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Microbial activation due to addition of electron donors/acceptors in deep groundwaters

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Publishable Summary

Microbial communities utilize many energy sources in the deep biosphere. These energy sources and the biogeochemical cycles of elements, such as carbon, nitrogen and sulphur, are multiply connected to each other. It is highly important to understand these connections and the most important sources of energy used by microbes in order to evaluate the risks these microbial communities cause to the geological long-term storage of used nuclear fuel. Deep subsurface microbial communities in steady-state do not cause unidentified risks. In Finland, the Olkiluoto final disposal site contains different groundwater types including sulphate rich, brackish chloride and highly saline waters and their mixtures. In addition, gases such as methane can be found extensively in some water types. Mixing of these groundwater types causes metabolic activation of the resident microorganisms and in some cases for example corrosive sulphide accumulation takes place. The aim here was to study and simulate the deep groundwater mixing and infiltration, which introduces previously limited electron donors and acceptors for microbial communities and generate community activation.

The two deep groundwaters from Olkiluoto, studied with redox sensing fluorescent dye CTC were very different from each other. OL-KR6 was a sulphate rich groundwater with high bacterial and archaeal diversity and clear activation with several electron donors and acceptors was detected. OL-KR15 on the other hand, had lower bacterial and archaeal diversity than OL-KR6 water and was activated with only acetate and acetate together with sulphate. Acetate was overall the most efficient activator of the studied microbial communities which indicates acetate's important role as an electron donor for different Olkiluoto deep subsurface groundwater communities.

Activation of deep subsurface microbial populations detected with fluorescent redox dyes was an efficient screening method to study induced microbial metabolism. The used redox dyes (CTC and RSG) stained a large part of the studied groundwater populations without additions, indicating high activity level of the studied communities. However, for this reason, not all activations induced by electron acceptor and donor amendments were possible to detect. Another tested approach to study microbial community activation, the reverse transcriptase quantitative PCR (RT-qPCR) was tested. Bacterial and archaeal 16S rRNA gene transcripts were detected in the original and in all activated groundwaters but *dsr*B, *mcr*A and *nar*G gene transcripts to detect sulphate and nitrate reducers and methanogens, were below detection limits.

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1 Introduction

Microbial communities utilize many energy sources in the deep biosphere. These energy sources and the biogeochemical cycles of elements, such as carbon, nitrogen and sulphur, are multiply connected to each other. It is highly important to understand these connections and the most important sources of energy used by microbes in order to evaluate the risks that these microbial communities might cause to the geological long-term storage of used nuclear fuel.

Microbes may form diverse communities in conditions with low levels of available energy and nutritional compounds. Such microbial communities may achieve relatively stable community structures over the course of time. The deep subsurface life in undisturbed condition exists in a balance that is maintained at steady-state by one or probably several limiting factors, such as energy or nutrient sources. Deep subsurface microbial communities in steady-state do not cause unidentified risks. However, the construction of the geological final disposal facility, bedrock disturbances and hydraulic gradients cause changes that affect the microbial steady-state. Conductive fracture zones in the Fennoscandian crystalline bedrock will mix different groundwater types that have different redox potential. In Finland, the Olkiluoto final disposal site contains different groundwater types including sulphate rich, brackish chloride and highly saline waters and their mixtures (Posiva, 2013). In addition, gases such as methane can be found extensively in some water types. Mixing of these groundwater types may cause metabolic activation of the resident microorganisms and in some cases for example corrosive sulphide accumulation takes place (Posiva, 2013).

Measurement between microbial viability, activity, dormancy and death is quite complicated (Hammes et al., 2010; Del Giorgio and Gasol, 2008; Lennon and Jones, 2011) and especially in the deep subsurface, where the physiological state of microbes is poorly explained by laboratory cultures (Hoehler and Jørgensen, 2013). There are several approaches to study microbial state of viability and activity and all these methods have their benefits and drawbacks in terms of different ecosystems, populations and applications (Hammes et al., 2010; Del Giorgio and Gasol, 2008). These techniques include targeting microbes' morphological/membrane integrity, membrane potential, efflux pump activity, respiratory activity, enzymatic activity, substrate uptake and cellular energy. In addition, fast developing sequencing, especially transcriptomics, provides new possibilities for studying microbial activation. Easier and simpler option for activation research is the quantitative PCR of the specific chosen transcript genes. Overall mixing of different approaches presents wider image of the studied diverse microbial communities than applying only one technique.

CTC, 5-cyano-2,3-ditolyl tetrazolium chloride, is a redox sensing fluorescent colourless dye that is incorporated into cells by metabolically active microbes and reduced by the electron transport system components to red fluorescent formazan (Créach et al., 2002). It has been widely used to study metabolic activity of aerobic bacteria but also facultative anaerobic and obligate anaerobic bacteria have shown to reduce CTC to intracellular CTC-formazan crystals (Bhupathiraju et al., 1999). RSG (RedoxSensor Green) is a fluorogenic redox indicator dye that turns into green fluorescent by microbial reductases of the electron transport systems

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(Kalyuzhnaya et al., 2008). RSG has been suggested as an alternative for CTC as it does not suppress cellular metabolism of some species, unlike CTC (Kalyuzhnaya et al., 2008).

The aim here was to study and simulate the deep groundwater mixing and infiltration, which introduces previously limited electron donors and acceptors for microbial communities and generate community activation in bedrock. The study approach included several techniques for the microbial activation measurement: cellular energy measurement with ATP, microbial respiratory activity measurements with CTC and RSG and transcript analysis with qPCR. These techniques were tested in practice and the most applicable ones were used in full-scale experiments supported with other general microbial techniques.

2 Experiment design

2.1 Studied deep fracture groundwaters

The Olkiluoto site has previously been extensively described (Posiva, 2013). Here two fracture fluids from Olkiluoto drillholes OL-KR6 and OL-KR15 were sampled in Spring 2017 (Table 2-1) and one groundwater from Olkiluoto underground rock characterisation facility (ONKALO) in Autumn 2017. The drillhole fracture water samples were collected overnight into sterile, anaerobic glass bottles and the ONKALO groundwater over period of nine days. The main fracture fluid hydrogeochemical characteristics are described in Table 2-1.

Table 2-1: OL-KR6 and OL-KR15 drillhole fracture fluid hydrogeochemical characteristics from Olkiluoto, Finland. na: not analysed

Drilhole	OL-KR6	OL-KR15	ONK-PV8
Sampling date	23/07/2017	31/01/2017	23-31/08/2017
Depth (m)	- 94	-434	- 279
Ec (mS m^{-1})	1270	2700	na
pH	7.8	8.1	7.8
HCO_3 (mg L^{-1})	134	12	27
NPOC (mgL^{-1})	4	11	2.2
NO_3^-	<0.2	<0.4	< 0.2
N_{tot} (mg L^{-1})	0.35	1.3	<0.25
NH_4^+ (mg L^{-1})	0.34	0.04	0.03
S_{tot} (mg L^{-1})	140	2.7	66
SO_4^{2-} (mg L^{-1})	435	6.3	191
S_2^- (mg L^{-1})	na	0.25	4.4
Fe_{tot} (mg L^{-1})	0.3	0.06	2.6
Fe(II) (mg L^{-1})	na	0.07	3.1
K (mg L^{-1})	19	11	6.2
Mg (mg L^{-1})	173	34	47
Ca (mg L^{-1})	620	2050	1050
Cl (mg L^{-1})	3650	9050	4690
Na (mg L^{-1})	1510	3230	1810
Acetate (mg L^{-1})	<0.3	<0.6	<0.3

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2.2 Activation of fracture fluids with electron donors and acceptors

Experiment I

The deep fracture waters of OL-KR6 and OL-KR15 were starved for 6 weeks before the activation experiments to diminish available electron acceptor and donor amounts and the microbial baseline activity. The major difference in the studied waters was the high concentration of sulphate in the OL-KR6 groundwater whereas in the OL-KR15 water the sulphate concentration was low. For this reason, in experiments with OL-KR15 water, sulphate was added as an additional electron acceptor compared to OL-KR6 experiments. Electron donors tested included acetate that is a corrosive compound used and produced in several microbial processes, hydrogen and methane that arise from geological and biological processes and sulphide that has been detected to accumulate in certain groundwaters. Nitrate was studied as an electron acceptor in addition to sulphate, as there seems to be microbes in Olkiluoto groundwaters, that are able to use it even though nitrate is normally under detection limit in Olkiluoto. The electron donors and acceptors studied in activation experiments are shown in Table 2-2.

All experiments were performed anaerobically with all reagents sterilized and rendered anoxic with nitrogen gas purging. The used redox sensing fluorescent dyes CTC (5-Cyano-2,3-ditolyl tetrazolium chloride, Polysciences) and RSG (RedoxSensor Green, Molecular Probes) were diluted in distilled sterile water according to the manufacturers' instructions. Experiments were performed in 20 mL sterile, glass microcosms in an anaerobic glove box containing nitrogen gas. The redox sensing dye was added to the sample water, mixed well and divided into duplicate microcosms. Activating compounds (Table 2-2) were then added into the microcosms. As an exception, hydrogen containing microcosms were at first purged with a gas mixture consisting of hydrogen and nitrogen (10:90 %) after which the microcosms were sealed with a gas tight butyl rubber stopper. Sample water containing the redox sensing dye was then injected into the microcosms through the butyl rubber stopper using a hypodermic needle and syringe. Microcosms were incubated at 15 °C in dark for 3 h, after which DAPI stain was added to the water and the microcosms were incubated for an additional 15 min. Each microcosm water was frozen into triplicate cryo-tubes containing 10% of GlyTE at -80 °C. In addition to the activation microcosms, original sample waters were stained with CTC, RSG or DAPI, incubated for 3 h and frozen in 10% GlyTE buffer at -80 °C. ATP amounts of all experiments were measured with the ATP Biomass Kit HS (BioThema) before DAPI staining.

The number of microbial cells (DAPI), activated microbial cells (CTC, RSG) and uncoloured particles were determined from the thawed microcosm waters using flow cytometry (BD FACSAria IIu, Becton Dickinson, NJ, USA). Un-dyed samples were analysed first, in order to determine the background fluorescence. The samples dyed with DAPI alone were then analysed to determine the cell fraction of the water samples. Finally, the double-stained samples (DAPI+CTC or DAPI+RSG) were run to detect the metabolically active cell population. For each sample, 10 000 events were acquired. All experiments were run duplicate. The activation of the microbial communities in the groundwater was measured by comparing the number of fluorescent CTC or RSG dyed cells to the total number of microbial cells stained with DAPI.

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Table 2-2: Activation experiments and electron acceptors and donors added to OL-KR6 and OL-KR15 fracture fluids.

Activating electron donors and acceptors final concentration in water	**Experiment I**		**Experiment II**
	OL-KR6	**OL-KR15**	**ONK-PVA8**
No activation addition	X	X	X
Electron donors			
Acetate 1 mM	X	X	X (2 mM)
Hydrogen 0.08 mM	X	X	X
Sulphide 0.15 mM	X	X	
Methane 1 mM	X	X	X (0.3 mM)
Electron acceptors			
Sodium nitrate 1 mM	X	X	X (2 mM)
Sodium sulphate 1 mM		X	
Combination of electron donors and acceptors			
Acetate 1 mM and Sodium sulphate 1 mM	X		
Hydrogen 0.08 mM and Sodium sulphate 1 mM		X	
Hydrogen 0.16 mM and Carbon dioxide 27 mM			X
Hydrogen 0.08 mM and Acetate 2 mM			X
Methane 1 mM and Sodium sulphate 1 mM		X	
Methane 1 mM and Nitrate 1 mM		X	X (0.3; 2 mM)

Experiment II

A separate experiment was performed for the ONKALO (ONK-PVA8) fracture water. This water was a sulphate rich water where also sulphide (4.4 mg L^{-1}) has accumulated and the water was assumed to have an active microbial community. Activation of the water community was studied by reverse transcriptase qPCR of the extracted RNA. The experiments were performed like in the experiment I, but in bigger sterile glass bottles (600 mL) and without redox sensing and DAPI dyes. Studied activations are shown in Table 2-2.

2.3 Nucleic acid isolation and estimation of microbial community sizes

Community biomass for DNA and RNA groundwater extractions were collected by filtrating the fracture fluids on nitrocellulose acetate filters from 2 × 1L (OL-KR6 and OL-KR15) and 600 mL of ONK-PVA8 water. DNA was extracted using the NucleoSpin Soil DNA extraction kit (Macherey-Nagel) and RNA with RNeasy PowerWater Kit (Qiagen). The size of the microbial community of original groundwater was determined by epifluorescence microscopy of DAPI stained cells as described in Purkamo et al. (2013). Quantitative PCR (qPCR) was applied to estimate the number of bacterial and archaeal populations (16S rRNA gene), sulphate reducers (*dsr*B), nitrate reducers (*nar*G) and methanogens (*mcr*A). The abundance of bacterial 16S rRNA genes was determined by qPCR as described by Tsitko et al. (2014) and the abundance of archaea as described in Bomberg et al. (2016). Bacterial nitrate reducers (*nar*G) and archaeal methanogens (*mcr*A) were determined according to Rajala et al. (2015) and Bomberg et al. (2015), respectively. Reverse transcriptase qPCR was conducted similarly as above described qPCRs but from RNA with the SensiFAST SYBR No-ROX One-Step Kit (Bioline).

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2.4 Amplicon library preparation and sequence analysis

Amplicon libraries for high throughput sequencing with Ion Torrent PGM were prepared by PCR from the DNA samples and sent to Bioser (Oulu, Finland) for fragment purification and sequencing. The Ion Torrent sequence data was analysed with Mothur v. 1.39.5 (Schloss et al., 2009) using the Silva v. 132 sequence database as reference (Quast et al., 2013) at VTT.

3 Results and discussion

OL-KR15 water had a low sulphate content, a trace of sulphide and it originated from a greater depth with more saline conditions than OL-KR6 groundwater, which was a sulphate rich water (Table 2-1). The cell numbers in OL-KR6 and OL-KR15 groundwaters were 3.8 x 10^4 and 4.0 x 10^4 cells mL^{-1}, respectively, based on DAPI staining before starvation.

Activations performed for these two slightly alkaline groundwaters were successful. Both CTC and RSG were actively transported into cells and reduced by the microbial electron transport system becoming fluorescent in the studied groundwaters. In the 6 weeks starved groundwaters, the percent of fluorescent cells ranged from 36% to 54 % and from 75% to 90 % after staining with CTC or RSG, respectively (Figures 3-1 and 3-2). However, this high activation hampered the detection of further activation with added electron donors and acceptors. Based on the lower activation with CTC the further activation experiments were performed with only CTC. In addition to fluorescent redox dyes, microbial ATP amounts were measured to compare the microbial activation. However, ATP amounts were relatively small in these groundwaters and no reliable results of activation was received.

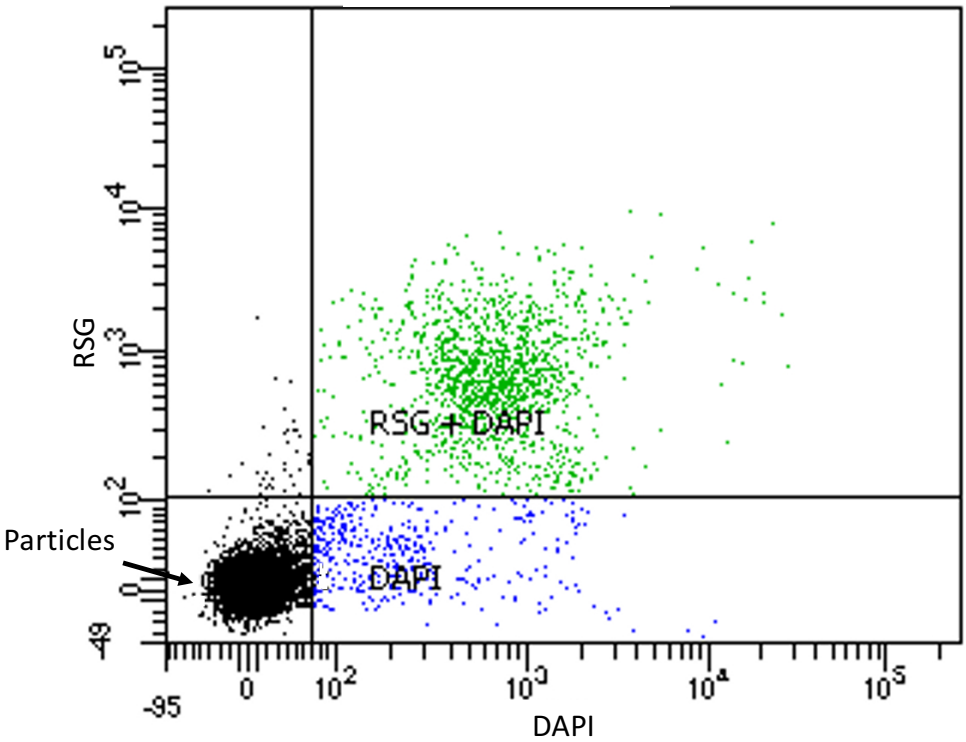

Figure 3-1. RSG (RedoxSensor green) and DAPI stained OL-KR6 unactivated groundwater. 10 000 events measured with flow cytometry: black dots are particles, blue dots are cells stained with DAPI and green dots are cells stained with both DAPI and RSG.

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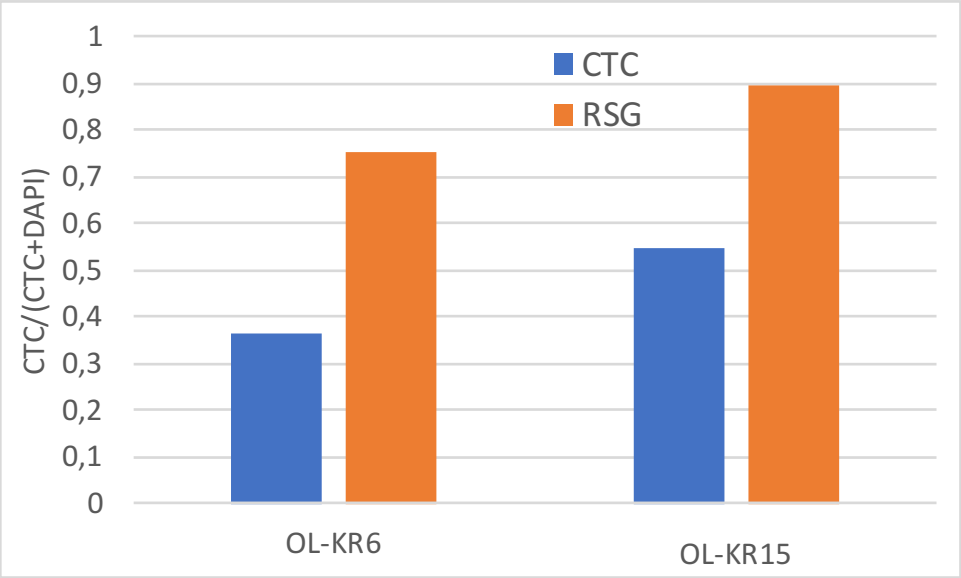

Figure 3-2. Share of fluorescent cells in starved groundwaters of OL-KR6 and OL-KR15 with CTC (5-Cyano-2,3-ditolyl tetrazolium chloride) and RSG (RedoxSensor Green) redox sensing fluorescent dyes measured with flow cytometry.

The OL-KR6 groundwater was activated with all tested additions (Figure 3-2). Acetate and nitrate were the most efficient activators. Acetate activated especially sulphate reducers (SRB) that were probably limited by electron donor shortage. SRBs were abundantly detected from the original groundwater in the qPCR assay (Figure 3-3). In addition, sequencing results (Figure 3-4) showed that there was between 5 to 7 % of Deltaproteobacteria that include sulphate reducers. We also know, that relatively rapid sulphate reduction and sulphide formation started in this water after acetate addition, detected with labelled sulphate ($^{35}SO_4$). In addition, no sulphate reduction could be detected without acetate addition again measured with the labelled sulphate. Other abundant bacterial groups that could utilise acetate in the OL-KR6 water, based on sequencing, consisted mainly of *Sulfurimonas* spp. (33 %) (Labrenz et al., 2013) and Dehalococcoidia (4 %) (Löffler et al., 2013). Based on sequencing of the archaea only few sequences of known aceticlastic archaea like *Methanosaeta* spp. and *Methanosarcina* spp. were detected. In addition, Hadesarchaea and Bathyarchaeota that were found to contribute with around 2-3% of the archaeal sequences each (Figure 3-5), are found to contain genes encoding for acetyl-CoA synthetase for conversion of acetate into acetyl-CoA (Baker et al., 2016; Evans et al., 2015) and may be activated by acetate addition.

The efficiency and attractiveness of nitrate as an activator and electron acceptor is not surprising as it offers higher oxidising potential than sulphate which was abundantly present in the water. The concentration of nitrate is normally below the limit of detection in these deep Olkiluoto groundwaters (Posiva, 2013). However, it is possible that nitrate is used immediately after it is formed in the deep subsurface and it cannot be measured. Some nitrate may be formed e.g. by radiolysis of NH_3 in anaerobic water (Silver et al., 2012). Here nitrate seems to be quite effectively used as an electron acceptor and the microbes have the potential for its utilisation. Nitrate reductase (*nar*G) genes were also detected in the original groundwater with qPCR. Furthermore, an abundant part of the bacterial sequences belonged to Epsilonproteobacteria, mainly to *Sulfurimonas* spp. (33 %). They are known to oxidise reduced sulphur compounds (Han and Perner, 2015) with oxygen, nitrate or nitrite. In

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addition, some gammaproteobacterial *Pseudomonas* spp. that were found at around 6 % relative abundance in the bacterial sequences are known for nitrate reduction (Sias and Ingraham, 1979; Bedzyk et al., 1999)

Methane was an average activator and it is probable that methanotrophs were activated. Anaerobic oxidation of methane (AOM) could take place as ANME-2d archaea are quite often found in Olkiluoto deep groundwaters and recently AOM coupled with sulphate reduction by archaea (Weber et al., 2017) was detected also in deep subsurface granitic environments (Ino et al., 2018). In this OL-KR6 groundwater ANME-2d archaea were detected, however, their abundance was low (0.6 %) based on archaeal sequence data. Bathyarchaeota are found to have genes for methane metabolism (Evans et al., 2015) and they may be activated by methane addition. Nitrate addition together with methane increased the amounts of activated cells which may be connected with AOM coupled with nitrate reduction (Haroon et al., 2013). On the other hand, it is possible that methane addition inhibited the activation level of microbes activated by sole nitrate addition. However, the difference in the activation capacity between nitrate and nitrate together with methane remained within the standard deviations.

Interestingly, sulphide and hydrogen were the least effective activators. OL-KR6 fracture water contained a lot of sulphur oxidisers as already mentioned. However, sulphide activated only a minor part of the community and a shortage of adequate electron acceptor was possibly a limiting factor. For the low activation effect of hydrogen, the reason may be that there was no carbon dioxide available and thus methanogens were not activated. It also seems that hydrogen was not the first choice for electron donor for sulphate reduction by the SRBs and at least some groups preferred acetate (Desulfobacteraceae, Desulfarculaceae). Overall it is also good to remember that one third of the community was already active before addition of any substrates, so these electron donor and acceptor additions only target cells that are not already actively incorporating the CTC stain.

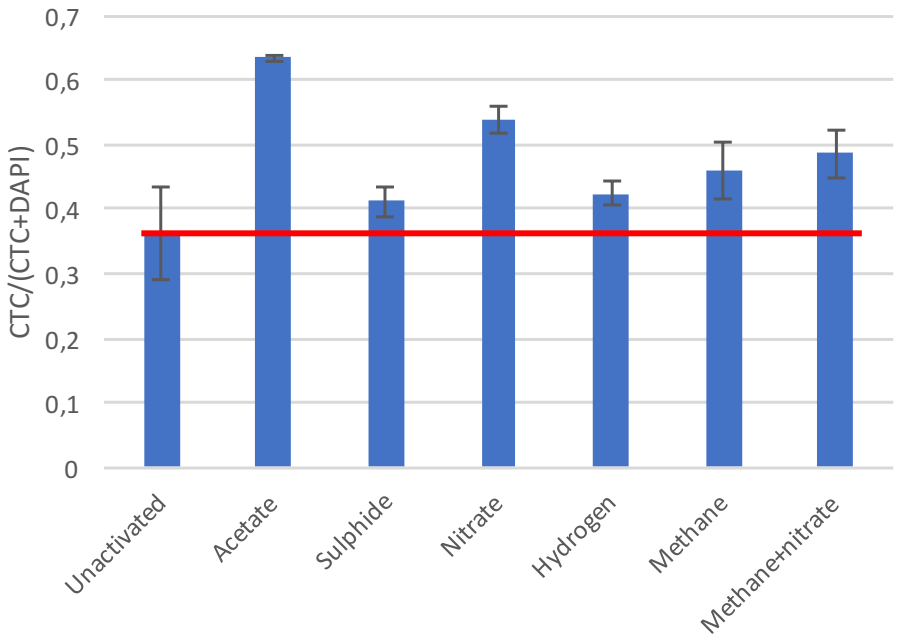

Figure 3-2. Comparison of microbial community activation with different electron donors and acceptors detected with fluorescent redox dye CTC by flow cytometry in OL-KR6 groundwater.

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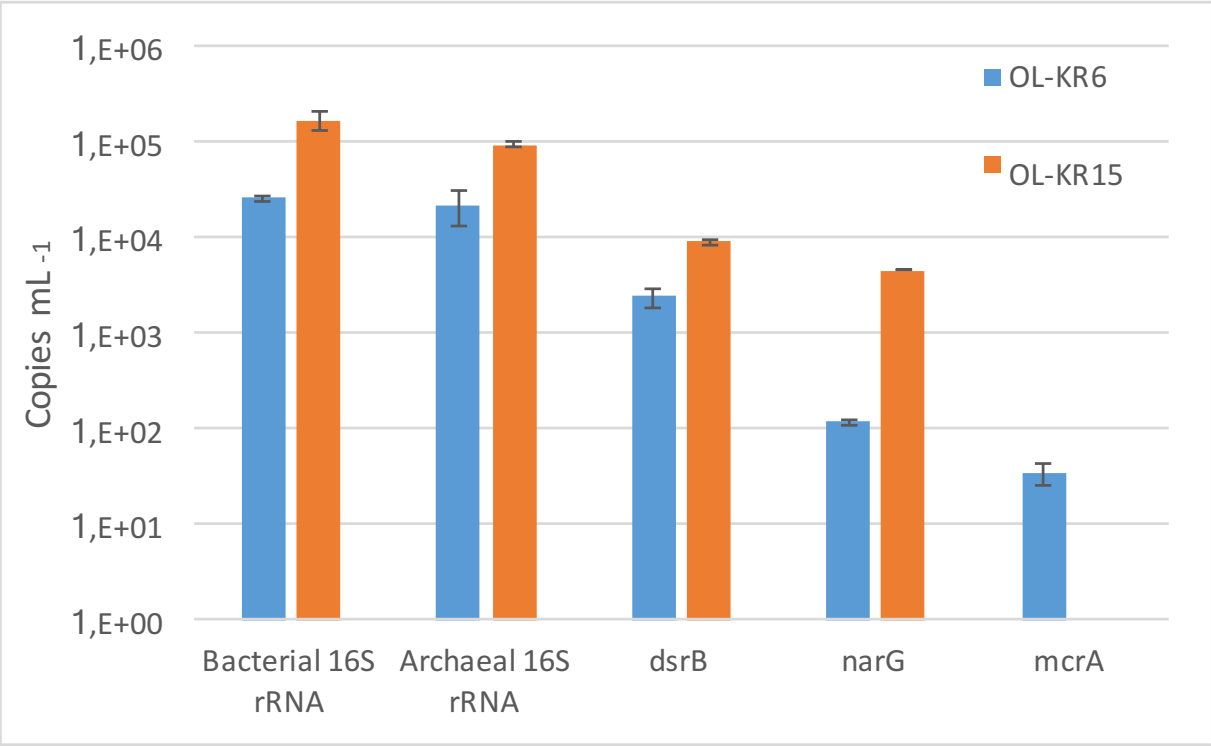

Figure 3-3. The number of bacterial, archaeal, dsr*B,* nar*G and* mcr*A gene copies determined by qPCR from OL-KR6 and OL-KR15 groundwaters (mL^{-1}).*

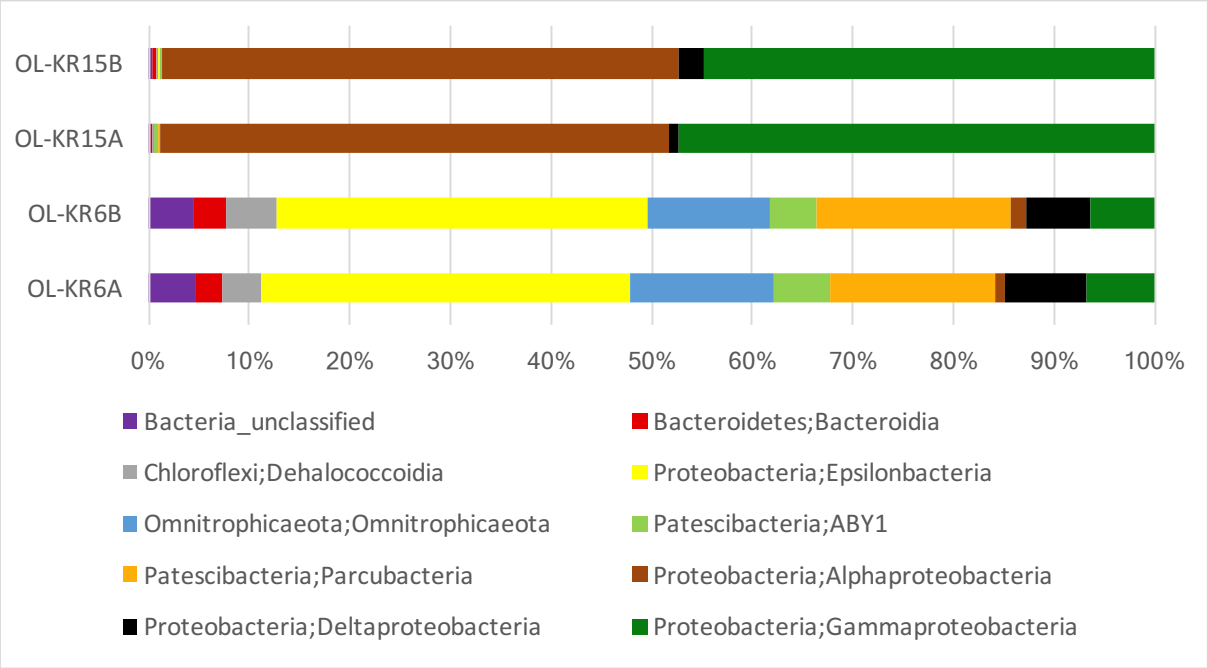

Figure 3-4. Relative distribution of dominant bacterial phyla in OL-KR6 and OL-KR16 groundwaters obtained by Ion Torrent sequencing.

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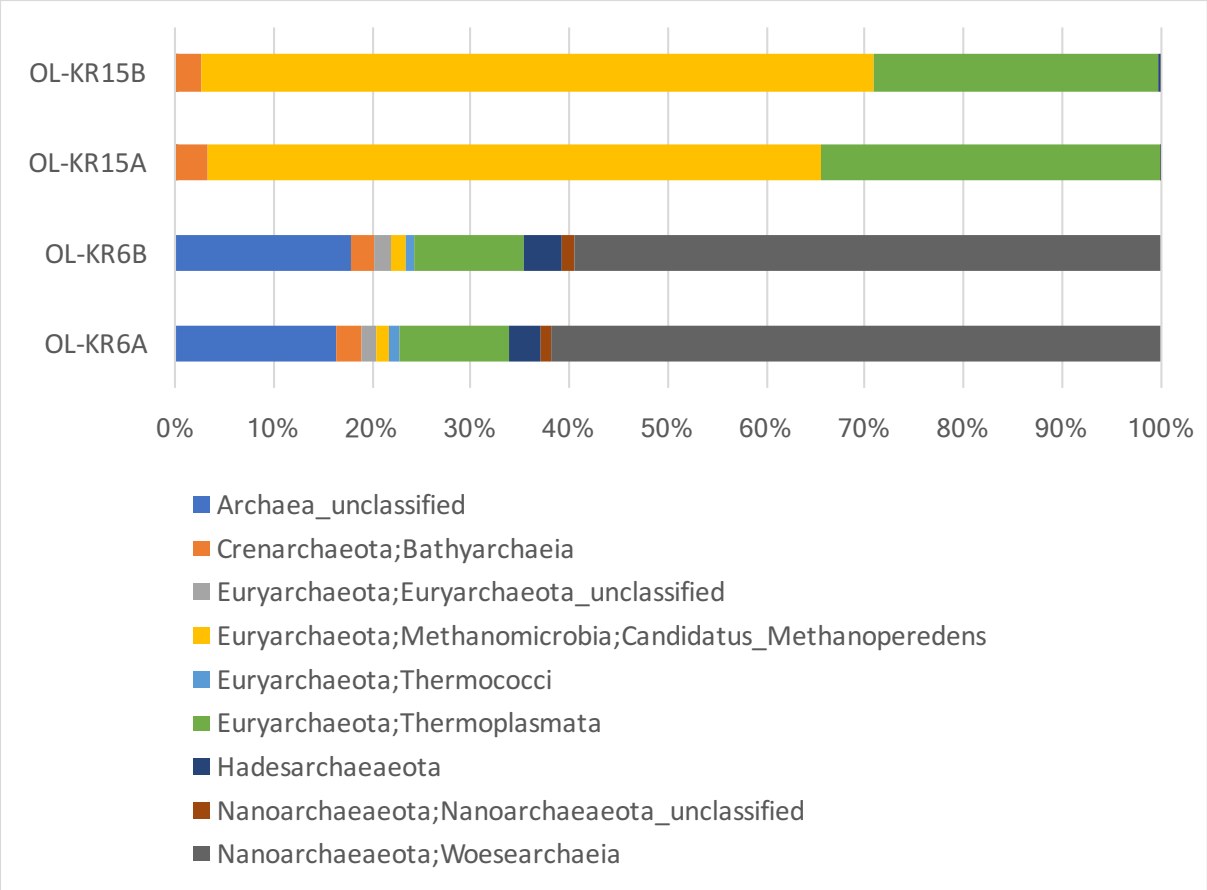

Figure 3-5. Relative distribution of dominant archaeal phyla in OL-KR6 and OL-KR16 groundwaters obtained by Ion Torrent sequencing.

The microbial community in OL-KR15 groundwater behaved very differently from that of the OL-KR6 groundwater. The active share of the community in the baseline water was over 50% (Figure 3-6), which made it difficult to study the effect and activating ability of the added electron donors and acceptors. As the aim was to study especially sulphur reactions, sulphate (1 mM) was added as an electron acceptor to many activation experiments as its concentration was initially low (0.07 mM) and after the 6-week starvation even lower. Only acetate and acetate together with sulphate activated the OL-KR15 microbial community, with approximately similar effect. As in the case with OL-KR6, it seems that acetate was a limiting electron donor for SRBs and aceticlastic archaea even though their abundance was low based on sequencing. It seems probable that also other acetate oxidisers or microbes using acetate as a carbon source have been activated. Alphaproteobacterial *Brevundimonas* spp. sequences were abundantly found (38-42 %). This bacterial group has previously been found from deep subsurface environment and been shown to especially benefit from acetate amendment (Purkamo et al., 2017). qPCR results showed overall higher copy numbers for bacterial and archaeal 16S rRNA, *dsr*B and *nar*G genes in OL-KR15 than in OL-KR16 groundwater. Only the copy numbers of *mcr*A we lower and below detection in the OL-KR15 sample (Figure 3-3).

Sulphate and sulphide additions had little effect on the community activity, which may indicate that they were not limiting sulphate reduction or sulphide oxidation. Nitrate addition had, surprisingly, a slightly inhibiting effect on the community activity. Based on qPCR, there was a relatively high number of *nar*G copies detected in the water, but no activation occurred.

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Hydrogen addition with or without sulphate had also an inhibitory effect on community activity. Again, as in the case of OL-KR6 groundwater, hydrogen with carbon dioxide might have activated methanogens in the archaeal community, and SRBs were probably not activated with hydrogen.

In the case of OL-KR15, the major part (60-66 %) of the archaeal community was found to consist of ANME-2d archaea based on sequencing (Figure 3-5). However, no *mcr*A genes were detected with qPCR, as the used primers did not probably detect *mcr*A genes of ANME archaea. In addition, all methane additions with or without sulphate or nitrate clearly inhibited the community activity. This indicates that the ANME-2d archaea were not oxidising methane coupled to nitrate or sulphate reduction, were dormant or inactivated during starvation, or possible suffered from CTC stain toxicity. CTC has some drawbacks, like toxicity to some species causing inhibition of their metabolism and respiration, and some strains poorly incorporate it into the cell (Hatzinger et al., 2003). Additionally, it may be abiotically reduced (Bhupathiraju et al., 1999). For this reason, all tests showing inhibition may be affected by the CTC. The other tested activity stain RSG could be more suitable, but it demands groundwaters with lower activity or a longer starvation period which changes the community composition.

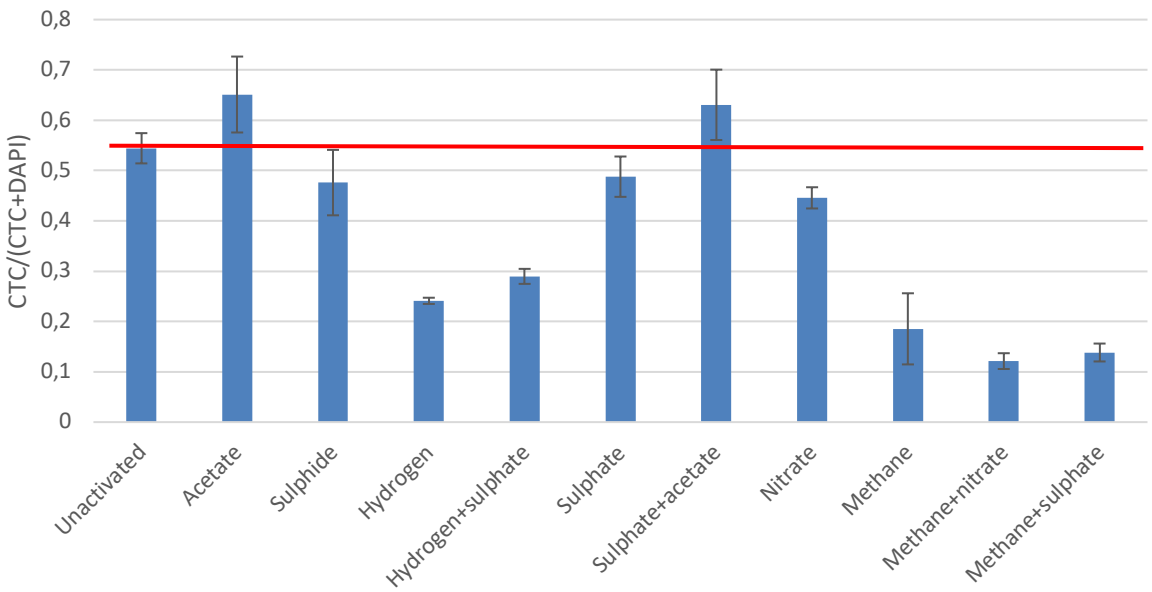

Figure 3-6 Comparison of microbial community activation with different electron donors and acceptors detected with fluorescent redox dye CTC by flow cytometry in OL-KR15 groundwater.

The second activation experiment with ONKALO ONK-PVA8 groundwater, was not that successful experiment. All activations based on the transcripts of the studied genes were at the same level or lower than in the untreated initial groundwater. Bacterial and archaeal 16S rRNA gene transcripts were detected in the original and in all activated groundwaters but *dsr*B, *mcr*A and *nar*G genes were below detection limit. It is possible that the microbial communities in this groundwater were not activated by the tested electron donors and acceptor during the 3 h activation period but a more likely reason for low activation is the water contamination with oxygen during sampling, that lasted for 9 days. For this reason, sensitive anaerobic

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microbes may have either died, inactivated or injured so that they were not responsive for the activations.

4 Conclusions

Activation of microbial populations detected with fluorescent redox dyes was an efficient screening method to study induced microbial metabolism. However, the used redox dyes (CTC and RSG) stained a large part of the studied groundwater populations indicating high activity level of the studied communities. For this reason, not all activations induced by electron acceptor and donor amendments were possible to detect. In addition, the CTC stain may be toxic or inhibit microbial metabolism of some species. Another possibly more reliable and bias free way to study bacterial and archaeal activation would be the quantitative reverse transcription PCR (RT-qPCR) or RNA sequencing. However, this demands much bigger sample volumes. The tested RT-qPCR based approach was not successful probably either due the oxygen contamination of the original groundwater before activations or the microbial communities were not activated by the tested electron donors and acceptors during 3 h activation period.

The two groundwaters studied with redox sensing fluorescent dye were very different from each other. OL-KR6 was a sulphate rich groundwater with high bacterial and archaeal diversity and clear activation with several electron donors and acceptors was detected. OL-KR15 on the other hand, had lower bacterial and archaeal diversity than OL-KR6 water and was activated with only acetate and acetate together with sulphate. Acetate was overall the most efficient activator of the studied microbial communities which indicates acetate's important role as an electron donor for different Olkiluoto deep subsurface groundwater communities.

5 Acknowledgement

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