correspondence plot of the microbial OTUs (blue, letters before the OTU name denoting the origin and the domain, i.e. D: derived from DNA, R: derived from RNA, B: Bacteria, A: Archaea), depths (black) and the geochemical parameters (red). Horizontal axis explains the 35 % of the variance of the data with statistical significance ($p < 0.01$), as vertical axis explains 27 % of the variance of the data ($p = 0.11$).

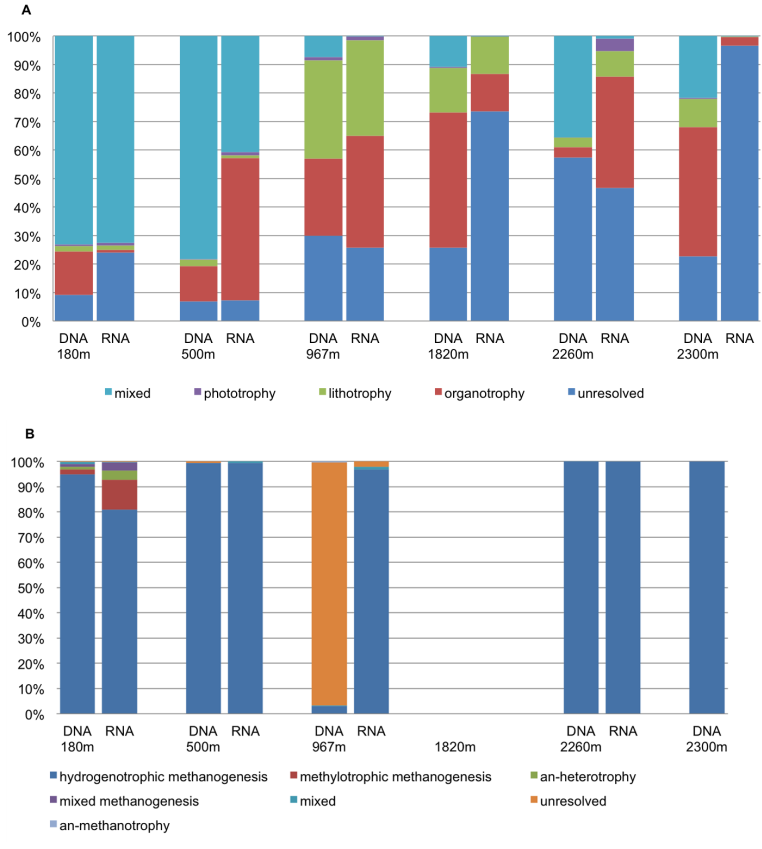

Figure 4. Binned **(a)** bacterial and **(b)** archaeal physiotypes according to the predominant metabolism of OTUs in the family level.

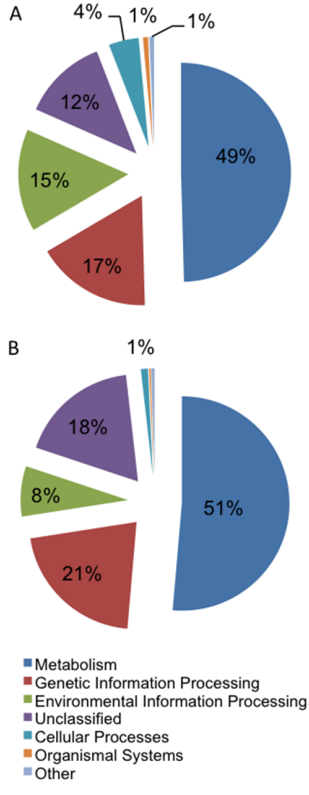

Figure 5. The average predicted functionality of all **(a)** bacterial and **(b)** archaeal metagenomes reconstructed from 16S rRNA gene sequences.

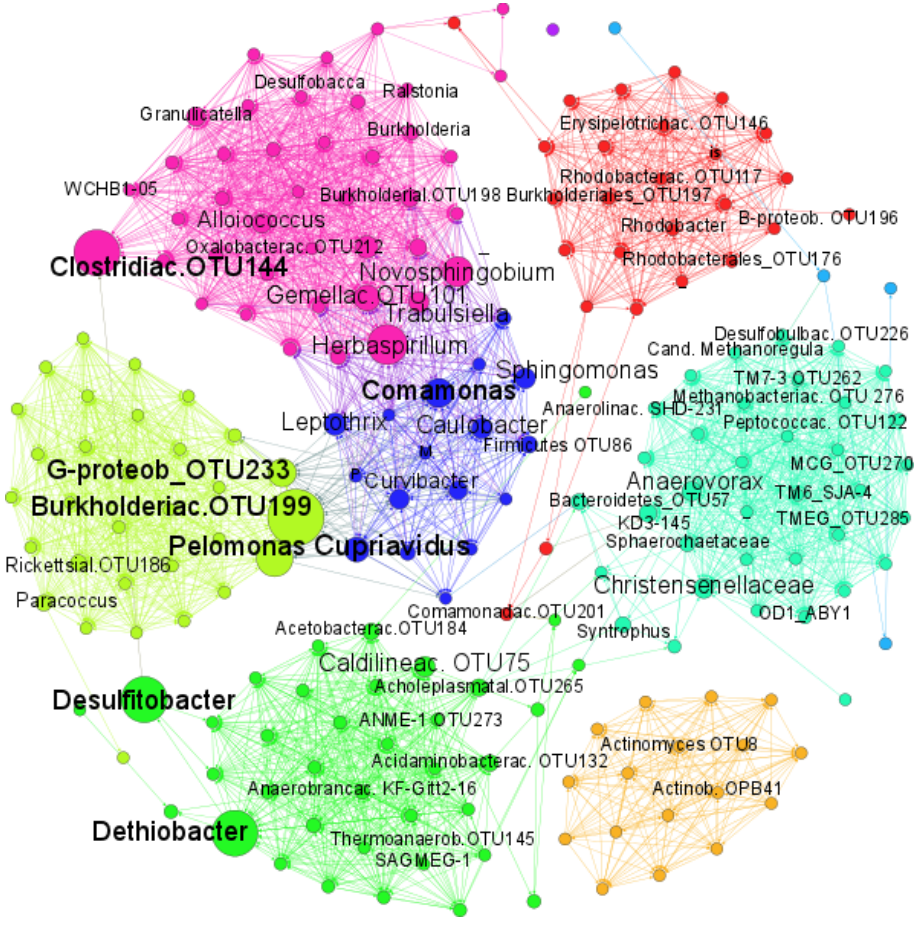

Figure 6. The co-occurrence network of the total microbial community in Outokumpu bedrock. The size of each node corresponds to the betweenness of centrality value of the OTU. The different modules are represented in different colours.

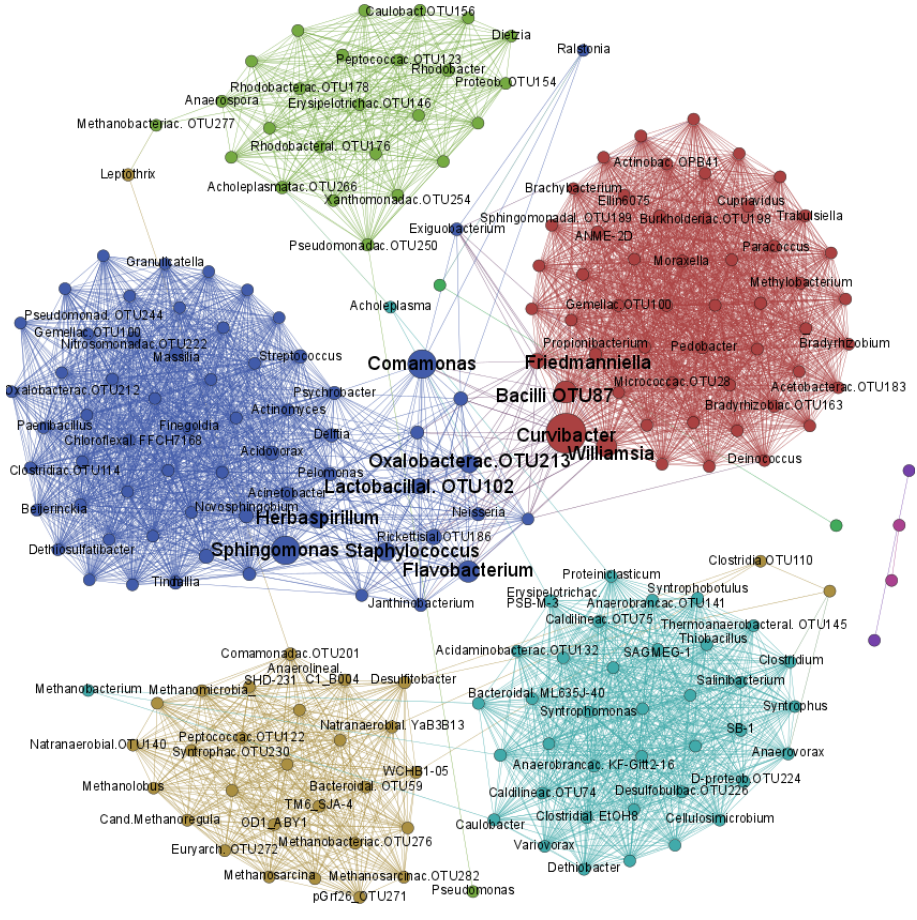

Figure 7. The co-occurrence network of the active microbial community in Outokumpu bedrock. The size of each node corresponds to the betweenness of centrality value of the OTU. Modules are represented by different colors.

Series title and number

VTT Science 116

Title	**Microbial ecology and functionality in deep Fennoscandian crystalline bedrock biosphere**
Author(s)	Lotta Purkamo
Abstract	Microbial life in the deep subsurface contributes significantly to overall biomass on Earth. Although the microbial communities inhabiting the deep subsurface are abundant, little is known about their diversity, activity, interactions and role in global biogeochemical cycles. The diversity of microbial life in the deep terrestrial subsurface of the Fennoscandian shield was studied with molecular biological methods. The Outokumpu Deep Drill Hole provides access to crystalline bedrock fluids that are estimated to be tens of millions of years old. Characterization of the indigenous bacterial and archaeal communities in addition to microbial communities with important functional properties in bedrock fluids was done from a depth range of 180 m to 2300 m. Microbial community profiling and assessment of possible functional processes was done with molecular fingerprinting, cloning and sequencing methods combined with suitable statistical and bioinformatics analyses. Low cell numbers but high diversity was characteristic to the microbial communities of the Outokumpu deep subsurface. The microbial communities in the fracture zones had in general fewer cells than those in the mixed fluids of the drill hole. *Comamonadaceae*, *Peptococcaceae* and *Anaerobrancaceae* were prevalent bacterial members of the microbial communities in the fracture fluids. Archaea were a minority in microbial communities. Sulfate-reducing bacteria and methanogens were detected at several depths. Microbial communities resembled those detected from other deep Fennoscandian Shield subsurface sites. Furthermore, sulfate reducing communities and archaeal communities resembled those found from the deep subsurface of South Africa. Investigation on carbon assimilation strategies of the microbial communities revealed that mainly heterotrophic *Clostridia* were responsible for CO_2 fixation in this habitat. Representatives of *Burkholderiales* and *Clostridia* formed the core microbial community and these were also identified to be the keystone genera. The microbial communities of Outokumpu fractures share similarity with those of serpentinization-driven ecosystems. Energy and carbon substrates formed in serpentinization reactions of ophiolitic rocks in Outokumpu may sustain the microbial communities in this deep subsurface environment.
ISBN, ISSN, URN	ISBN 978-951-38-8370-6 (Soft back ed.) ISBN 978-951-38-8371-3 (URL: http://www.vttresearch.com/impact/publications) ISSN-L 2242-119X ISSN 2242-119X (Print) ISSN 2242-1203 (Online) http://urn.fi/URN:ISBN:978-951-38-8371-3
Date	December 2015
Language	English, Finnish abstract
Pages	86 p. + app. 82 p.
Name of the project	
Commissioned by	
Keywords	terrestrial deep biosphere, crystalline bedrock, microbial communities, functional genes, bacteria, archaea, sulfate reducers, methanogens
Publisher	VTT Technical Research Centre of Finland Ltd P.O. Box 1000, FI-02044 VTT, Finland, Tel. 020 722 111

Julkaisun sarja ja numero

VTT Science 116

Nimeke	**Mikrobien ekologia ja toiminnallisuus syvällä Fennoskandian kiteisessä kallioperässä**
Tekijä(t)	Lotta Purkamo
Tiivistelmä	Syvällä maanpinnan alla elävät mikrobit muodostavat merkittävän osan maapallon kokonaisbiomassasta. Vaikka syvällä elää runsaasti mikrobeja, niiden monimuotoisuudesta, aktiivisuudesta, vuorovaikutuksista ja roolista maailmanlaajuisissa biogeokemiallisissa kierroissa tiedetään vielä vähän. Mikrobielämän monimuotoisuutta Fennoskandian kilven kallioperässä tutkittiin käyttäen molekyylibiologisia menetelmiä. Outokummun syvä kairareikä tarjoaa mahdollisuuden tutkia kiteisen kallioperän vesiä, joiden on arvioitu olevan kymmeniä miljoonia vuosia vanhoja. Kallioperän endeemisten bakteeri- ja arkeoniyhteisöjen koostumusta ja merkittäviä toiminnallisia ominaisuuksia luonnehdittiin syvyysvälillä 180–2300 m. Mikrobiyhteisöjen toiminnallisten ominaisuuksien arviointi toteutettiin molekyylibiologisin menetelmin käyttäen sormenjälki-, kloonaus- ja sekvensointimenetelmiä sekä sopivia tilastollisia ja bioinformatiikan analyysejä. Matala solumäärä mutta korkea monimuotoisuusaste leimasivat mikrobiyhteisöjä Outokummun syvässä kallioperässä. Rakovyöhykkeissä oli yleisesti vähemmän soluja kuin sekoittuneessa kairareiän vesipatsaassa. *Comamonadaceae*-, *Peptococcaceae*- ja *Anaerobrancaceae*-sukujen bakteerit olivat vallitsevia rakovyöhykkeiden mikrobiyhteisöissä. Arkeonit olivat vähemmistönä yhteisöissä. Sulfaattia pelkistäviä bakteereita ja metaanintuottajia havaittiin useissa eri syvyyksissä. Mikrobiyhteisöt muistuttivat muista Fennoskandian syvistä ympäristöistä havaittuja mikrobiyhteisöjä. Lisäksi sulfaatinpelkistäjät sekä arkeoniyhteisöt olivat samankaltaisia Etelä-Afrikan syvän kallioperän mikrobiyhteisöjen kanssa. Mikrobien hiiliaineenvaihdunnan tutkimus paljasti heterotrofisten klostridien toimivan pääasiallisina hiilidioksidin sitojina näissä elinympäristöissä. *Burkholderiales*- ja *Clostridia*-bakteeriryhmien edustajat muodostivat nk. Outokummun kallioperän ydinmikrobiston, eli näiden ryhmien jäseniä löytyi kaikista syvyyksistä. Lisäksi näiden ryhmien edustajien todettiin olevan yhteisöjen avainlajeja. Outokummun kallioperän rakovyöhykkeiden vesissä elävät mikrobiyhteisöt ovat samankaltaisia kuin ne yhteisöt, joita löytyy ekosysteemeistä joiden energia ja hiili ovat lähtöisin ofioliittisten kivien serpentinisaatiosta. Serpentinisaatioreaktioissa muodostuvat energian- ja hiilenlähteet voivat ylläpitää mikrobiyhteisöjä myös Outokummun syväbiosfäärissä.
ISBN, ISSN, URN	ISBN 978-951-38-8370-6 (nid.) ISBN 978-951-38-8371-3 (URL: http://www.vtt.fi/julkaisut) ISSN-L 2242-119X ISSN 2242-119X (Painettu) ISSN 2242-1203 (Verkkojulkaisu) http://urn.fi/URN:ISBN:978-951-38-8371-3
Julkaisuaika	Joulukuu 2015
Kieli	Englanti, suomenkielinen tiivistelmä
Sivumäärä	86 s. + liitt. 82 s.
Projektin nimi	
Rahoittajat	
Avainsanat	Mikrobiyhteisöt, bakteerit, arkeonit, syväbiosfääri, kiteinen kallioperä, toiminnalliset geenit, sulfaatinpelkistäjät, metanogeenit
Julkaisija	Teknologian tutkimuskeskus VTT Oy PL 1000, 02044 VTT, puh. 020 722 111

Microbial ecology and functionality in deep Fennoscandian crystalline bedrock biosphere

The need for a comprehensive understanding of the microbial processes in deep subsurface and the environmental factors regulating these activities has recently been growing simultaneously with the industrial interest of utilization of deep bedrock for material or energy source or storage space. In addition to the assessment of the microbial risks of these operations, microbial ecological research of the deep subsurface may provide innovations for different types of industries in form of novel microbes, enzymes or bioactive compounds. Furthermore, research on microbial ecology of the deep subsurface will improve our ability to understand the origin of life on Earth and possibility of extraterrestrial life on other planetary bodies.

The microbial communities in Outokumpu deep crystalline bedrock fluids share common features with other deep ecosystems.Thus, this thesis contributes to the biogeographic trend that different deep subsurface sites host microbial communities with structural similarities often related with depth. The geochemical composition and the sources of carbon and energy in the deep, ancient fluids in bedrock are likely the most important factors determining the community structure in Outokumpu subsurface.

ISBN 978-951-38-8370-6 (Soft back ed.)
ISBN 978-951-38-8371-3 (URL: http://www.vttresearch.com/impact/publications)
ISSN-L 2242-119X
ISSN 2242-119X (Print)
ISSN 2242-1203 (Online)
http://urn.fi/URN:ISBN:978-951-38-8371-3