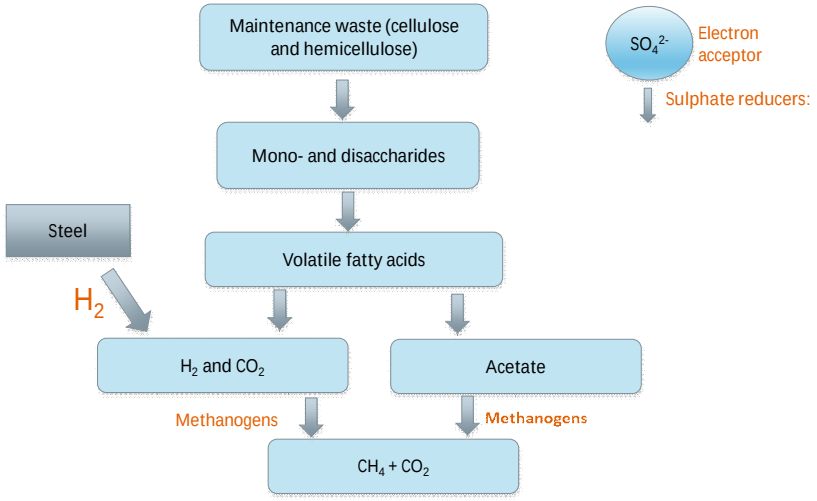
RESEARCH REPORT

VTT-R-00151-22



The influence of chemical conditions on gas generation in the disposal of low level maintenance waste (KaMu)

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Summary

In Finland, low-level maintenance waste (LLW) originated in nuclear power plants is disposed of in the repositories situated ca.100 meters below the sea-level. A large-scale *in situ* Gas Generation Experiment (GGE) was established in 1997 in Olkiluoto, Finland, to simulate the gas generation from LLW under geological repository conditions. The gas generation started already during the first year of the GGE as a result of microbial degradation of waste and steel corrosion. The experiment is operated by Teollisuuden Voima Oyj (TVO) and it has been regularly analysed for chemistry, gas generation and composition of gas. VTT has been responsible for microbiological analyses of the GGE.

The aim of KaMu project was to induced disturbances to the GGE and study the influences on gas generation, gas composition, chemical parameters, and microbiology. The disturbances were induced by adding sulphate and raising the pH. The addition of sulphate simulates sulphate-rich flow of groundwater to the repository and higher pH value the influence of concrete in the repository. The results obtained in this project can be used for safety assessments and for modelling of gas generation.

After sulphate addition, quantitative PCR analysis showed a moderate increase in the number of sulphate reducers and a decrease in the number of methanogens. The diversity of methane-producing microbes stayed similar during the disturbance but certain sulphate reducing bacteria emerged to the microbial population in the tank water. Despite of these microbial changes the sulphate reduction was not enhanced significantly and no changes in gas generation or gas composition were detected.

Similarly, the increase of tank water pH from 6.7 to 8.1 did not influence the rate of gas generation. However, some changes in gas composition were detected. CO_2 concentration in the released gas was reduced because CO_2 was partly absorbed into the more alkaline tank water. In addition, soluble iron concentration in the tank water was reduced indicating precipitation of iron.

Confidentiality	Public

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Contents

ABBREVIATIONS

ASV	Amplicon sequence variant
bp	Base pair
chao1	Abundance-based estimator of species richness
DAPI	4,6-diamidino-2-phenylindole
DIC	Dissolved inorganic carbon
Eh	Electrical potential
DIC	Dissolved inorganic carbon
DNA	Deoxyribonucleic acid
DOC	Dissolved organic carbon
FAPTOTAX	Functional Annotation of Prokaryotic Taxa
GGE	Gas generation experiment
qPCR	Quantitative PCR
*dsr*B	Dissimilatory sulphite reductase β-subunit
*mcr*A	Methyl coenzyme M reductase
NGS	Next generation sequencing
SRB	Sulphate reducing bacteria
TNC	Total number of cells

1. Introduction

In Finland, low-level maintenance waste (LLW) originated in nuclear power plants is disposed of in the repositories situated ca.100 meters below the sea-level. A large-scale *in situ* Gas Generation Experiment (GGE) was established in 1997 in Olkiluoto, Finland, to simulate the gas generation from LLW under geological repository conditions. The GGE is operated by Teollisuuden Voima Oyj (TVO) and is monitored for generated gas, water chemistry and microbiology. A full description of the GGE together with data concerning gas generation, the aqueous chemistry and microbiological studies has been published by Small et al (2008, 2017) and Vikman et al. (2019).

Sixteen 200 dm^3 carbon-steel waste drums were emplaced in a concrete box, as used in the repository, which was enclosed in an acid-proof steel tank. Drums were filled with representative maintenance waste from nuclear power units including paper, cardboard, cotton, and other materials (polyethylene [PE], polyvinylchloride [PVC], glass fibre, latex gloves, natural rubber, polycarbonate cloth, metal). After closure, the repository will be filled with water, which was simulated by filling the GGE with locally sourced untreated river water.

The microbial degradation of cellulose- and hemicellulose-based waste, together with the utilization of hydrogen generated by metal corrosion, resulted in gas generation (Fig. 1). A significant observation from the GGE has been that the pH conditions were heterogeneous (pH 11 to 6), providing optimal neutral pH niches for microbial activity from the outset of the experiment. Over the extended time scale of the experiment, chemical conditions were stabilized and differences in the microbial abundances and community structure in various GGE compartments have become less significant. In recent years, gas generation has stayed at a constant level. Because termination of the GGE is under consideration by TVO, KaMu project was initiated to study the influences of certain disturbances on the gas generation.

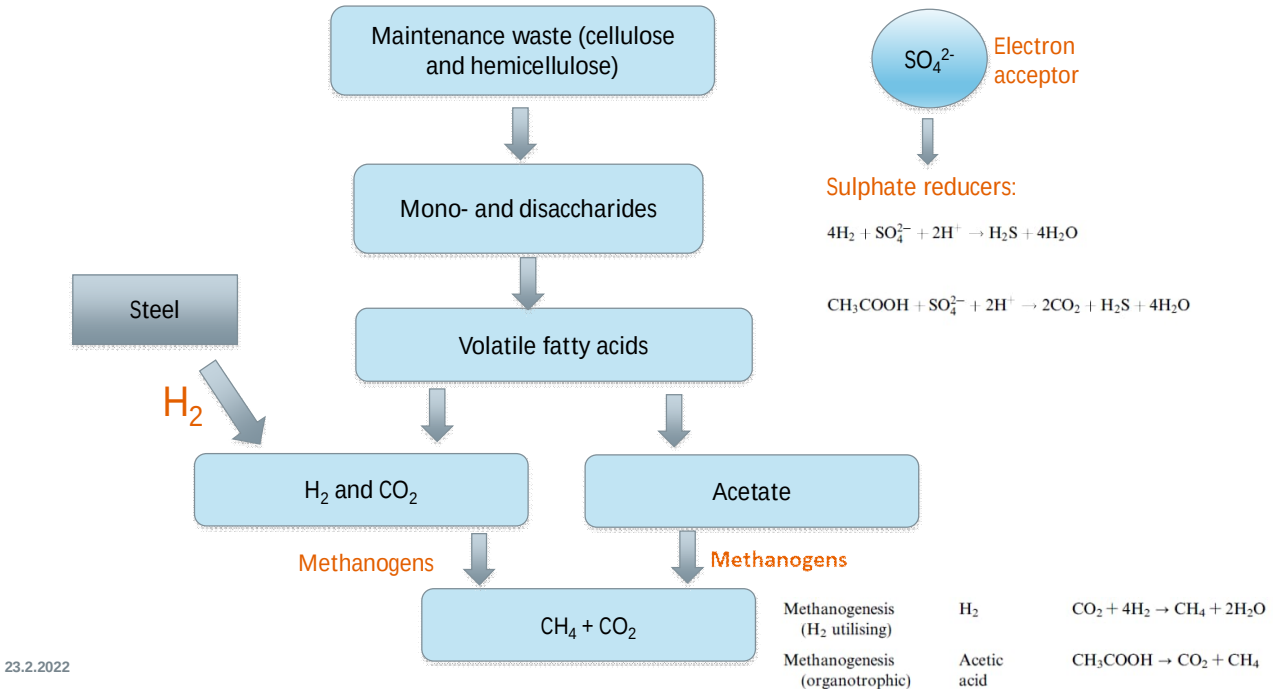

Figure 1. Schematic presentation of microbiological processes in the GGE.

2. Objectives

The aim of KaMu project was to induce disturbances to the Gas Generation Experiment and study the influences on gas generation, gas composition, chemical parameters and microbiology. The disturbances were:

a) The addition of sulphate
   - Sulphate concentration in the GGE gas been below detection limit. Sulphate reducers (SRBs) are bacteria which use sulphate as electron acceptor and can compete with methanogens for electron donors, such as hydrogen and acetate.
   - Sulphate concentration in the repository can increase because of the flowing ground water.
   - The increase of sulphate can lead to increase of activity of sulphate reducers and consequently bigger proportion of electron donors can be utilized by SRBs. This could decrease the activity of methanogens and methane formation.

b) The increase in pH
   - Alkaline environment created by concrete surroundings in final disposal conditions are expected to reduce microbial activity.
   - During the GGE pH of the tank water has decreased to close 7 as a result of microbial activity.
   - The aim was to increase pH from 6.7 to between 8 and 9 (measured by on-line analyser).

3. Methods

3.1 Induced disturbances and samplings from the GGE

During KaMu project two disturbances (sulphate addition and raise of pH) were induced to the GGE. These disturbances and samplings from the GGE are summarized in the Table 1.

K_2SO_4 and KOH were selected to induce disturbances because tank water already contained considerable amount potassium-ions. K_2SO_4 was powder, which was dissolved to tank water from the GGE before addition to the tank by pumping (concentration of added solution ca. 120 g/L). The aim was to increase sulphate concentration from below detection limit to 100 mg/L, which theoretically meant the addition of 2.97 kg K_2SO_4. The addition of sulphate was performed 9th of June, 2020. Tank water was taken from the inlet 123 to sterile glass bottles, mixed with K_2SO_4 in oxic conditions using a magnetic stirrer, and added to the tank by pumping using the inlet 123. The measurement of sulphate and potassium ion concentrations in tank water after the addition revealed that sulphate concentration was higher at the bottom of the tank compared to the lid level of the drums, and mixing of the tank water by pumping was initiated on 12th of October, 2020. Tank water was pumped from inlet K01/K08 situated on top of the tank to the inlet 222 situated at the bottom of the tank.

KOH was added as 45 w-% solution, which was first (sampling 1st of June, 2021) added directly to the tank but was mixed with groundwater after that to enhance mixing with tank water. Because pH was increased at the bottom to very high value (see Table 4), KOH was added to the tank in three different occasions.

The layout of the sampling points in the GGE are presented in Figure 2. Samples were taken from the following sampling points:

- valve 123 (the lid level of the tank)
- valve 122
- valve 121 (the bottom of the tank)
- valve 223 (the bottom of the tank, below concrete)
- below window (not marked)

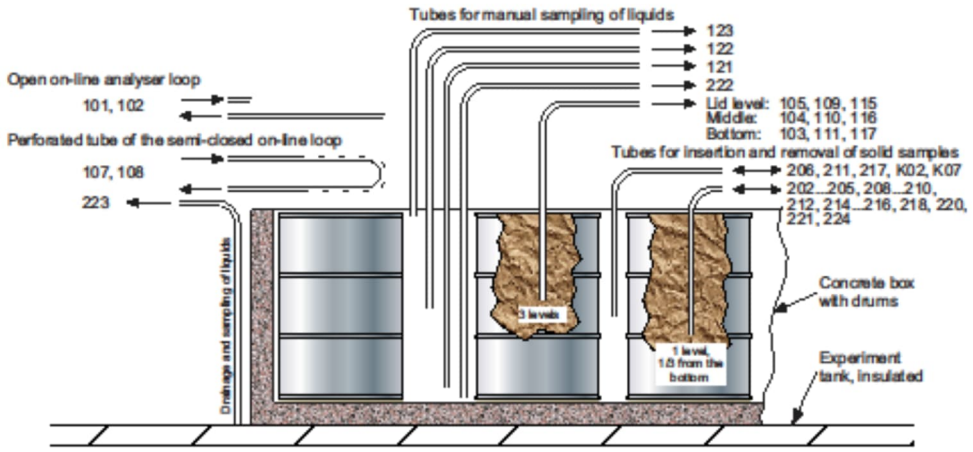

Figure 2. The layout of sampling points in the GGE.

Microbiological samples were taken anaerobically as described in Vikman et al. (2019).

Table 1. Induced disturbances and samplings from the GGE during KaMu project in 2020 and 2021.

Date	Action	Samplings from tank water	Results of the action, remarks
9.6.2020		Chemical, gas, microbiological	
9.6.2020	Addition of K_2SO_4		Theoretical amount of added K_2SO_4 should increase sulphate concentration from 0 to 100 mg/L
22.6.2020		Chemical	Sulphate and potassium concentration was higher at the bottom of the tank compared to the lid level
6.7.2020		Chemical	"
16.9.2020		Chemical	"
12.10.2020	Mixing of the tank water by pumping was started		
14.10.2020		Chemical	Sulphate concentration 100 mg/L at the bottom and in the lid level of the tank.
20.10.2020		Microbiological	
2.12.2020		Chemical	
18.-20.1.2021		Chemical, microbiological	
27.5.2021		Chemical	
1-6-2.6.2021	Addition of KOH + mixing by pumping		Mixing of the KOH evenly to the tank water took several days.
10.6.2021		pH measurements	
16.6-17.6.2021	Addition of KOH + mixing		KOH was mixed with ground water before addition.
11.8.2021	Addition of KOH + mixing		KOH was mixed with ground water before addition.
13.8.2021		pH measurements	
23-25.8.2021		Chemical, Microbiological	
2.9.2021		pH measurements	
10.11.2021		Chemical	
16.11.2021		Microbiological	

3.2 Chemical analyses and gases

Chemical parameters were analysed by TVO's laboratory. Ca, K, Mg, Fe, Na and SO_2 were analysed by ICP-OES, S^{2-} and NH_4, dissolved P, and total P spectrophotometrically, SO_4, NO_2, NO_3, Cl by ion chromatographically. Volatile fatty acid analyses were carried out at VTT by capillary electrophoresis (Vikman et al., 2019). Gas concentrations in the head space samples were analysed by gas chromatography at TVO and VTT.

3.3 Microbiological analyses

3.1.1 Total cell counts

The total number of cells (TNC) was determined by fluorescent staining with 4,6-diamidino-2-phenylindole (DAPI) [21] with slight modifications. A 5 mL subsample of each tank water sample was stained with DAPI (1 μg mL^{-1}) for 20 min at room temperature in the dark and collected on black polycarbonate Isopore Membrane filters (0.2 μm GTBP, Millipore, Ireland) with the Millipore 1225 Sampling Manifold (Millipore, USA) under low vacuum. The filters were rinsed with 1 mL filter sterilized 0.9% NaCl prior to and after filtration. Fluorescent cells were visualized under UV light with an epifluorescence microscope (Zeiss) and 1000x magnification. The number of cells was calculated from 30 random microscopy fields according to the magnification factor, filtered volume, and the surface area of the filter used.

3.1.2 DNA extraction

Collected samples were filtered on 0.22 µm pore-size filtered Corning filters (Corning Inc., New York, USA). Sample size ranged from 100 mL to 150 mL.

DNA from Corning filters was extracted using the DNeasy PowerWater Kit DNA extraction kit (Qiagen, Hilden, Germany). The filters were cut and placed in bead tubes of the DNA extraction kit and DNA extraction was done according to the manufacturer's instructions. The tubes were centrifuged in an Eppendorf 5810R benchtop. The DNA was eluted in 100 µL elution buffer. Triplicate DNA extraction was done for each sample. The DNA concentration was measured with a Qubit fluorometer (Invitrogen, Waltham, USA)

3.1.3 Abundance of indicator microbial groups detected with quantitative PCR (qPCR)

Quantitative PCR (qPCR) of the bacterial and archaeal 16S rRNA genes and bacterial *dsr*B and archaeal *mcr*A genes was used as a proxy to estimate the microbial community sizes and the abundance of sulphate reducers and methanogens.

The size of the bacterial communities was estimated using primers S-D-Bact-0341-b-S-17/S-D-Bact-0785-a-A-21 (Herlemann et al., 2011), producing a 400-bp fragment covering the variable region V3-V4 of the bacterial 16S rRNA gene. Archaeal *mcr*A gene was amplified with primers ME1 5′-GCMATGCARATHGGWATGTC-3′ and ME3r TGTGTGAAWCCKACDCCACC-3′ modified from Hales et al., 1996 and bacterial *dsr*B gene with primers 2060F (Geets et al., 2006) and dsr4R (Wagner et al., 1998). The abundance of bacterial 16S, bacterial *dsr*B, and archaeal *mcr*A genes was determined by qPCR with KAPA SYBR Fast 2x Master mix for Roche LightCycler 480 (Kapa Biosystems, Inc., Boston, MA, USA). Reactions were performed in triplicate for each sample. Each reaction contained 1 μL of extracted DNA as template and 5 pmol of both forward and reverse primers. The qPCR was performed on a Roche

LightCycler 480 (Roche Applied Science, Germany) on white 96-well plates (Roche Applied Science, Germany) sealed with transparent adhesive seals (4titude, UK). The qPCR conditions consisted of an initial denaturation at 95°C for 10 minutes followed by 45 amplification cycles of 15 seconds at 95°C, 30 seconds at 55°C, and 30 seconds at 72°C with a quantification measurement at the end of each elongation. A final extension step of three minutes at 72°C was performed prior to a melting curve analysis. The melting curve analysis consisted of a denaturation step for 10 seconds at 95°C followed by an annealing step at 65°C for one minute 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. The number of gene and transcript copies was calculated by comparing the amplification result (Cp) to that of a dilution series of plasmids containing 16S bacterial, 16S archaeal, *mcr*A or *dsr*B genes.

The archaeal community size was tested with primers A344F (Bano et al., 2004) and A744R, modified from Barns et al., 1994 flanking an approximately a 400-bp fragment containing the V3-V4 regions of the archaeal 16S rRNA gene according to the method described by Bomberg and Miettinen, 2020. The amplification was detected with a FAM-labelled probe, A516F (Takai et al., 2000) using the KAPA Probe Fast qPCR kit (Kapa Biosystems, Inc., Boston, MA, USA).

3.1.4 Archaeal and bacterial community analysis with next generation sequencing (NGS)

DNA elutions extracted from 30 samples taken from the GGE were sent to Illumina Miseq sequencing of the bacterial and archaeal V3-V4 16S regions to Eurofins (Eurofins Genomics, Konstanz, Germany) (Figure 3). First, bacterial 16S libraries were constructed with Nextera (Illumina, Inc., San Diego, CA, USA) two-step PCR with bacterial primers 357F 5`-TACGGGAGGCAGCAG-3` (Turner et al., 1999) and 800 R 5`-CCAGGGTATCTAATCC-3´ (Kisand et al., 2002) (443 bp) and archaeal primers 340F 5`-CCCTAYGGGGYGCASCAG-3` (Ganther et al., 2011) and 806R 5`- GGACTACNVGGGTWTCTAAT-3` (Apprill et al., 2015) (466 bp). The libraries were sequenced at Eurofins by 2 × 300 bp paired-end sequencing on the MiSeq platform. DNA extraction negative controls and positive control ZymoBIOMICS Microbial Community Standard (Zymo Research, Irvin, CA, USA) were included in the sequencing set.

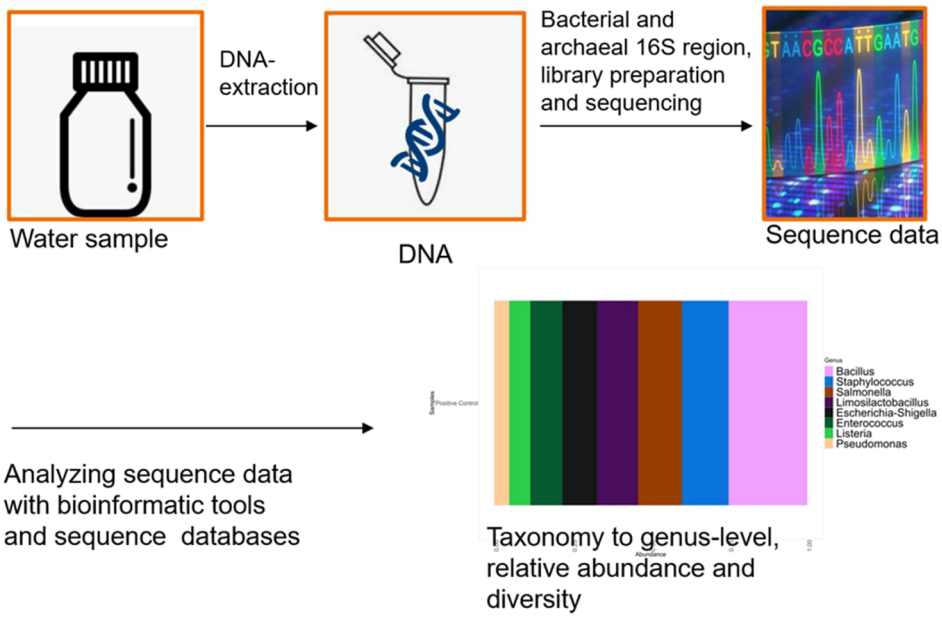

Figure 3. Graphical description of the methods used to analyse the microbial population in the GGE.

The sequence reads obtained from Illumina Miseq sequencing were subjected to sequence analysis using the DADA2 software package version 1.14 (Callahan et al., 2016) and DADA2 Pipeline Tutorial 1.16 with some modifications. DADA2 package was run in RStudio (RStudio, Boston, MA, USA, version 1.4.1106) with R version 4.0.4. First, the sequences were prefiltered to remove ambiguous bases (Ns) that could affect accurate mapping. Then, primers were identified from the sequences and removed using the cutadapt tool (Martin et al., 2011). The quality of the sequence reads was checked according to the DADA2 workflow. Next sequences were filtered and trimmed using DADA2 filterAndTrim function. Filtering parameters maxN = 0, maxEE = c(2, 2), truncQ = 2, truncLen=c(280,225) were used. With truncLen the low-quality tails of the sequences were trimmed. The maximum possible error rates were calculated using the learnErrors command. Identical reads were de-replicated (unique sequences). Amplicon sequence variants of the sequence data were identified using the DADA2 pipelines core sample inference algorithm. Denoised paired reads were merged according to the DADA2 pipeline, and an amplicon sequence variant table (ASV) table was constructed. Subsequently, chimeric sequence reads were removed from the dataset with the removeBimeraDeNovo function, using the consensus option. Finally, taxonomy from the domain to the species level was assigned to ASVs with DADA2's native implementation of the naive Bayesian classifier method. Taxonomy was assigned against Silva reference taxonomy version 138 (Quast et al., 2012). All images of the sequencing data were constructed with R using the packages phyloseq (McMurdie et al., 2013) and ggplot2 (Wicham et al.,2016). In addition, Functional Annotation of Prokaryotic Taxa (FAPROTAX) was used to annotate 16S rRNA sequencing data to evaluate the functional diversity (Louca et al., 2016).

Two samples were chosen for metagenomic shotgun sequencing where all the microbial genomes were sequenced. DNA samples from two samples A44a = 1_Lid_a and A149a = 2_Lid_a were sent to Eurofins for 2 × 150 bp paired-end sequencing on the Illumina HiSeq-2000. Metagenomes were analyzed at Eurofins where first sequence quality assessment was done and then poor-quality bases, adapters and primers were removed. Taxonomic profiling was done using NCBI database of bacterial, archaeal fungal, protozoan and viral genomes. Raw data of the metagenomes was received, and further analysis of the metabolic functions is planned for later.

4. Results

4.1 The influence of disturbances on chemical parameters

On-line measurements of pH, conductivity, and electrical potentiality Eh were shown in the Figure 4. As expected, conductivity showed increased values after the addition of sulphate and KOH. Tank water pH showed decreasing trend during the experiment but was increased from 6.7 to 8.1 after addition of KOH.

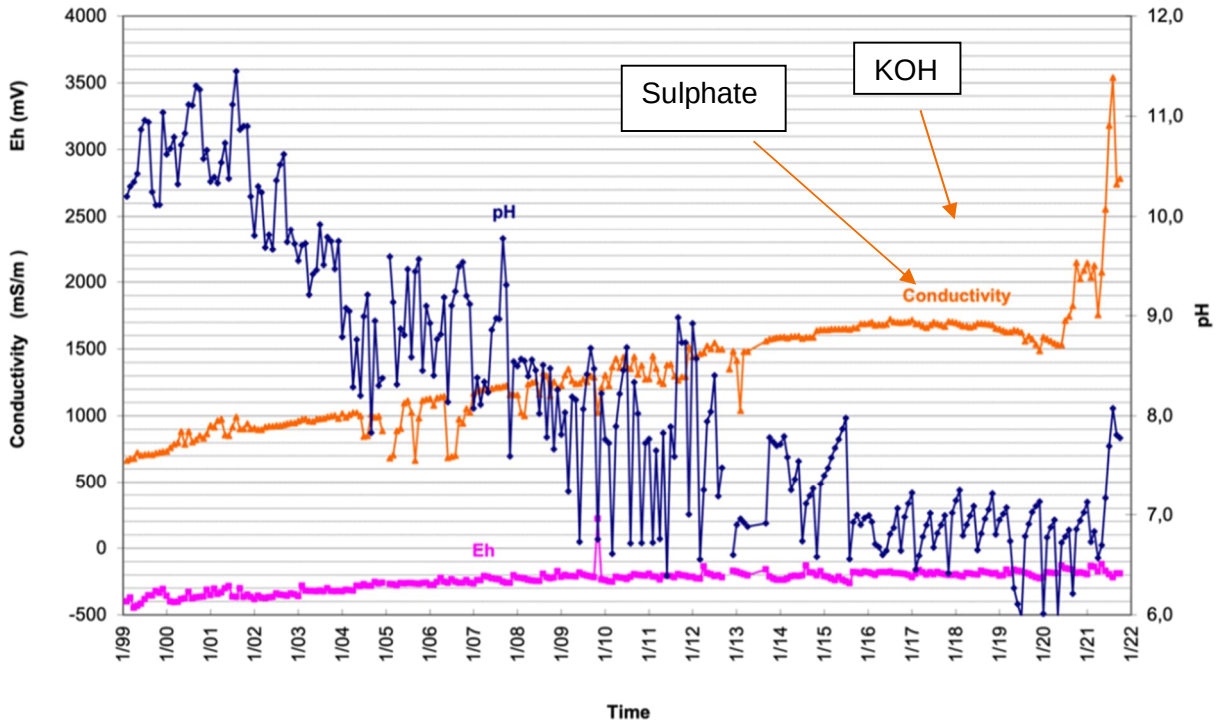

Figure 4. On-line measurements of pH, conductivity and redox potential Eh in the GGE.

The changes in chemical parameters in the lid level of the tank and at the bottom of the tank are collected to the tables 2 and 3. After sulphate addition 22nd of June, 2020, the amount of sulphate ions were increased to 990 mg/L at the bottom but were 350 mg/L in the upper part of the tank. The similar behaviour of potassium ions was also detected. In order to mix tank water more properly mixing by pumping was initiated 12.10.2020. After the pumping both sulphate and potassium concentration showed similar values at the bottom and in the lid level, and the target value of 100 mg/L was reached. Sulphate concentration in the tank water stayed at the same level during the experiment. Sulphide concentration increased from below the detection limit to 0.11 mg/L.

After the addition of KOH (Table 4) pH was gradually increased to pH 8.1 measured by online analyser measuring pH at the upper level of the tank. Occasionally pH was higher at the bottom of the tank (maximum value of 10.1 measured 11th of August, 2021) before tank water was properly mixed using the pump. Gradually pH value of tank water was decreased to 7.9 (on-line analyser). The amount of dissolved iron was reduced in the tank water which indicated precipitation of iron. Other significant changes in chemical parameters were not detected.

Table 2. Chemical parameters at the bottom of the tank (inlet 121). Sulphate addition 9th of June 2020 and KOH addition after 27th May, 2021.

Date		9.6.2020	22.6.2020	14.10.2020	18.1.2021	27.5.2021	23.8.2021	10.11.2021
Al					7.5	<4		
NH_4	mg/L	7.2			7.9	7.5	7.6	7.5
P	mg/L	0.31			0.27	0.33		0.31
K	mg/L	**250**	**990**	**338**	**340**	**340**	**560**	**510**
Ca	mg/L	62			66	67	62	57
Cl	mg/L	96			91	98	89	99
DIC	mg/L	180			171	200	240	230
DOC	mg/L	31			30	30	32	29
Mg	mg/L	8,7			9	9	9	9
Na	mg/L	120			120	120	120	120
Fe, total	mg/L	54			55	**69**	**28**	**12**
Fe^{2+}	mg/L	54			52	**66**	**25**	**12**
S, total	mg/L	0,23			33	34	28	29
SO_4	mg/L	**<0.2**	**940**	**107**	**98**	**110**	**90**	**98**
S^{2-}	mg/L	**<0.03**	**<0.03**		**0.11**	**0.11**	**0.07**	**0.07**
N, total	mg/L	8.5			8.3	8.7	8.2	8.7
HCO_3	mg/L	915			830	910	1100	1100
Conductivity	mS/cm	1.8	3.9		2.0	2.0	2.4	
pH		6.8	6.8		6.8	6.8	7.4	7.4

Table 3. Chemical parameters in the lid level of the tank (sampling valve 123). Sulphate addition after 9.6.2020 and KOH addition was started after 27.5.2021.

Date		9.6.2020	22.6.2020	14.10.2020	18.1.2021	27.5.2021	23.8.2021	10.11.2021
Al					5,1	<4		
NH_4	mg/L	7.1			7.9	7.4	7.6	7.4
P	mg/L	0.3			0.27	0.32		0.31
K	mg/L	**240**	**350**	**333**	**340**	**340**	**550**	**500**
Ca	mg/L	59			66	66	60	55
Cl	mg/L	96			90	96	88	99
DIC	mg/L	180			179	200	240	230
DOC	mg/L	30			30	30	31	29
Mg	mg/L	8.5			9	8.7	9	8.3
Na	mg/L	120			120	120	120	120
Fe, total	mg/L	55			61	**60**	**27**	**15**
Fe^{2+}	mg/L	54			63	**55**	**27**	**13**
S, total	mg/L	0.24			33	33	28	29
SO_4	mg/L	**<0.2**	**130**	**98**	**98,0**	**110**	**89**	**97**
S^{2-}	mg/L	**<0.03**	**<0.03**		**0.1**	**0.1**	**0.06**	**0.06**
N	mg/L	8,6			8.5	8.7	8.3	8.8
HCO_3	mg/L	854			832	860	1100	1100
Conductivity	mS/cm	1.8	2.1		2.0	2.0	2.4	
pH		6.8	6.8		6.8	6.8	7.5	7.4

Table 4. pH in various sampling points during the 2nd disturbance (the addition of KOH).

Date	KOH addition	pH on-line	Tank bottom (121)	Middle of the tank (122)	Below window	223
1.6.2021		6.7				
1.6 - 2.6.2021	after KOH addition	7.0	8.9	7.2	-	7.1
10.6.2021		7.1	7.2	7.2		
16.6-17.6.2021	after KOH addition	7.6	9.5	8.7		7.2
11.8.2021		7.7				
11.8.2021	after KOH addition	7.8	10.1	8.9	8.7	
13.8.2021		8.1	7.8		7.5	
24.8.2021		7.7	7.0		7.0	
2.9.2021		7.8	7.5		7.4	
16.11.2021		7.9	7.6		7.5	

4.2 Gas generation and composition

The addition of sulphate and the increase in pH did influence of the gas generation (Fig.5). The increase of pH influenced the gas composition by decreasing CO_2 concentration in the gas phase (Table 5).

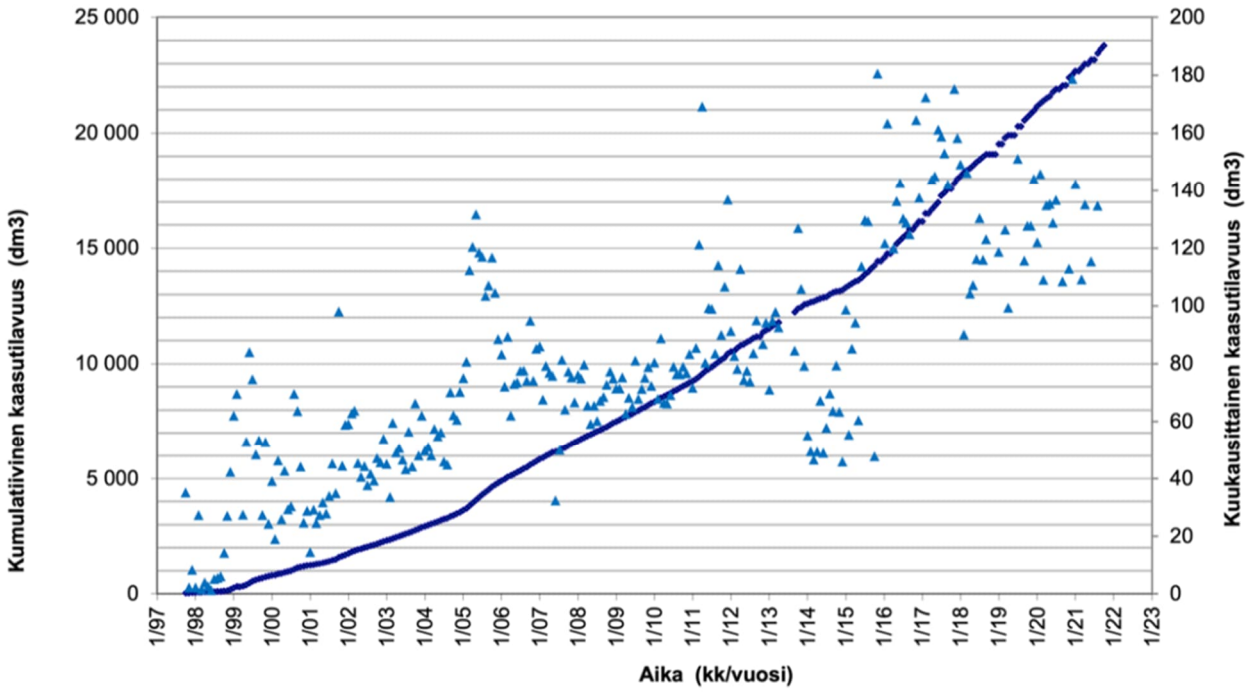

Figure 5. Cumulative gas generation in the GGE.

Table 5. The gas composition of the released gas in the GGE after induced disturbances.

Date	H_2 (t-%)	O_2 (t-%)	CH_4 (t-%)	CO_2 (t-%)	N2 (t.-%)
13.1.2020	<0.1	<0.1	90		0.48
15.5.2020	<0.1	<0.1	90		0.38
17.6.2020	0	1.7	82.9	10.4	5
8.7.2020	0	0.1	87.2	12.2	0.5
20.8.2020	<0.1	0.3	86.5	11.9	1.4
13.8.2020			87.5	10.2	
13.8.2020			89.2		
17.9.2020			86.8	10.0	
17.9.2020	<0.1	<0.1	90.3		<2.0
21.10.2020			87.7	10.0	
20.1.2021	<0.1	<0.1			<2.0
20.1.2021			87.5	10.1	
28.5.2021			88.6	11.1	
26.8.2021	<0.1	<0.1			2
26.8.2021			91	4.4	
10.11.2021			91.6	5.7	<2
3.1.2022			88.9	6.1	<2

4.3 Changes in microbial communities

4.3.1 Total cell counts and abundance of indicator microbes

The total number of microbial cells detected with the Dapi staining method was in a similar level, ~10^6 cells/mL at all sampling times (Figure 6).

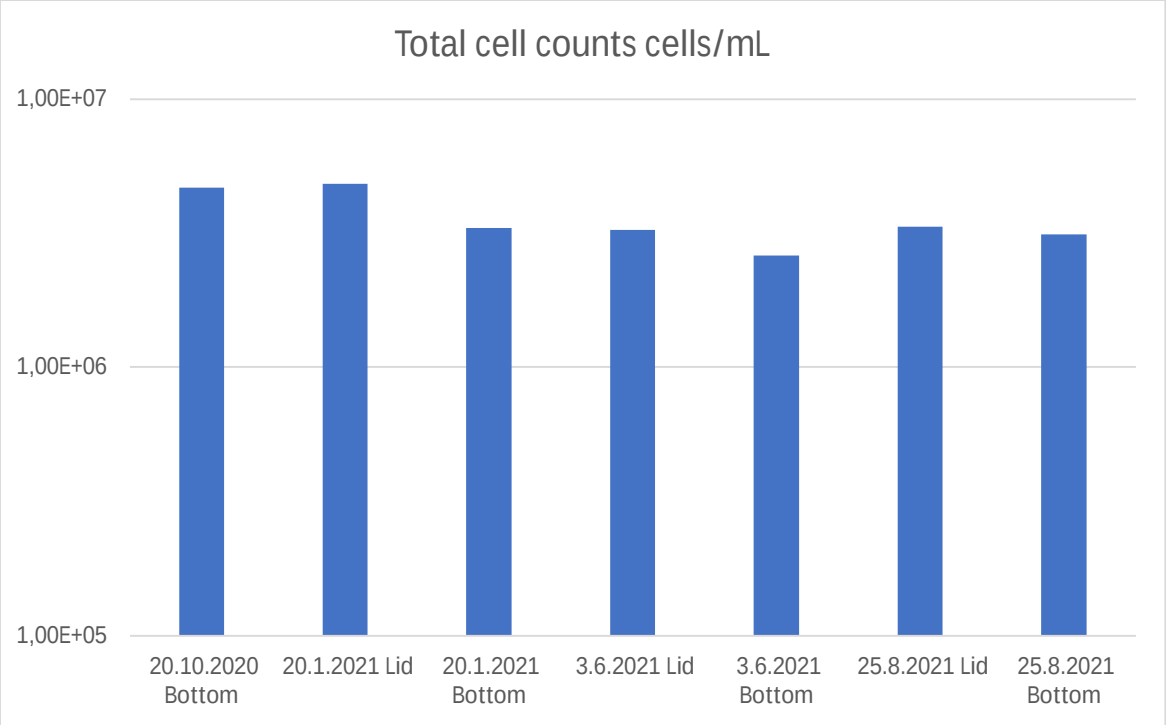

Figure 6. Total cell counts (cells/mL) of the samples taken from bottom level and lid level of the tank at different sampling times.

The size of the bacterial and archaeal communities in the GGE were studied with quantitative PCR. In addition, bacterial *dsr*B and archaeal *mcrA* genes were used as a proxy to estimate the abundance of sulphate reducers (*dsr*B) and methanogens (*mcr*A). After the addition of sulphate into the water in the GGE experiment, the number of sulphate-reducers increased, and the number of methanogens decreased (Figure 7 A and B). The results were similar in the bottom and on the surface of the tank. Total archaeal community size decreased after sulphate addition except in the fourth sampling (3.6.2021) where the highest number of archaea was detected. Total bacterial community size decreased after sulphate addition but started to increase after that.

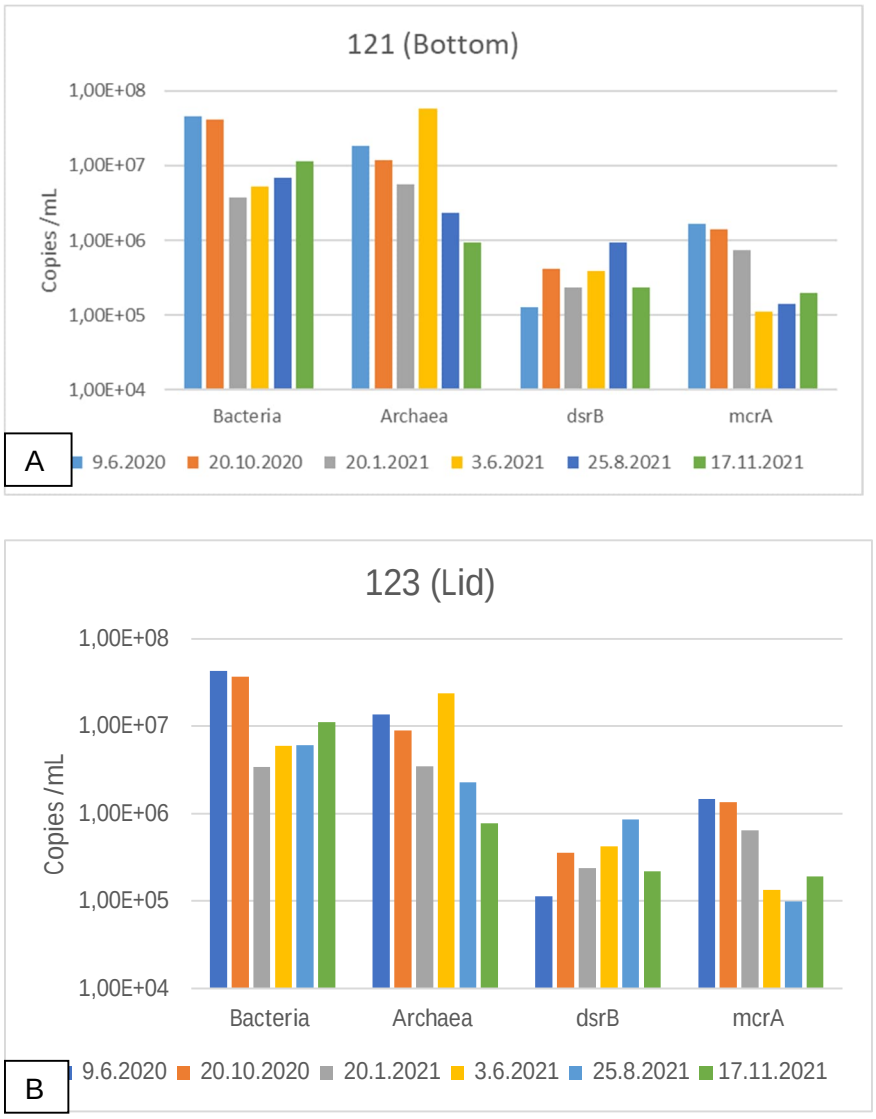

Figure 7. Abundance of bacteria, archaea, sulphate-reducers (dsrB) and methanogen (mcrA) in the GGE in different sampling times at the bottom of the tank (A) and at the lid (B) presented as copies/mL. Sulphate was added after the first sampling and KOH after 4th sampling.

4.3.2 Archaeal and bacterial community analysis

The archaeal community including methanogens remained stable in the GGE during the sampling period of 2020-2021 (Figure 8). The most abundant methanogen (archaeal genus) was *Methanosaeta* which formed 60-74 % of the archaeal community. Species belonging to *Methanosaeta*-genus metabolize acetate as their sole source of energy. Another synonym of the *Methanosaeta* genus is *Methanotrix*. The most abundant methanogen group that utilizes H_2 and CO_2 to produce methane was *Methanoregula* which formed 21-28 % of the archaeal community. Other minor archaeal groups detected were *Methanospirillum* and *Methanobacterium*.

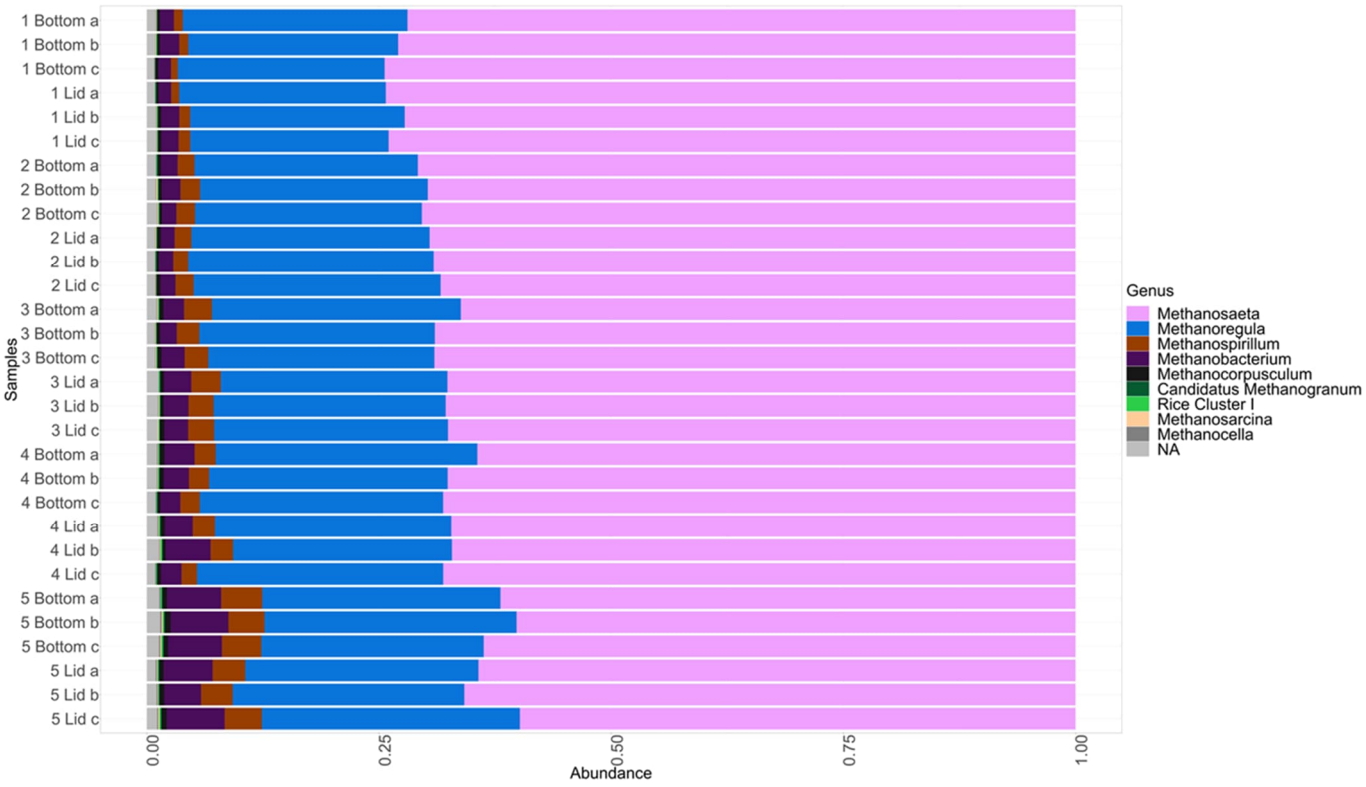

Figure 8. Relative abundances (%) of archaeal genera present in the GGE in five sampling times as determined by NGS. Samples were taken both from the bottom and from the lid of the tank. NA: archaeal ASVs not identified to genus level.

The FAPROTAX analysis of the potential archaeal function indicated mainly methanogenesis using H_2 and CO_2 and some methanogenesis using formate and methyl compounds (Figure 9). In addition, *Methanosaeta* uses acetate to produce methane. The FAPROTAX database uses the name of *Methanotrix* instead of *Methanosaeta* and this is the reason the FAPROTAX analysis did not indicate methanogenesis using acetate. A low percentage of other functions like nitrogen fixation and hydrogen oxidation was indicated. The functions were similar in all samples taken from the GGE.

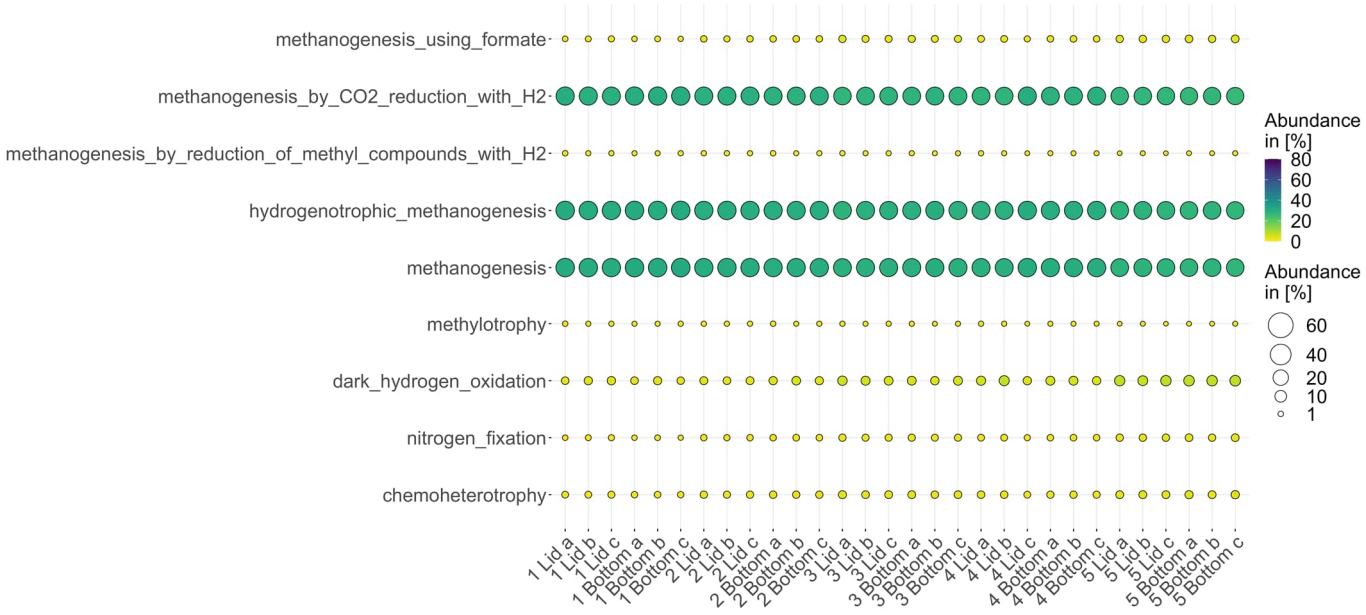

Figure 9. Relative abundance of different metabolic functions (Y axis) of the archaeal communities (including methane producing methanogens) predicted with FAPROTAX analysis in the GGE samples. Both the size of the balloon and the color indicate the relative abundance of taxonomy-based functional capacities of the microbial community. Lid= lid level of the tank, Bottom= Bottom of the tank.

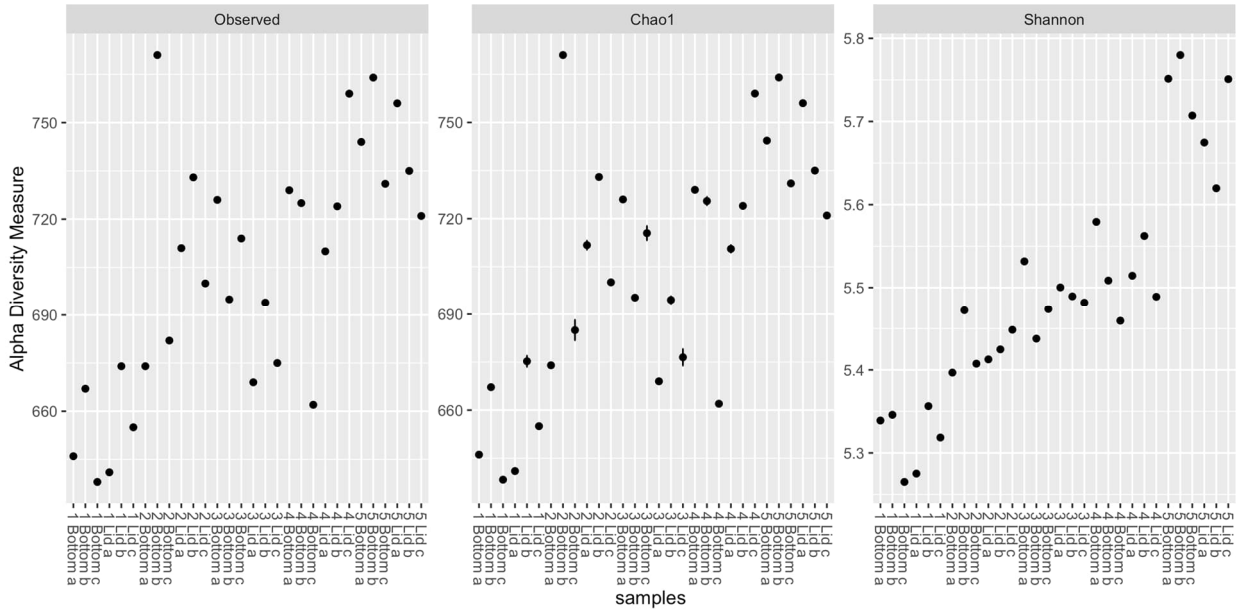

Figure 10. The alpha diversity indexes of the archaeal community: the number of amplicon sequence variants ASVs (observed), Chao1 estimated number of ASVs (total richness) and Shannon's diversity index (richness and evenness). The bigger the Chao1 and Shannon diversity indexes are, the more diverse microbial communities in the sample are. Alpha diversity represents diversity within an ecosystem or a sample, in other words, what is there and how much is there in term of species. ASV represents the unique sequences present in the original sample.

In total, 1161 archaeal ASVs were detected in the sample set. The number of observed ASVs in the samples varied between 638 and 771 (Figure 10). When comparing the Chao1 ASV richness estimate

values to true observed ASV numbers, all of the estimated archaeal ASVs were obtained from the sequence data (Figure 10), meaning that sequencing depth was sufficient to fully characterize the archaeal communities in all of the samples. The Shannon index indicated that the archaeal diversity increased after sulphate and KOH addition.

The identified bacterial community in the GGE changed after the sulphate was added and after an increase in pH (Figure 11). After sulphate addition, the sulphate-reducing bacterial genus *Desulfovibrio* became the dominant identified bacterial genus that comprised 10-21 % of the community after the sulphate addition. Before sulfate addition, *Desulfovibrio* was detected only as a minor group (0.4%) and dominant identified bacterial genera were *Synthropus* and *Smithella* that grow only in the presence of H_2 or formate-utilizing partners (methanogens) in syntrophic associations. They are known to enhance the growth of methanogens.

After an increase in pH in addition to *Desulfovibrio* another sulphate-reducing bacteria genus, *Desulfocapsa* was detected in the bacterial community. In the first sampling after the KOH addition (4th sampling), *Desulfocapsa* formed 2 % of the bacterial community but in the 5th sampling, *Desulfocapsa* comprised 9-10 % of the bacterial community.

Many of the bacterial ASVs could not be identified to genus level. This could be because of a low number of sequences from similar environments in sequence databases. In addition, species from anoxic environments are more difficult to cultivate and thus identify and deposit to sequence databases.

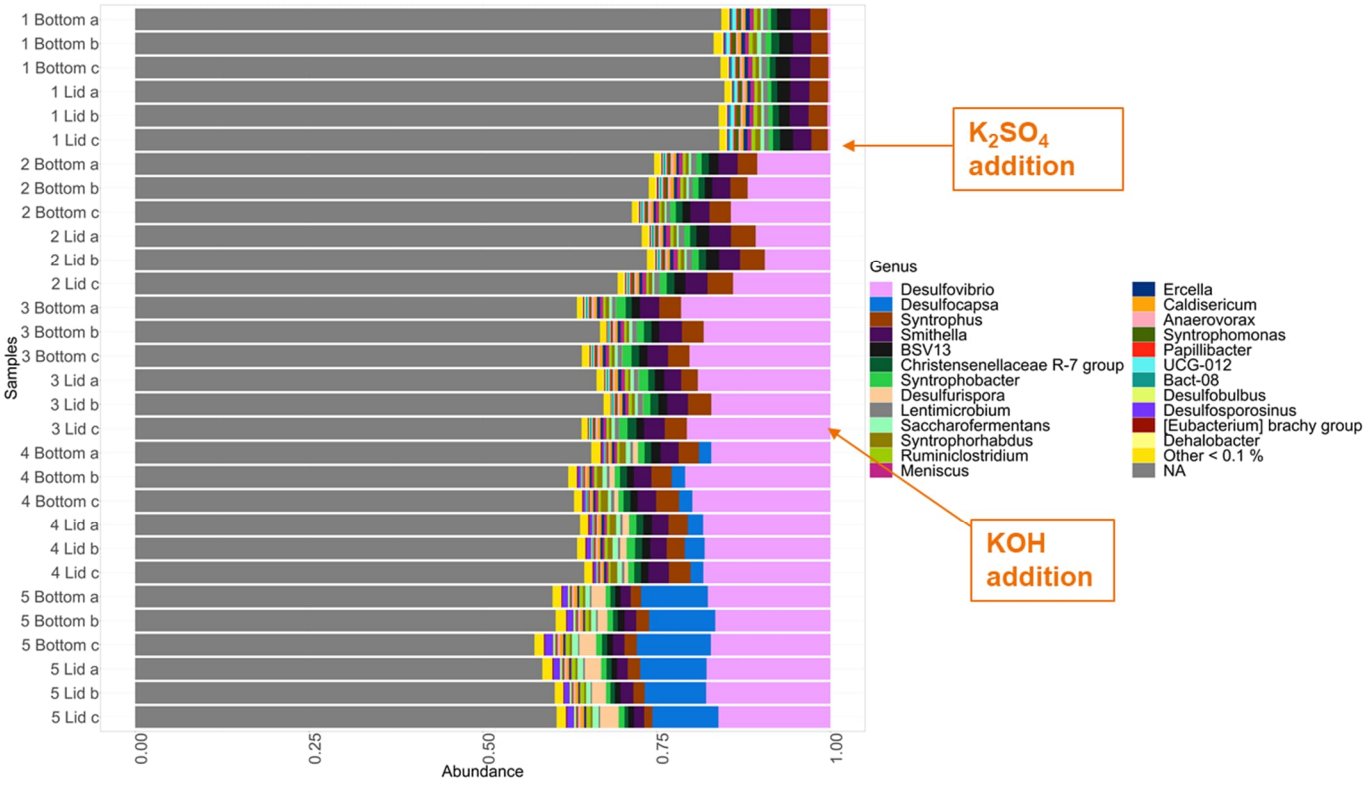

Figure 11. Relative abundances (%) of bacterial genera present in the GGE in five sampling times as determined by NGS. Samples were taken both from the bottom and from the lid of the tank. NA: bacterial ASVs not identified to genus level.

The FAPROTAX analysis of the potential bacterial function indicated mainly sulphate-reduction and respiration of other sulphur compounds (Figure 12). In addition, different functions in the nitrogen cycle

like nitrate reduction and nitrogen fixation were indicated. The iron reduction was indicated in the first three samplings and iron reduction in the last sampling.

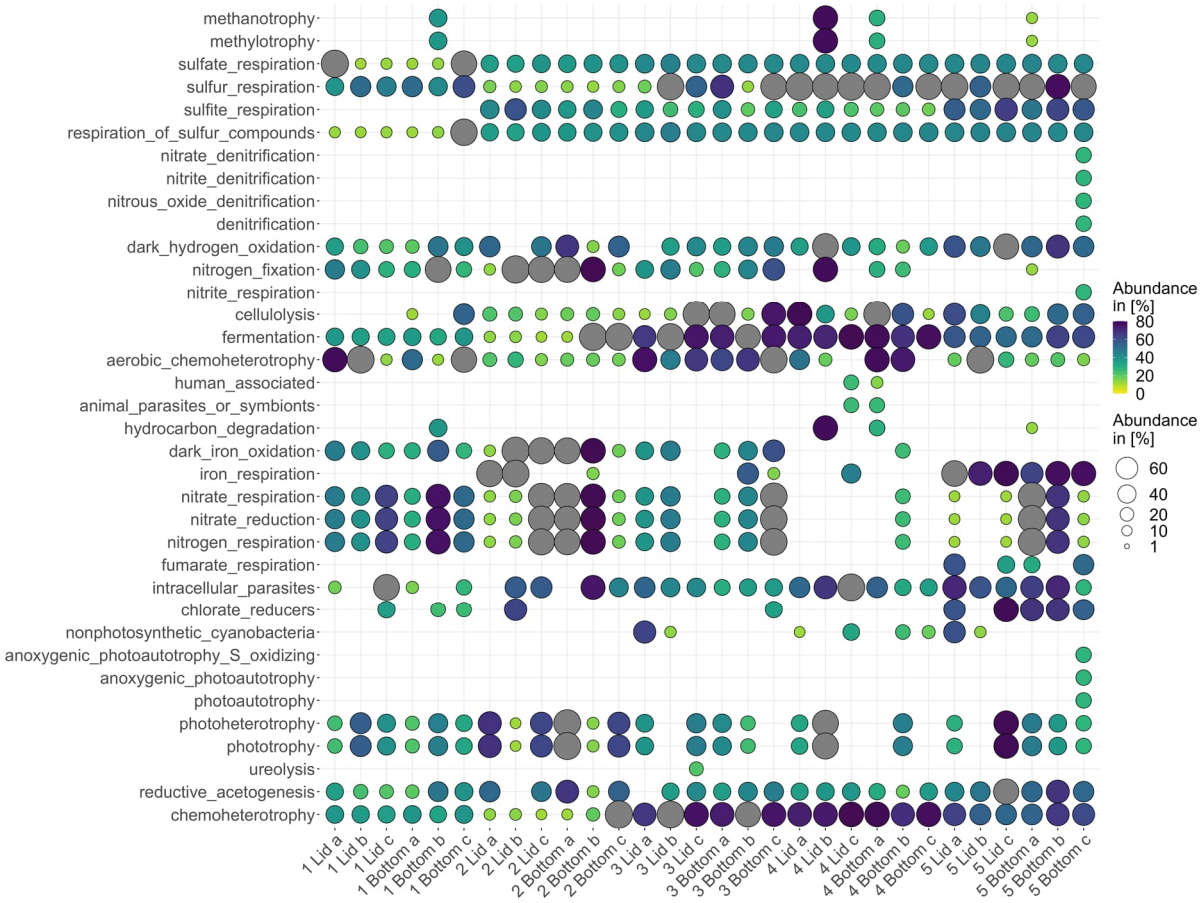

Figure 12. Relative abundance of different metabolic functions (Y axis) of the bacterial communities predicted with FAPROTAX analysis in the GGE samples. Both the size of the balloon and the color indicate the relative abundance of taxonomy-based functional capacities of the microbial community. Lid= lid level of the tank, Bottom= Bottom of the tank.

In total, 805 bacterial ASVs were detected in the sample set. The number of observed ASVs in the samples varied between 224 and 349 (Figure 13). When comparing the Chao1 ASV richness estimate values to true observed ASV numbers, all of the estimated bacterial ASVs were obtained from the sequence data, meaning that sequencing depth was sufficient to fully characterize the bacterial communities in all of the samples. The Shannon index indicated that the bacterial diversity decreased after sulphate and KOH addition.

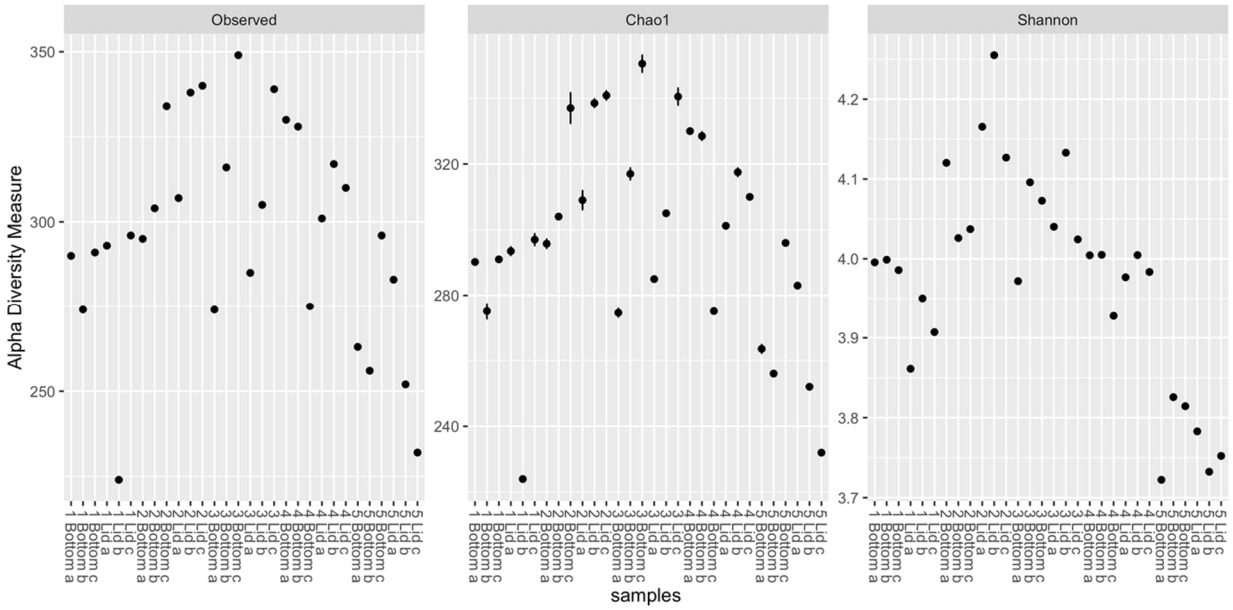

Figure 13. The alpha diversity indexes of the bacterial community: the number of amplicon sequence variants ASVs (observed), Chao1 estimated number of ASVs (total richness) and Shannon's diversity index (richness and evenness). The bigger the Chao1 and Shannon diversity indexes are, the more diverse microbial communities in the sample are. Alpha diversity represents diversity within an ecosystem or a sample, in other words, what is there and how much is there in term of species. ASV represents the unique sequences present in the original sample.

4.3.3 Metagenomic analysis

In the metagenomic analysis, most of the sequences belonged to bacterial (51.8%) and archaeal (46.8%) kingdoms (Table 6). Only a small percentage of sequences belonged to other kingdoms like fungi (0.33%) and viruses (0.01%).

Table 6.The number of sequence reads assigned to different kingdoms.

Kingdom	A149aMeta		A44aMeta	
Archaea	298,347	46.78 %	224,308	55.87 %
Bacteria	330,414	51.81 %	171,214	42.64 %
Eukaryota	497	0.08 %	300	0.07 %
Fungi	2,128	0.33 %	1,254	0.31 %
Viruses	72	0.01 %	50	0.01 %
Ambiguous	6,242	0.98 %	4,368	1.09 %

With metagenomic analysis, sequences were identified to genus and species level (Figure 14). The genus-level identification was similar to identification with targeted amplicon 16S NGS. The species-level identification showed that species belonging to sulphate-reducing *Desulfovibrio* genus was *Desulfovibrio mexicanus*. The dominating methanogens were identified as *Methanoregula formicicum*, *Methanoregula boonei* and *Methanotrix soehngenii* (syn. *Methanosaeta*).

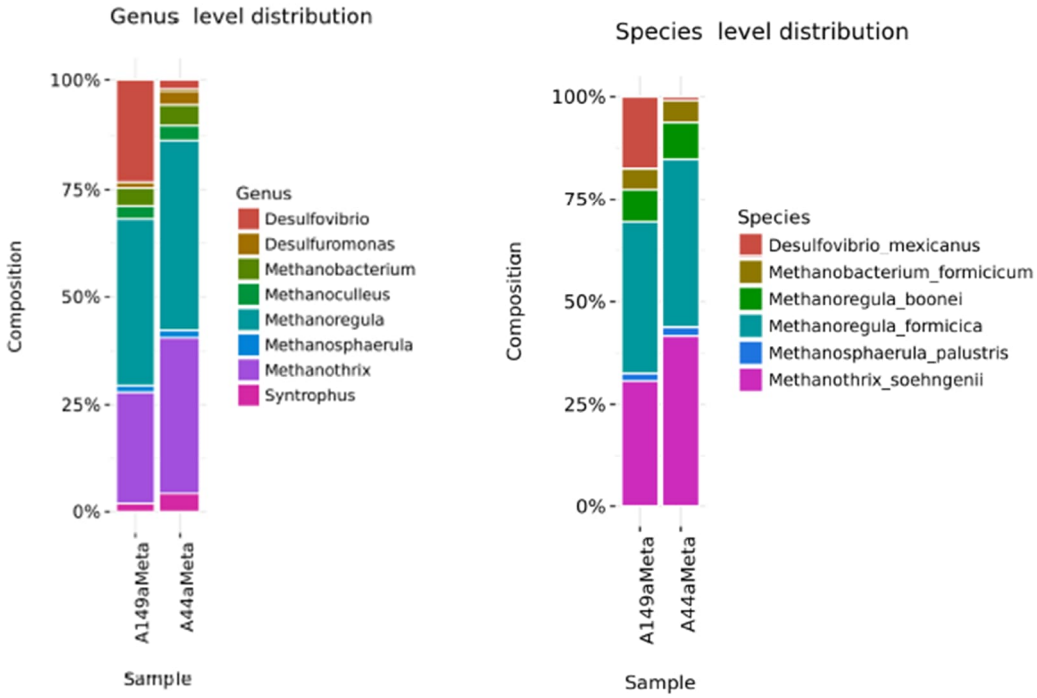

Figure 14. Genus and species-level distribution of metagenomes in samples 1 Lid a (A44a) and 2 Lid a (A149a).

5. Conclusions

The main conclusion from KaMu project is that gas generation in the GGE is stable and neither the addition of sulphate nor increased pH did not influence gas generation rate. The obtained results can be used to verify modelling and to improve safety assessment related to the final disposal of low-level radioactive waste in Finland.

Methane producing microbes and sulphate reducing bacteria SRBs compete for same electron donors, e.g. acetate and hydrogen. As sulphate concentration in the GGE has been below detection limit, the addition sulphate can enhance activity of SRBs thus potentially decreasing activity of methanogens and gas generation. In this project K_2SO_4 was added to the GGE tank to increase sulphate concentration from 0 to 100 mg/L. After sulphate addition, the sulphate-reducing bacterial genus *Desulfovibrio* became the dominant identified bacterial genus that comprised 10-21 % of the community. Before sulphate addition, *Desulfovibrio* was detected only as a minor group (0.4%) and dominant identified bacterial genera were *Synthropus* and *Smithella* that grow only in the presence of H_2 or formate-utilizing partners (methanogens) in syntrophic associations. They are known to enhance the growth of methanogens. In addition, qPCR analysis showed a moderate increase in the amount of sulphate reducers and a decrease in the amount of methanogens. Despite the changes in microbial population no significant influences on the gas generation and gas composition were detected. In addition, sulphate concentration stayed in a high level in the tank water and sulphide concentration was not increased significantly. The diversity of methane producing microbes stayed similar after the sulphate addition.

Similarly, the higher pH did not influence the gas generation rate in the GGE. Some changes in the gas composition were detected as CO_2 concentration in the released gas was reduced because CO_2 was partly

absorbed into the more alkaline tank water. In addition, iron concentration in the tank water was reduced as a result of its precipitation. In addition to *Desulfovibrio,* another sulphate-reducing bacteria genus, *Desulfocapsa* was detected in the bacterial community. No significant changes in methane producing microbes were detected after raising of pH.

The most abundant archaeal genus in the GGE in all samplings was *Methanosaeta,* which formed 60-74 % of the archaeal community. Species belonging to *Methanosaeta*-genus metabolize acetate as their sole source of energy. The most abundant methanogen group that utilizes H_2 and CO_2 to produce methane was Methanoregula which formed 21-28 % of the archaeal community. These findings were similar to those already reported by Vikman et al. (2019).

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